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Enzymatic Browning and Its Prevention

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Foreword

THE ACS SYMPOSIUM SERIES was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of this series is to publish comprehensive books developed from symposia, which are usually "snapshots in time" of the current research being done on a topic, plus some review material on the topic. For this reason, it is necessary that the papers be published as quickly as possible.

Before a symposium-based book is put under contract, the proposed table of contents is reviewed for appropriateness to the topic and for comprehensiveness of the collection. Some papers are excluded at this point, and others are added to round out the scope of the volume. In addition, a draft of each paper is peer-reviewed prior to final acceptance or rejection. This anonymous review process is supervised by the organizer(s) of the symposium, who become the editor(s) of the book. The authors then revise their papers according to the recommendations of both the reviewers and the editors, prepare camera-ready copy, and submit the final papers to the editors, who check that all necessary revisions have been made.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previously published papers are not accepted.

Preface

POLYPHENOL OXIDASE (PPO) ACTIVITY OF PLANTS is desirable in the processing of certain foods such as prunes, black raisins, black figs, zapote, tea, coffee, and cocoa, and it probably protects plants against attack by insects and microorganisms. However, its action leads to major economic losses in some fresh fruits and vegetables such as potatoes, lettuce and some other leafy vegetables, apples, peaches, apricots, grapes, bananas, strawberries, and many other tropical fruits. A corn (maize) fungus, *Ustilago maydis*, produces dark pigmented mycelia that are considered an excellent food in some countries, but this also can lead to major economic losses in the corn industry.

For many years, the food industry all over the world used sulfur dioxide extensively to prevent browning. Sulfites are a potent browning inhibitor and are very economical. However, because of recent changes in our scientific knowledge and life style as related to our food and health, the consumer's demand for more natural foods and safer, fewer added chemicals in processed foods has been increasing. A few years ago, the U.S. government banned the use of sulfites in fresh fruits and vegetables because of its health effects in some people. Therefore, the food industry has been looking for alternate ways to prevent browning. Our scientific community was forced to provide new information to meet consumers' and the industry's demands.

Much research has been done in recent years to find effective and economical ways to prevent enzymatic browning in various fruit and vegetable products. An emerging new scientific development is in the field of molecular biology to control PPO and its substrates in order to prevent browning in foods. Several researchers have been focusing on gene manipulation to inhibit PPO in transgenic plants by expressing PPO in an antisense orientation. Cloning of the genes for PPO offers the possibility of understanding the essential function of PPO in plants, which has remained a puzzle for years.

The symposium upon which this book is based was organized to bring together many of the world's experts on PPO and enzymatic browning to discuss these issues. The authors of the chapters, from 10 different countries, represent many of the major laboratories working on the structure and mechanism, expression and regulation in vivo, industrial utilization, and prevention of browning caused by PPO.

The objectives of this volume are to provide a broad but detailed treatment of the current knowledge of PPO, including structure and function, molecular biology, biosynthesis and regulation, chemistry of formation of brown products, and prevention of browning in fruits and vegetables. No book devoted to all of these subjects has been published; therefore, this book provides valuable information and useful research tools for chemists, biochemists, and food scientists in the academic community and the food industry.

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Chapter 1

Recent Advances in Chemistry of Enzymatic Browning

An Overview

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Polyphenol oxidase (PPO) is important in the beneficial coloration of some of our foods, such as prunes, dark raisins and teas. However, in most cases, PPO is the most damaging of enzymes in color deterioration (browning) of plant foods, with resulting losses of up to 50% for tropical fruits and others. Preventing PPO activity in postharvest fruits and vegetables has enormous economic and quality benefits, but current prevention methods are not ideal. Through an understanding of the structure and mechanism of action of PPO, and the chemistry of enzymatic browning, better prevention methods can be used, including decrease in PPO biosynthesis *in vivo* by the antisense RNA method. PPO can be used commercially in the biosynthesis of L-DOPA for pharmaceutical uses and for production of other polymeric products. PPO is stable in water-immiscible organic solvents, facilitating specific oxidation reactions with water-insoluble organic compounds. Melanins for use as sun blockers can be produced readily by PPO genetically engineered into *Escherichia coli*.

Polyphenol oxidase (PPO) is a generic term for the group of enzymes that catalyze the oxidation of phenolic compounds to produce brown color on cut surfaces of fruits and vegetables. Based on the substrate specificity, Enzyme Nomenclature (*1*) has designated monophenol monooxygenase, cresolase or tyrosinase as EC 1.14.18.1, diphenol oxidase, catechol oxidase or diphenol oxygen oxidoreductase as EC 1.10.3.2, and laccase or *p*-diphenol oxygen oxidoreductase as EC 1.10.3.1. PPO is found in animals, plants and microorganisms. The role of PPO in animals is largely one of protection (pigmentation of skin, for example), while the role of PPO in higher plants and microorganisms is not yet known with certainty. Intensive efforts to show that it is involved in photosynthesis and/or energy induction have failed to date.
    The action of PPO leads to major economic losses in some fresh fruits and vegetables, such as Irish potatoes, lettuce and some other leafy vegetables, apples, apricots, bananas, grapes, peaches and strawberries (*2*). In some tropical fresh fruits, up to 50% can be lost due to the enzyme-caused browning. Browning also leads to off-flavors and losses in nutritional quality. Therefore, the consumer will not select fruits

and vegetables that have undergone browning. Black spots in shrimp are caused by PPO-catalyzed browning; the "browned" shrimp are not acceptable to the consumer and/or they are down-graded in quality. PPO activity in plants is desirable in processing of prunes, black raisins, black figs, zapote, tea, coffee and cocoa and it probably protects plants against attack by insects and microorganisms (*3*).

PPO was first discovered by Schoenbein (*4*) in 1856 in mushrooms. Subsequent investigations showed that the substrates for the enzyme are O_2 and certain phenols that are hydroxylated in the *o*-position adjacent to an existing -OH group (Equation 1), further oxidized to *o*-benzoquinones (Equation 2) and then nonenzymatically to melanins (brown pigments).

$$(1)$$

p–Cresol   4–Methylcatechol

$$(2)$$

Catechol   *o*–Benzoquinone
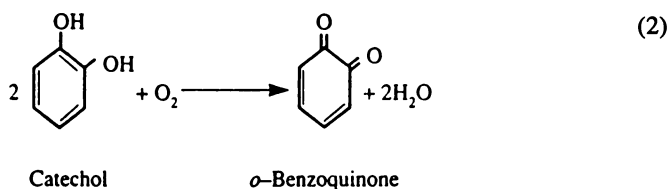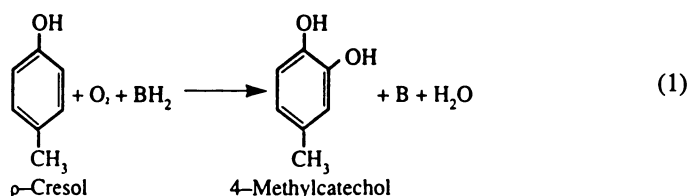
Millions of dollars are spent each year on attempts to control PPO oxidation; to date none of the control methods are entirely successful. It is said that Napoleon offered a sizable financial reward for the replacement of $NaHSO_3$, to which he was very sensitive, in wines to prevent browning with an innocuous compound. To date, the reward has not been claimed.

The objectives of this overview chapter are to provide a broad, general treatment of the current knowledge of PPO, including structure and function, molecular biology, biosynthesis and regulation, chemistry of formation of brown products and prevention of browning, as well as suggestions of future research needs.

Structure, Function and Molecular Biology of PPO

Purification to homogeneity of the enzyme required before detailed structure and function studies has been difficult, in large part because the required disintegration of tissues leads to formation of *o*-benzoquinones (first product formed); the *o*-benzoquinones rapidly react non-enzymatically to form melanins, leading to modifications of proteins, including PPO. Most of the earlier purification was done on mushroom PPO, which occurs in multiple forms (isozymes and artifacts) with different ratios of cresolase to catecholase activities. Mushroom PPO is a multi-subunit protein which associates to give dimeric to octameric polymers. The purification of PPO from

higher plants continues to be a problem (*5*), compounded by the presence of some bound and/or inactive forms of PPOs, whose nature is poorly understood.

Rapid advances were made in understanding the structure and function of PPO when *Neurospora crassa* PPO, a monomeric protein, was purified (*6*). During the past decade, much progress has been made in understanding the nature of the active site and interrelation of the mechanisms of hydroxylation (cresolase activity) and dehydrogenation (catecholase activity), activation and inactivation of the enzyme by reducing compounds, as well as its inhibition by pseudosubstrate-type compounds.

The primary structures of 12 PPO's from plants (tomato, potato, fava bean, grape and apple (Boss, P.K., Gardner, R.C., Janssen, B.-J. and Ross, G.S., unpublished, 1994)), microorganisms (*Neurospora crassa, Streptomyces glaucescens, A. antibioticus* and *Rhizobium meliloti*) and animals (human, mouse and frog) have been determined, largely by cDNA sequencing techniques (*7*). It is expected that several more primary sequences of PPO will be known shortly, because of the major interest in this economically important enzyme. Within closely related organisms, such as tomato and potato there is ~91% exact homology between the PPO's, but between tomato and fava bean PPOs there is only 40% exact homology, for example (*7*). While the overall homology in primary amino acid sequences among the 12 PPO's is limited, there are two regions around the active site that are highly conserved, especially with respect to five of the six histidine residues that ligand the two Cu^{2+} at the active site. This active site sequence has appreciable homology with the O_2-binding site of hemocyanins (*8*).

Nothing is known about the tertiary structures of the PPO's. However, the close resemblance of the PPO active sites with respect to amino acid sequence, the five histidine residues and their coordination to Cu^{2+}, among others, to that of domain 2 of subunit of *Panulirus interruptus* (spring lobster) hemocyanin (*8*) may give clues as to the tertiary structures of the PPO's. Except for mushroom PPO, which is thought to contain four subunits (MW of 128 kDa), all other PPO's studied are probably single polypeptide enzymes of 31 to 63 kDa (*7*).

Polyphenol oxidase is found in many plants (*9*), where PPO is localized in the plastids (*10*). PPO is expressed as a proenzyme, with various sizes of N-terminal signal peptides in different organisms which are removed to give the mature, active enzymes of 40-60 kDa. Despite the continuing hypotheses that plant PPO is an essential component of photosystem I or II, PPO biosynthesis in Irish potato has been largely repressed by expressing mRNA for PPO in an antisense orientation without any detectable disadvantages to the potato plant (*11*), but with potentially major economic benefits to the potato industry.

Chemistry of Enzymatic Browning

Control of enzymatic browning in fruits and vegetables and in juices and wines requires chemical knowledge of the types of phenolic substrates present in a particular plant, the level of reducing compounds, such as ascorbic acid and sulfhydryl compounds, the level of O_2 accessibility, nature of co-oxidizable compounds present and the pathways of polymerization and degradation of the *o*-benzoquinones. It is also essential to understand the level of PPO and substrates available at different stages of plant development. Above all, it is important to distinguish between enzyme-caused browning and non-enzyme-caused browning (the Maillard reaction) in foods.

Some PPO's hydroxylate monophenols to give *o*-dihydroxyphenols, which are then further oxidized enzymatically to *o*-benzoquinones (see Equations 1 and 2). The yellowish *o*-benzoquinones are very reactive and unstable. Further nonenzymatic reactions with O_2 lead to additional reactions to give complex products such as indole-

5,6-quinone from tyrosine for example with further polymerization to melanin and reaction with nucleophiles, such as amino groups of proteins. The o-benzoquinones can react covalently with other phenolic compounds by Michael addition, to give intensely colored products that range from yellow, red, blue, green and black (*12*). o-Benzoquinones also react with aromatic amines and thiol compounds, including those in proteins, to give a great variety of products, including higher molecular weight protein polymers (*13*).

The mechanism of action of *N. crassa* PPO has been extensively investigated and there is a plausible and detailed theory explaining its catalytic activation. (Figure 1; (*14, 15*)). The proposed mechanisms for hydroxylation (Equation 1) and dehydrogenation (Equation 2) reactions with phenols probably occur by separate pathways but are linked by a common *deoxy* PPO intermediate (deoxy in Figure 1).

The proposed mechanism of dehydrogenation, with intermediates, is shown in Figure 1A. O_2 is bound first to the two Cu(I) groups of *deoxy* PPO (deoxy) to give *oxy* PPO in which the bond distance of O_2 bound to the two Cu(II) groups is characteristic of a peroxide (*15*). The two Cu(II) groups of *oxy* PPO then bind to the oxygen atom of the two hydroxyl groups of catechol to form the O_2·catechol·PPO complex.

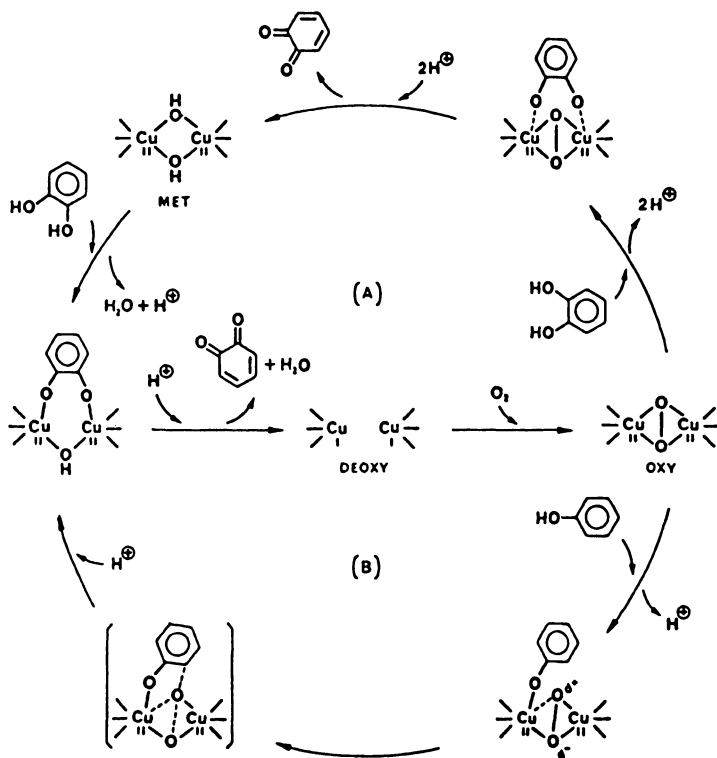

Figure 1. Proposed kinetic scheme depicting the mechanisms of oxidation of o-diphenol (catechol; top (A) and monophenol; bottom (B)) for *Neurospora crassa* polyphenol oxidase. (Adapted from ref. (*14*) and (*15*)).

The catechol is oxidized to *o*-benzoquinone and the enzyme is reduced to *met* PPO. Another molecule of catechol binds to *met* PPO, is oxidized to *o*-benzoquinone and the enzyme reduced to *deoxy*- PPO, completing the cycle.

The mechanism of *o*-hydroxylation of a monophenol by PPO is shown in Figure 1B. *In vitro*, the reaction begins with *met* PPO (at about 11 o'clock on the A portion of the diagram). *Met* PPO must be reduced by a reducing compound BH_2 (Equation 1; catechol is BH_2) if a lag period is to be avoided, to give *deoxy* PPO. *Deoxy* PPO binds O_2 to give *oxy* PPO, the monophenol is bound to one of the Cu(II) groups via the oxygen atom of the hydroxyl group to give the O_2·monophenol·PPO complex. Subsequently, the *o*-position of the monophenol is hydroxylated by an oxygen atom of the O_2 of the O_2·monophenol·PPO complex to give catechol, which then dissociates to give *deoxy* PPO, to complete the cycle. Only the first cycle of hydroxylation of a monophenol requires starting at the *Met* PPO; all subsequent cycles begin with *deoxy* PPO.

Inhibition of Enzymatic Browning

In theory, PPO-catalyzed browning of fruits and vegetables can be prevented by heat inactivation of the enzyme, exclusion or removal of one or both of the substrates (O_2 and phenols), lowering the pH to 2 or more units below the pH optimum, by reaction-inactivation of the enzyme or by adding compounds that inhibit PPO or prevent melanin formation. Hundreds of compounds have been tested as inhibitors of enzymatic browning (*16, 17*).

Exclusion and/or separation of O_2 and phenols from PPO prevents browning of intact tissues; commercial utilization of these methods are being examined by numerous researchers (*18*). Fruits and vegetables have "skins" (waxes, and other surface layers) that exclude O_2 as long as there is no damage to the skins. PPO is physically compartmentalized from phenols in the intact cell. Commericaly, O_2 can be excluded from or reduced in concentration in fruits and vegetables by controlled atmospheric storage, packaging techniques, etc. Phenols can be removed from fruit and vegetable juices by cyclodextrins or by treatment of cut surfaces with O_2-impermeable coatings. PPO activity can be decreased by modifying the pH; the pH optima of most PPO's are near 6, although there are some exceptions.

Reducing compounds, such as ascorbate, sodium bisulfite and thiol compounds, decrease browning by reducing the *o*-benzoquinones back to *o*-dihydroxyphenols or by irreversible inactivation of PPO (*19*). Maltol does not inhibit PPO, but it prevents browning by its ability to conjugate with *o*-benzoquinones, while kojic acid is effective in preventing browning by both reacting with PPO and with *o*-benzoquinones (*20*). Competitive inhibitors, such as benzoic acid and 4-hexyl-resorcinol, are useful in controlling browning in some food products. 4-Hexylresorcinol is a very good inhibitor of enzymatic browning of shrimp, apples and Irish potatoes.

Summary

Enzymatic browning due to PPO in our plant foods is controlled in the food processing industry by use of ascorbate, sodium bisulfate and lowering the pH (addition of citric acid for example). However, chemical control is not fail-safe, not acceptable to some consumers and cannot be used to prevent browning in intact fruits and vegetables. Through better understanding of the mechanism of action of PPO and its essential or nonessential metabolic role(s) in plants, it is expected that genetic engineering techniques will be important in preventing unwanted enzymatic browning. Breeders

have been working to decrease the level of PPO in apples, bananas, mushrooms, peaches and other plants over many years. The genetic engineering approach provides a more precise method of decreasing PPO expression, while retaining the desirable genetic traits of plants. Its utility has already been demonstrated for preventing browning in potatoes (*11*).

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Chapter 2

Enzymatic Browning in Fruits

Its Biochemistry and Control

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Enzymic browning in fruits is usually caused by catecholase (*ortho*-diphenol oxidase) enzymes whilst laccases (*para*-diphenol oxidase) are present in some fruits and many phytopathogenic fungi. The biochemical properties these enzymes is reviewed. Inhibition of catecholase as a means of prevention of enzymic browning is discussed and the properties of different classes of inhibitors reviewed. The possible roles of some of these inhibitors in the food industry is evaluated. Tests with selected substrates and inhibitors have been developed to differentiate catecholases and laccases.

When most, but not all, fruit and plant tissue is bruised, cut or damaged in any way, it rapidly turns brown or even black. This discoloration is an enzyme-catalysed reaction which is usually highly undesirable from the point of view of the food processor or, equally, for the biochemist trying to investigate plant enzymes and/or organelles such as chloroplasts or mitochondria. However in some other situations such as the manufacture of tea, coffee, cocoa or cider this enzymic browning reaction is an essential part of the processing. Enzyme-catalysed browning occurs in unheated plant tissues and must be distinguished from non-enzymic browning resulting from the Maillard reaction that occurs when mixtures of amino acids and carbohydrates are heated together (Figure 1).

This paper will discuss some aspects of the biochemistry of diphenol oxidases as related to enzyme-catalysed browning and its control during the processing of various foodstuffs of plant origin; the emphasis on apples reflects the author's research interest in this fruit. There are useful reviews of food browning as a polyphenol reaction by Mathew and Parpia *(1)*, Mayer *(2)*, Mayer & Harel *(3)*, Walker *(4)*, Vámos-Vigyázó *(5)*, Zawistoski et al. *(6)* and in the book by Macheix et al. *(7)*.

Enzymes Involved in Browning

It is now generally accepted that enzymic browning in plants and fruits is brought about by the action of an enzyme system variously known as catecholase, diphenol oxidase, phenolase, polyphenoloxidase or tyrosinase. The correct biochemical title for this enzyme is O_2:o-diphenol oxidoreductase (E.C. No. 1.10.3.1); however it

will be referred to as catecholase or *o*-diphenol oxidase (*o*-DPO) throughout this review to distinguish it from its close relative laccase, or *p*-diphenol oxidase (*p*-DPO); the reactions of these enzymes are compared in Figure 2. This latter enzyme, which catalyses the oxidation of *p*-dihydroxyphenols, was first discovered in the sap of the Japanese lac tree (*Rhus vernicifera*) and is widely distributed in Basidiomycetes and many phyto-pathogenic fungi. It is not common in fruits although laccase-like enzymes have been reported from peach fruit *(8)*, and more recently we have found it in apricots *(9)*.

Isolation of Diphenol Oxidases. For the biochemist the isolation of active DPO's from plant tissues is fraught with problems because both the enzyme and its substrate are present, but do not react, in the *intact* cell. However, as soon as the cell's internal organization is damaged then enzyme and substrate interact to yield reactive quinones which subsequently react with DPO and other proteins and enzymes present thereby causing their inactivation. It is considered by many workers, including the author, that this *o*-DPO-mediated interaction is a key part of the plant's defense mechanism against disease causing micro-organisms. Thus, during enzyme isolation from plant tissue, it is usually necessary to adopt special precautions to prevent this enzyme inactivation and it is customary to add reducing agents such as ascorbic acid, cysteine or sodium metabisulphite when attempting to isolate DPO's *(10, 11)*. Similarly it is necessary to add DPO inhibitors, such as metabisulfite and polyvinylpyrrolidone (PVP or PVPP) etc. and/or phenolic adsorbents (such as PVPP), when isolating other plant enzymes and organelles *(10)*. Many of these protective reagents have also been used, or suggested for use, to control enzymic browning in foods.

Mode of Action of *o*-Diphenol Oxidase. Early workers investigating the formation of natural brown pigments found enzyme preparations from mammalian tissue which catalysed the oxidation of the amino acid tyrosine to melanin via 3:4-dihydroxy-phenylalanine (DOPA) so naturally this enzyme was called "tyrosinase" a name still commonly mis-applied to all *o*-DPO's. Many, but not all, *o*-DPO's are able to catalyse the insertion of a hydroxyl group at the 3-position ('cresolase' activity) and this reaction may be important in the biosynthesis of phenolic compounds.

Catecholases contain copper as the prosthetic group and for the enzyme to act on its substrate phenols the Cu^{2+} must first be reduced to Cu^+ in which state the enzyme can then bind O_2. The phenolic substrates bind only to the oxy-DPO moiety and, as a result of this binding, hydroxylation of the monophenol or oxidation of the diphenol occurs *(6, 12)*; see Sawistowski et al. *(6)* for detailed reaction mechanisms. It is interesting to note that laccase operates by a different mechanism.

The major expression of *o*-DPO activity in plants is the oxidation of a range of *o*-dihydroxy-phenols to the corresponding *o*-quinones as shown in Figure 2. Note that the first stage of the reactions is reversible and it is for this reason that ascorbic acid and other reducing agents can prevent enzymic browning by reducing the colourless *o*-quinones back to their parent phenols. Unfortunately, once all the ascorbic acid is oxidised, the *o*-quinone is no longer reduced and so it then can undergo oxidative polymerisation to yield brown-black melanin pigments. Nevertheless the addition of ascorbic acid (Vitamin C) is still one of the commonest and safest methods for the commercial control of enzymic browning in fruit juices and similar products. These *o*-quinones are highly reactive compounds and may also couple with amino acids and proteins *(13)* which enhances the final brown

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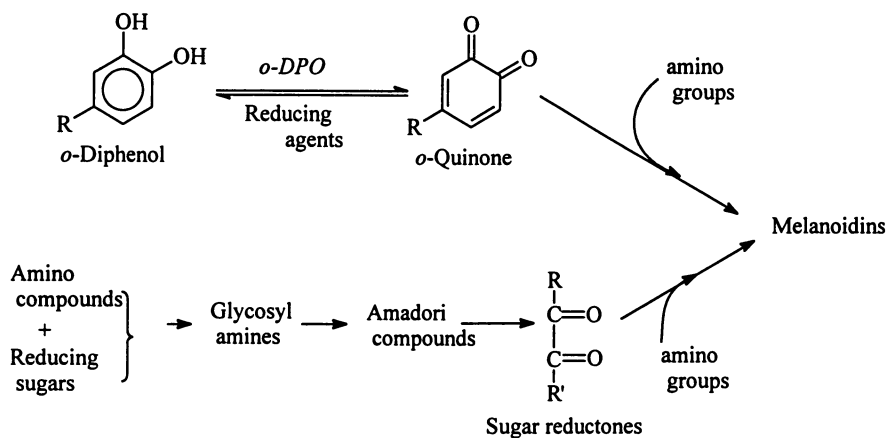
ENZYMIC BROWNING

Figure 1. Comparison of enzymic and non-enzymic (Maillard) browning reactions.
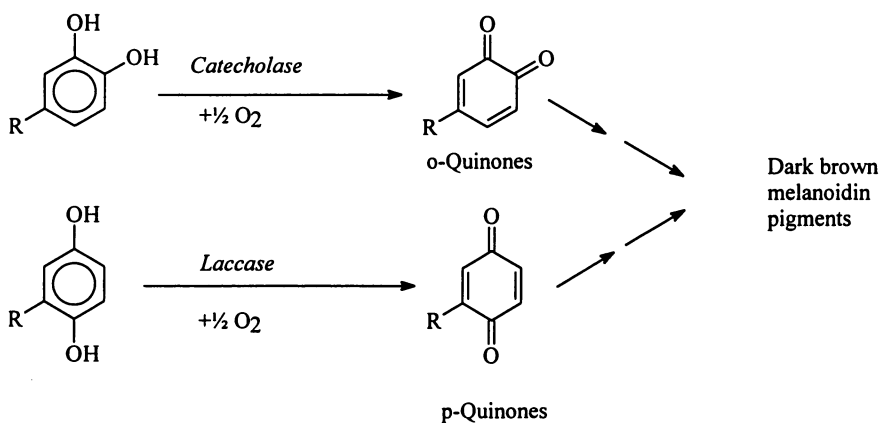

Figure 2. Comparison of reactions catalysed by catecholase (*o*-DPO) and laccase (*p*-DPO).

coloration produced; for example, mushrooms contain large amounts of proline which combines with the enzymically-produced *o*-quinones to form their characteristic chocolate-brown color. Figure 3 summarizes some of the reactions of *o*-DPO and its reaction products; however for more detailed discussion of the mechanism of *o*-DPO action the reader is referred to the reviews by Walker *(14)*, Vámos-Vigyázó *(5)*, Sawistowski et al. *(6)* and Macheix et al. *(7)*.

Laccases. The *p*-DPO's or "laccases" are similar in many respects to *o*-DPO's. They also are Cu-based enzymes which oxidise *o*- and *p*-dihydroxyphenols, and also compounds such as *p*-phenylene diamine and syringaldazine, to colored end-products. They differ in that *p*-DPO's are unable to accomplish the hydroxylation of monophenols.

These enzymes may be differentiated from *o*-DPO's by differences in their substrate specificity and differences in their sensitivity to various enzyme inhibitors *(14)*. In my laboratory we have found the oxidation of guaiacol and syringaldazine to be very useful as a sensitive and specific assay for laccases.

It may be noted in passing that many plants such as squash (*Cucurbita pepo*) also possess another Cu-enzyme, ascorbic acid oxidase, which oxidises ascorbate to dehydroascorbate. It is also necessary to take care to distinguish catecholase from peroxidase activity when examining crude plant extracts.

Assay of Diphenol Oxidases. It is of key importance for any study of the kinetic properties of an enzyme that the assay procedure should measure the *initial* rate of the reaction and for enzymes, such as DPO's, which suffer product inactivation this may pose difficult problems.

The simplest, but least accurate, method of assaying DPO activity is to record the final color yield when the enzyme is incubated with a suitable substrate such as catechol, DOPA, or 4-methylcatechol. Unfortunately this simple procedure is wide open to error since it is measuring the end-product of a sequence of reactions rather than the initial reaction rate. Furthermore, different substrates yield different final colors so that valid kinetic comparisons between substrates are not possible. Nevertheless, despite these limitations, this simple assay technique has proved adequate for useful comparative studies of the browning levels of different fruit varieties *(15)* and similar problems. In order to try to overcome the above limitations several workers developed a "chronometric" assay which involved measuring the rate of loss of ascorbate in an *o*-DPO/phenolic substrate/ascorbate coupled system but this is a cumbersome method.

Nowadays the commonest method of DPO assay is by measurement of the rate of O_2-uptake by means of an O_2-electrode. The relative merits of these methods has been critically assessed by Mayer et al. *(16)* and it is now generally agreed that the polarographic O_2-electrode is the method of choice since it has rapid response and may be coupled to a potentiometric recorder for the immediate display of results. This method is routinely used by the author in his studies of apple *o*-DPO.

Substrate Specificity of *o*-Diphenol Oxidases. All *o*-DPO's require the basic *o*-dihydroxyphenol structure for oxidase activity so that catechol is the simplest possible, but not necessarily the best, substrate; 4-methyl catechol is usually the fastest! The structures of some natural and artificial diphenol oxidase substrates are shown in Figure 4. However the nature of, and position of, any substituent groups has profound effects on the rate of substrate oxidation and studies *(17, 18)* of these problems can help shed light on the nature of the interaction between the substrate and active-site for *o*-DPO's.
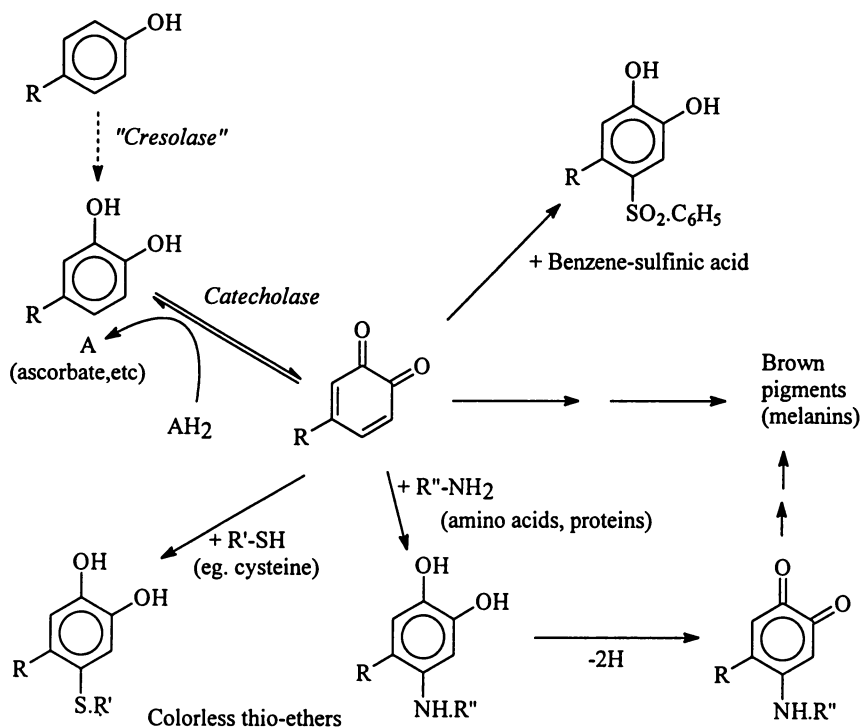
Figure 3. Reactions catalysed by catecholase (*o*-DPO).

Catechol   4-Methyl catechol   DOPA   Quinol   Toluquinol
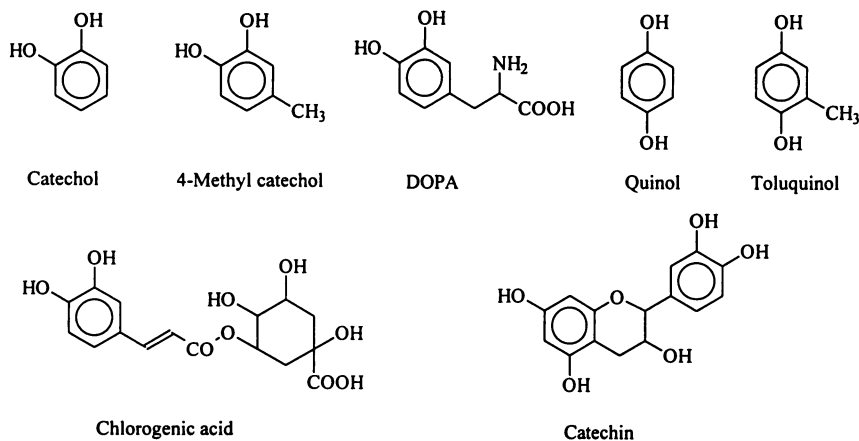
Chlorogenic acid   Catechin

Figure 4. Common substrates for catecholases and laccases.

Considering first the hydroxylation reaction; the commonest natural substrates are probably tyrosine and *p*-coumaric acid or their derivatives. Tyrosine is hydroxylated to yield DOPA as mentioned previously whilst several workers have demonstrated that *o*-DPO preparations from a number of plants hydroxylated *p*-coumaric acid to caffeic acid or *p*-coumarylquinic acid to yield chlorogenic (caffeoylquinic) acid. Thus it seems very probable that, *in vivo*, *o*-DPO's play an important role in the biosynthesis of plant phenolics *(19)*.

However, with respect to enzymic-browning it is the so-called "catecholase" activity that is important and the commonest natural substrate for this reaction is chlorogenic acid which is widely distributed in many plants; see reviews by Vámos-Vigyázó *(5)* and Macheix et al. *(7)*. Probably the next commonest substrates are catechin, epicatechin and DOPA which are also of widespread occurrence.

Many workers have investigated the substrate specificity of the *o*-DPO's from different plant sources. In the majority of cases chlorogenic acid, caffeic acid, catechin, 3:4-dihydroxyphenylpropionic (hydrocaffeic) acid and 4-methylcatechol were all readily oxidised but the rate of oxidation was much reduced with 3:4-dihydroxyphenylacetic acid and 3:4-dihydroxybenzoic (protocatechuic) acid whilst the isomeric 2:3-dihydroxybenzoic acid was not oxidised by *o*-DPO's from apples or other fruits. These observations help support the view that the nature of the side-chain is critical and therefore may play a part in the enzyme/substrate interaction.

It should also be noted that substrates such as DOPA were oxidised relatively slowly (40-60% of rate for chlorogenic acid) yet their final color yield was greater because of the darker nature of their polymeric end-product. Thus any investigations of substrate kinetics/specificity based on color yields would be highly suspect!

The common flavonoids are usually poor substrates for *o*-DPO but they may be oxidised by DPOs via coupled reactions *(20)*. For example anthocyanins and procyanidins are poor substrates for *o*-DPO but undergo significant breakdown in the presence of favorable substrates such as chlorogenic acid. These reactions could lead to serious losses of pigment in some fruits and have been demonstrated in sweet cherries *(21)*, grapes *(22)* and strawberries *(23)* egg plant *(24)*.

Naturally Occurring Substrates for Diphenol Oxidases. The distribution of phenolic compounds in fruits has been reviewed by Van Buren *(25)*, Vámos-Vigyázó *(5)* and Macheix et al. *(7)* and, as stated previously, the commonest natural substrates for *o*-DPO catalysed enzymic browning are chlorogenic acid and its isomers, plus catechin and epicatechin (Figure 3). However some fruits *o*-DPO's use other phenolic substrates; for example a relative of DOPA, 3:4-dihydroxyphenylethylamine (dopamine), is the major substrate in bananas and DOPA is the natural substrate in the leaves of broad beans (*Vicia faba* L.). Grape catecholase acts on *p*-coumaryl and caffeoyl-tartaric (caftaric) acids whilst dates contain an unusual combination of diphenol oxidase substrates including a range of caffeoyl-shikimic (dactyliferic) acids; these are analogous to the ubiquitous isomers of chlorogenic acid.

Inhibitors of Diphenol Oxidase Activity

Many reagents inhibit DPO activity and studies with them have provided valuable insight into the mode of action of this enzyme whilst other inhibitors may be used to differentiate *o*-DPO's from *p*-DPO's. However only a limited number of DPO inhibitors are considered acceptable on grounds of safety and/or expense for use to control enzymic browning during food processing and this is a fertile field for continuing research throughout the world.

Inhibitors of DPO may be grouped according to their mode of action (Table I) although some compounds may belong to more than one group. Inhibition may be caused by chelation of the prosthetic group, competition for the substrate, or by interaction with the products of reaction. Both o- and p-DPO's utilise Cu as a tightly bound prosthetic group so that the first category includes many Cu-chelating agents which will inhibit these enzymes. Thus carbon-monoxide, cyanide, Na diethyldithiocarbamate (DIECA), azide, tropolone (26), methimazole (27), mercaptobenzothiazole (28) and PVP (29) are all potent inhibitors of DPO's and are frequently used for this purpose by biochemists when endeavouring to isolate other plant enzymes and organelles.

Inhibition by Reducing Agents and Thiols. Probably the commonest method of controlling enzymic browning, both in industry and the laboratory, is by the addition of reducing agents such as SO_2, metabisulfite and/or ascorbic acid. These compounds prevent browning by reducing the enzymically formed quinones back to their parent o-diphenols; they are therefore consumed in the process (Figure 2). However although metabisulfite can act as a reducing agent it also can react with the quinone intermediates to form sulfoquinones and may irreversibly inhibit o-DPOs (6). Furthermore the first two compounds may give rise to off-flavors or corrosion problems if used to excess and recently have been implicated as the cause of some forms of asthma (30, 31). Nevertheless, these and/or ascorbate, are probably still the commonest anti-browning agents used in food processing operations.

By contrast -SH compounds can combine chemically with the o-quinones to form a stable, colourless product thus permanently preventing further oxidation to brown pigments. However, these thiols can only combine with o-quinones when the molar ratio exceeds a critical value (usually 1.5:1) but, provided this critical level is exceeded, then they should, in theory, provide permanent protection against enzymic browning (17). Experiments by Walker and Reddish (32) and later workers have confirmed this idea and the former showed that cysteine could be used to prevent the browning of apple products for over 24hr without introducing undesirable off-flavors.

It has become apparent that in any study of the control of enzymic browning it necessary to distinguish clearly between the actual inhibition of the DPO enzyme and the prevention of enzymic browning since the latter may be achieved without the former (eg, as with ascorbate or cysteine, etc.). Recent notable papers by Richard et al. (33) and Kermasha et al. (34) have drawn attention to apparent anomalies in results when various compounds have been assessed for their potential to prevent browning (by colorimetric assay) and their ability to inhibit DPO activity (by polarographic assay). Their results show that the degree and type of inhibition observed was markedly dependent upon the assay method used. Cysteine and other thiols appeared to control browning by formation of a colorless thiol-quinone adduct thus preventing the oxidative polymerisation of the reactive quinones without actually inhibiting the initial O_2-uptake reaction. By contrast cinnamic acids inhibited the initial O_2-uptake reaction. In the light of the above reports these authors suggest that only the polarographic method, which measures substrate utilization and is thus artifact-free, is the only reliable and valid method for the measurement of DPO activity.

Polymeric Diphenol Oxidase Inhibitors. During the past decade various forms of polyvinylpyrrolidone (PVP and PVPP) have been much used in the extraction of plant enzymes (see reviews by Loomis and Bataille (9), Anderson

(*11*)) because of their ability to inhibit enzymic browning and concomitant enzyme inactivation. Two processes seem to be involved, firstly with soluble PVP (MW 10-60 KDa) which acts as a competitive inhibitor of *o*-DPO (29) but may also form complexes with low MW phenolics; however the exact mechanism is still unclear. Secondly with PVPP (Poly-Clar AT), an insoluble high MW grade of PVP, which is also much used for the prevention of hazes in beer, but in this case browning is probably prevented because phenolic compounds are very firmly bound to Poly-Clar AT and are thus removed from the reacting system.

Polypeptide Catecholase Inhibitors. There have been two reports of the inhibition of diphenol oxidase activity by low MW polypeptides. The first of these was by Harel et al. *(35)* who reported that the fungus *Dactylium dendroides* secreted an inhibitor of galactose oxidase and that this small peptide also inhibited apple catecholase activity. Oszmianski & Lee *(36)* found a polypeptide non-competitive DPO inhibitor in honey. More recently *Lactobacillus helveticus* has been found to secrete a cyclotetrapeptide that inhibited *o*-DPO activity *(37)*. Tan & Kubo *(38)* reported that the roots of *Zea mays* secreted a 6.3KDa protein which inhibited catechin oxidase activity. The safety and utility of such polypeptides to control enzymic browning in foods has yet to be evaluated.

Inhibition of *o*-DPOs by Substrate Analogues. Several years ago the author noticed that apples that had been attacked by the blue-mold fungus (*Penicillum expansum*) did not display the brown, dead tissue characteristic of most diseased fruit. Investigation of this problem *(39, 40)* eventually revealed that the juice from the soft, rotted tissue contained *p*-coumaric, caffeic and ferulic acids together with patulin which is a toxic mold metabolite. Furthermore, the juice from the affected tissue inhibited enzymic browning and this prompted subsequent more detailed studies *(41)* of the inhibitory effects of a wide variety of phenolic acids upon apple *o*-DPO. It was hoped that these experiments could shed light on the mechanism of enzyme action and enzyme inhibition. Various cinnamic acids (Figure 5) were found to be powerful inhibitors of apple *o*-DPO and inhibitory action decreased in the order cinnamic acid > *p*-coumaric acid > ferulic acid > *m*-coumaric acid > *o*-coumaric acid >> benzoic acids. It was also significant that sinapic (3:5-dimethoxy-4-hydroxycinnamic) and hydro-cinnamic (phenylpropionic) acid together with the lower homologues of *p*-coumaric acid were all without inhibitory action; it therefore seemed that the unsaturated side-chain of the cinnamic acid derivatives was essential for inhibition of *o*-DPO action. Work by Pifferi et al. *(42)* and Janovitz-Klapp et al. *(18)* has shown that unsaturated dicarboxylic acids with at least two conjugated double bonds, such as sorbic acid, were good *o*-DPO inhibitors. More recently we have shown that oxalic acid secreted by fungal phytopathogens enhanced their pathogenicity by inhibiting the plant's *o*-DPO defense mechanism *(43)*.

  In the light of the earlier observations it is of interest to note that Finkle and Nelson *(44)* suggested the use of an *O*-methyltransferase preparation to methylate, and thus block, the natural substrates of various fruit *o*-DPO's and thereby inhibit browning. They subsequently demonstrated that this enzyme converted caffeic acid to ferulic acid and chlorogenic acid to feruloylquinic acid. By this process, the fruit's *o*-DPO was deprived of its substrate(s) which were converted to enzyme inhibitors; the only snag is that the methylating enzyme and one of its substrates (*S*-adenosyl-methionine) are prohibitively expensive!

Table I. Diphenol Oxidase Inhibitors

Group I. Reducing agents
    Ascorbic acid, SO_2, Na metabisulphite,
    Thioglycollate, 2-Mercaptobenzothiazole, Mercaptoethanol
Group II. Cu-chelating agents
    DIECA, Tropolone, Methimazole, Carbon monoxide, Cyanide
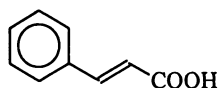*Group III. Quinone couplers*
    Cysteine, Glutathione, Penicillamine, Benzenesulphinic acid
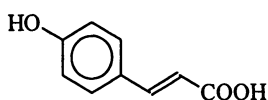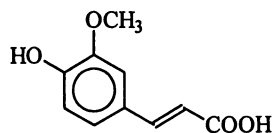*Group IV. Substrate analogues*
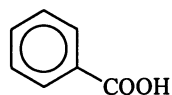   Cinnamic acid, *p*-Coumaric acid, Ferulic acid
Group V. Miscellaneous
    PVP, 4-Hexyl-resorcinol, SHAM.
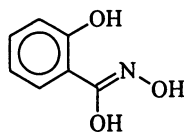

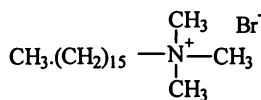Cinnamic acid   *p*-Coumaric acid   Ferulic acid

Benzoic acid   SHAM   CTAB

Figure 5. Inhibitors of catecholase and laccase activity.

Research in my laboratory found that salicylhydroxamic acid (SHAM) was a potent and highly selective inhibitor of catecholase but, as may be seen in Figure 6, appeared to be without effect on laccase *(45)*. Concentrations as low as 10µM were inhibitory so, on a molar basis, it was about 100 times more active than cinnamic acid. Rich *et al (46)* suggest that substituted hydroxamic acids act by competing with the substrate at the binding site but the exact mechanism of action still remains unclear.

Since 1991 much interest has focused on the use of 4-hexyl-resorcinol as a safe and effective inhibitor of enzymic browning *(31, 47)* but this topic is discussed by other contributors to this symposium.

Selective Inhibition of *o*- and *p*-DPOs. A major part of my research has been concerned with devising methods to distinguish *o*- and *p*-DPOs in diseased plant tissues since many fungal pathogens secrete *p*-DPOs (laccase). This can be achieved by comparison of substrate specificity and the use of selective inhibitors as summarized in Table II below; thus syringaldiazine is a particularly useful laccase-specific substrate for colorimetric assays whilst 4-methyl-catechol (1,2-dihydroxy-toluene) and toluquinol (1,4-dihydroxy-toluene) are appropriate substrates for quantitative O_2-electrode work.

Because of differences in the reaction mechanisms and the oxidation levels of the Cu in the active site catecholase and laccase differ in their responses to certain inhibitors (Table II). For example catecholases are inhibited by cinnamic acids and SHAM which are without effect on laccases *(41, 45)*. By contrast, laccases are selectively inhibited by quaternary ammonium compounds such as CTAB *(14)*; the reasons for this has still to be investigated fully but it could be related to the high level of protein glycosylation reported for most laccases. A major obstacle in this work is the difficulty of obtaining highly purified catecholase preparations.

Current Research at the University of Canterbury

Some recent work from the author's laboratory is reviewed below.

Enzymic Browning in Apricots. Most of the work described above was investigated using apple DPO although it is of general applicability. In our research with apricots *(9)* we used tests with specific substrates and inhibitors and found that apricots were unusual in that they contained both types of DPO activity (catecholase and laccase). Both enzymes acted on the naturally present substrates chlorogenic acid and catechin and both appeared to be bound to cell membranes.

From the commercial point of view we found the best way to prevent browning during the drying of apricots was by means of a pre-drying dip in 1% Na metabisulphite which left virtually undetectable residual SO_2 levels. By contrast use of cinnamic acid or ascorbic acid had little effect; the latter actually enhanced browning which leads us to suspect that non-enzymic, Maillard type browning is the main cause of darkening in commercial dried apricots *(48)*.

Phenolases and our Daily Bread. Although this review is concerned primarily with fruits our work on wheat phenolics may also be of interest. In this research it was found that both *o*-DPO activity and phenolic content varied widely between different red and white wheat cultivars and between different milling flour streams. In all cases these correlated significantly with flour color and bread crumb color. Thus there is a clear involvement of phenolics in the final color, and therefore customer acceptance, of bread and other wheaten products *(49)*. These phenolic compounds are also involved in the important problem of dormancy in wheat.
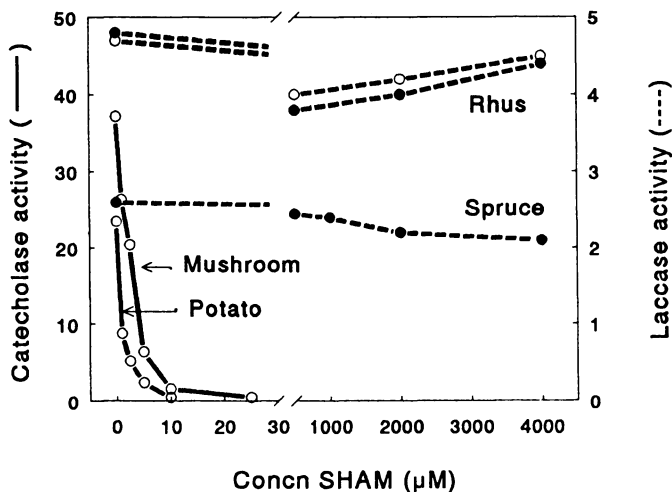
Figure 6. Selective inhibition of diphenol oxidases by SHAM. Catecholase (—
—), laccase (- - -). Substrates; open circles (O) = 4-methyl catechol, full
symbols (●) = toluquinol. (Adapted from ref. 45).

Table II. Differential Tests for Catecholase and Laccases

Test	Catecholase (o-DPO)	Laccase (p-DPO)
Substrate specificity:		
o-Dihydroxyphenols	oxidised	-
p-Dihydroxyphenols	nil or slow	oxidised
p-Cresol	oxidised (orange-red)	
Guiacol	-	oxidised
1-Naphthol	-	oxidised (purple)
p-Phenylene-diamine	-	oxidised
Syringaldazine	-	oxidised
Inhibitor specificity:		
Cinnamic, p-coumaric and ferulic acids	inhibition	nil
PVP	inhibition	nil
SHAM	inhibition	nil
4-Hexyl-resorcinol	inhibition	nil
CTAB (and other QACs)	nil	inhibition

Practical Control of Enzymic Browning in Food Processing

During the preparation of many fruits and vegetables for canning or other processing operations the prevention of enzyme-catalysed browning is a major problem and it is unfortunate that the majority of the diphenol oxidase inhibitors discussed in the previous sections are not safe nor suitable for use in foodstuffs. In practice food processors faced with this problem usually rely on an early heat-treatment stage to inactivate DPO's and other enzymes but some *o*-DPO's are relatively heat stable, for example, apple *o*-DPO typically possesses a half-life of about 12 min at 70 °C and Macheix et al. *(7)* cite a number of other examples. Nevertheless it is essential to take positive action to prevent oxidative browning until the DPO's are denatured. Adams and Blundstone *(50)* have provided a useful summary of the browning problems associated with fruit-canning.

The inhibitors of enzymic browning most frequently used in industry include acid or brine dips, ascorbic acid and SO_2, either as the gas or sodium metabisulphite. Ascorbic acid and its derivatives, either alone or with other additions such as citric acid etc., are widely used to prevent the oxidative browning of fruit juices prior to pasteurization and they may be also be added to acid dips used for the pretreatment of peeled or sliced fruit. Likewise SO_2, or metabisulphite, may be used to control the browning of sliced fruit and vegetables before drying, as for example in the manufacture of apple rings. However excess SO_2 may have undesirable effects such as the generation of off-flavors and is also a cause of concern with respect to some types of asthma *(30)*.

During the past two decades a number of novel procedures that avoid the addition of compounds such as SO_2 have been suggested. As mentioned earlier Finkle and Nelson *(44)* suggested the use of an enzyme *O*-methyltransferase to covert the *o*-dihydroxyphenolic *o*-DPO substrates to the corresponding methoxy-derivatives but this process does not appear to be economic for large scale use.

An alternative approach to the control of enzymic browning was proposed by Kelly and Finkle *(51)* who treated apple juice with protocatechuate 3:4-dixoygenase (PC-ase), a bacterial enzyme which catalyses the *ortho*-fission of aromatic rings. Unfortunately this would be a costly process and the authors admitted that chlorogenic acid was a poor substrate for this enzyme. It would be interesting to repeat their experiments with a *meta*-fission enzyme from bacteria since the *meta*-fission oxygenases generally show less specificity with respect to the side-chain of the aromatic ring. The commercial use of such a system would probably require the use of immobilized enzyme technology.

Subsequently the author *(52)* published a novel method for the prevention of enzymic browning in apple juice based on earlier fundamental studies of the inhibition of apple *o*-DPO by cinnamic acids. Different amounts of cinnamic, *p*-coumaric or ferulic acids were added to freshly prepared opalescent apple juice and the mixtures aerated to promote browning. As will be seen from Figure 7 additions of these acids, especially cinnamic acid, provided effective long-term control of browning. The exact quantity of inhibitor required will depend upon the level of phenolics of the particular fruit or variety but the minimum control level is readily estimated; typically less than 0.01% (=10ppm) of cinnamic acid or its more soluble sodium salt was enough to prevent the browning of the juice from Granny Smith apples (a high browning variety).

The author has not investigated the question of possible toxicity of these cinnamic acids but it is well known that they are widely distributed in nature; either free or as esters in essential oils (e.g. oil of cinnamon, etc.). It would therefore seem unlikely that there would be any health hazards associated with the use of cinnamic acids to control enzymic browning.
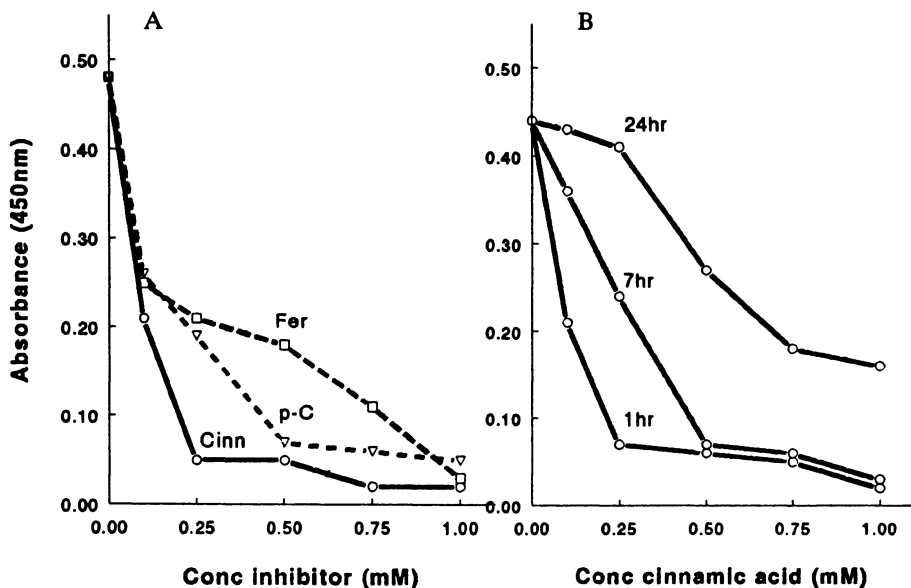
Figure 7. Control of enzymic browning of apple juice by cinnamic acids. Graph A shows effect of concentration (Cinn = cinnamic acid, p-C = *p*-coumaric acid, Fer = ferulic acid), graph B shows effect of concentration and holding time. (Adapted from ref. 52).

Beneficial Forms of Enzymic Browning

So far browning has been presented as a problem for the food processor but not all cases of enzyme-catalysed browning are undesirable. In certain instances such as the manufacture of tea, coffee, or cocoa, these reactions are essential to the manufacturing processes but only brief descriptions of these can be included in this review.

Probably the best studied example of beneficial browning is that concerned with the biochemical changes that take place during the manufacture of black tea *(6)*. These have been investigated in detail by a number of workers *(53, 54)* who have isolated and studied tea-leaf *o*-DPO and shown that it plays an important role in the "fermentation" stage of tea manufacture. Studies with tea-leaf *o*-DPO showed that its major natural substrates were catechin, epicatechin and epicatechin gallate and their *o*-quinone oxidation products were precursors of the more complex theaflavins and thearubigins *(20)*.

The beverage quality of coffee has been shown to be related to the level of *o*-DPO of the green coffee beans whilst *o*-DPO's also play a part in the development of the final color of processed cacao beans which contain large amounts of phenolic constituents such as epicatechin. Powerful *o*-DPO's were found in fresh cacao beans and their pericarp.

Enzymic browning is responsible in whole, or in part, for the characteristic brown color of certain dried fruits such as dates, prunes and sultanas. During the manufacture of cider and perry (fermented pear juice) and white wine the action of

o-DPO's upon the naturally occurring phenolics and tannins leads to their subsequent condensation and polymerisation; reactions which may have an important role in aiding subsequent clarification processes as well as removing unwanted astringency.

In conclusion it is still appropriate to quote from the earlier review by Mathew and Parpia (*1*) who concluded that "That the greatest challenge for food technologists is to evolve more economical and easier methods of preventing undesirable browning during the commercial handling and processing of food".

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Chapter 3

Phenolic Browning: A Perspective from Grape and Wine Research

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Previous oxidation studies with wine phenols, both enzymic and nonenzymic, are briefly reviewed and placed in context. With three grape-derived polyphenoloxidase preparations and commercial mushroom tyrosinase, oxygen uptake is studied for various substrates with and without glutathione or other sulfhydryls present. Ascorbic acid, but not glutathione, acts as a direct reductant for the PPO-generated *ortho*-quinones. These quinones react with nucleophiles such as glutathione and phloroglucinol to produce adducts with regenerated hydroquinone structure and lowered oxidation-reduction potential compared to the original phenol. The ones tested are not direct substrates for PPO, but can reduce the parent quinones becoming oxidized by such coupling and thereby regenerating parent hydroquinones for substrate. The quinones inactivate the enzyme. Other proteins either added or present in impure enzyme lessen this PPO inactivation. Although glutathione in high proportion prevents browning, it stimulates oxygen uptake in the reaction with caffeic acid, reaching the theoretical maximum of 0.5 O_2 per caffeic acid equivalent. With coumaric acid and grape PPO, glutathione depresses oxygen uptake. With gallic acid, glutathione decreases the total oxygen uptake and essentially eliminates production of CO_2. With catechin or caffeic acid plus phloroglucinol, high levels of glutathione lower total O_2 uptake to about 0.5 O_2/unit of substrate phenol. The significance and interpretation of the results are briefly discussed.

Oxygen uptake and browning reactions by natural phenols have great practical importance in grapes and wines. Raisin production involves thorough browning for the sun-dried and complete prevention for golden or shade dried green products.

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Browning is a major cause of rejection of fresh table grapes, if it occurs before the grapes reach the customer, and is additionally expensive if storage costs have been incurred. In converting grapes to white wines for table use or as base wines for sparkling wines, oxidative browning has been minimized as undesirable or encouraged by "hyperoxidation" to remove the phenolic substrates and prevent later browning. With certain wines, sherries for example, their character depends on considerable post-fermentation oxidation and browning. In red wines, oxidative reactions may contribute to color loss and yet a shift toward "brick" red and limited oxidation are important aspects of maturation and aging desirable in older wines. Any browning and the associated flavor changes are undesirable in the freshest "picnic" style wines, red or white. Clearly the desirability of oxidation and browning is variable, depending upon the situation, but is critical during processing and delivery worldwide of the largest fruit crop.

Owing to this importance, it is not surprising that the polyphenol oxidase (PPO) from grapes has been one of the most studied from higher plants (*1, 2, 3, 4, 5*). It is apparently quite typical of PPO's in general including mushroom tyrosinase (*6, 7*), and therefore findings with grape PPO have broad applicability. Although enzymes from different sources can have somewhat different specific reactivities, natural vicinal dihydroxy phenols such as caffeic acid are readily oxidized by the "catecholase" action and analogous monophenols such as *p*-coumaric acid produce the same products via the "cresolase" action of the PPO. Grape PPO is particularly active with caffeic and coumaric acids (*1*). In this paper we propose to correlate previous research, particularly that from out laboratory, and present new studies to clarify oxygen uptake catalyzed by PPO acting on phenols typical of grapes or wines. The reaction rather than the enzyme's characteristics will be emphasized by using high levels of the PPO preparation to approach completeness in relatively short times.

Materials and Methods

A total of four enzyme preparations were used in the new studies: two acetone powders prepared from juice from Grenache or Clairette blanche grapes (*8*), an enriched one from Thompson Seedless grapes, and purchased mushroom tyrosinase (Sigma Chemical Co., St. Louis, MO). The two acetone powders behaved very similarly and, to simplify, only the results from the Grenache PPO are reported. The acetone powders contained insolubles, but with care for uniform suspension, reproducible results were obtained. The Thompson Seedless PPO was obtained from Dr. D. O. Adams' group and had been solubilized by Triton X100, precipitated with ammonium sulfate, and retained during dialysis. Substrates and other chemicals were purchased from Sigma, Aldrich Chemical Co. (Milwaukee, WI), or Fisher Scientific Co. (Fair Lawn, NJ).

All solutions were prepared in 0.03 M potassium phosphate buffer adjusted to pH 5.0. Oxygen uptake was measured in a Warburg-type (Gilson Medical Electronics, Middletown, WI) differential respirometer with the bath temperature

maintained at 22.0 °C, shaking rate 140 cycles/min., and air as the oxygen source. To check for CO_2 generation, 0.2 mL of 5% KOH was placed with a filter paper wick in a center well in the reaction flasks. Ordinarily, substrates were prepared at about 5 micromoles per mL, 1-20x the typical level for grape juice. Glutathione, cysteine, and dithiothreitol were used at about 6x the molar content of the phenol, and enzymes at a level equivalent to 4-20 g of fruit per mL or 2000u per mL for the tyrosinase. Corrections of the mm^3 of oxygen taken up were based on temperature at the manometers, and most results were calculated as micromoles of O_2 taken up per micromole of phenol.

Results and Discussions

Nonenzymic Oxidation. In the course of fermentation and early clarification of wines PPO is greatly decreased. Remaining active polyphenol oxidase (PPO), whether natural or added, survives at most a few weeks even in wines stored in cool cellars and not treated with sulfur dioxide (*9*). If wine free of PPO is saturated with oxygen from air, about 6 mL/L at 20 °C, this oxygen is consumed within about a week or less. This oxygen uptake can be repeated several to many times before the wine's capacity is reached. The capacity of a wine to consume oxygen is large and roughly proportional to its phenolic content (*10*). At wine pH, about 3.4, oxygen uptake to completion is too slow and risky of microbial contamination to follow satisfactorily, but represents several saturations even for the lightest wines. If, on the other hand, a sample is made alkaline, the reaction is fast and at pH 9+, near or above the phenolic pK, is complete in about 30 min. This has been made the basis of an assay of a wine's oxygen capacity (*10, 11*). Measured in this way the capacity of a wine that has been allowed to consume oxygen under its normal acidic condition is decreased, but not proportionally to the oxygen already consumed (*11, 12*). For example, a white wine that had an alkaline oxygen capacity of 89 mL/L before exposure to consumption of 63 mL/L under normal acidity, afterward still had 76 mL/L of alkaline capacity. Study of these phenomena indicated that the reaction we have named regenerative polymerization (quinone + adduct ⇒ hydroquinone-adduct) followed by reoxidation (hydroquinone-adduct + O_2 ⇒ quinone-adduct) accounts for the relatively modest loss of alkaline oxidation capacity in proportion to oxygen uptake while acidic.

When oxidizable phenols are rapidly all converted to quinones or other oxidation products as under alkaline conditions, regenerative dimerization is limited. During the slow oxygen uptake under acidic conditions, additional polymerization can regenerate a considerable portion of oxidizable substrates and even incorporate previously inoxidizable phenols into dimers and polymers both more readily oxidized and contributing increased capacity for oxygen (*12*). The course of oxidation under acidic wine conditions is undoubtedly different from that in the same wine under alkaline conditions not only for reasons of relative speed allowing less regenerative polymerization under alkaline conditions. Electrophilic substitution by a quinone onto a nucleophilic phenol would certainly be affected by whether or not the phenol

or the phenolate ion was present. Other nucleophiles such as amines would also be affected, but differently, by pH.

Direct autoxidation differs from enzymatic oxidation and alkaline from acidic oxidation in another important aspect. As an o-dihydroxy phenol reacts with O_2 to produce its quinone, only one atom of oxygen is needed and the second appears as hydrogen peroxide (13). Under acidic conditions, this hydrogen peroxide oxidizes additional material, including ethanol in wines, which would not otherwise readily autoxidize. Under alkaline conditions, hydrogen peroxide behaves as an acid and accumulates as its salt (14). Under PPO oxidation, hydrogen peroxide is not produced and only half as much oxygen should be consumed per quinone produced.

Study of the reaction of gallic acid (14) and of caffeic acid (15, 16) under alkaline conditions has shed light on autoxidation processes and illustrated products to be considered in regard to PPO oxidation. Nonenzymic oxidation appears to be proportional to the phenolate content at the pH in question (15). The reaction between a phenolate ion and O_2 is not a spin-forbidden process, whereas most other possible direct O_2 reactions are (12, 17). The type of products (but not necessarily their relative proportions) appeared the same down to pH 4, the lowest pH that could be followed in a reasonable time (15). First order rate constants were determined and the Arrhenius activation energy was 49.0 ± 6.4 kJmol^{-1} for caffeic acid oxidation at pH 8.0. Several of the same products observed in model system oxidation were found in white wine treated with esterase to hydrolyze caftaric acid to caffeic acid and then oxidized at pH 7 (18). Thus, the alcohol and other components of the wine did not overturn conclusions from model system study. The importance of phenolate content even though very low at wine pH was again shown and differences in phenolate content with the normal wine pH range were invoked to explain why wines from hot vineyards or picked late (high pH) over-oxidize readily and do not age well (18).

Wine Browning and Oxidation. There was a highly significant correlation between the brown color produced in a white wine or a model system and the caffeic acid consumed during oxidation under any one set of conditions (18). In developing a test for a white wine's maximum relative capacity to brown it was shown that all the browning was due to oxidation (19). Heavy treatment with bentonite to remove proteins was used to prevent precipitation of the brown pigments. Below 52 °C, sugar did not participate in the browning. Additions of amino acids did not, but catechin did enhance the maximum capacity to brown in tested wines. Applying this test to wines prepared from white grapes with increasing pomace extraction during fermentation showed that increased flavonoid content from such extraction (largely flavan-3-ols, catechins, and derivatives) increased the browning capacity (20, 21). Figure 1 shows browning capacity is directly correlated with flavonoid content. The results cover wines from six varieties, two harvest of one, and show only the extreme cases. The other five examples gave nearly parallel lines between these extremes.
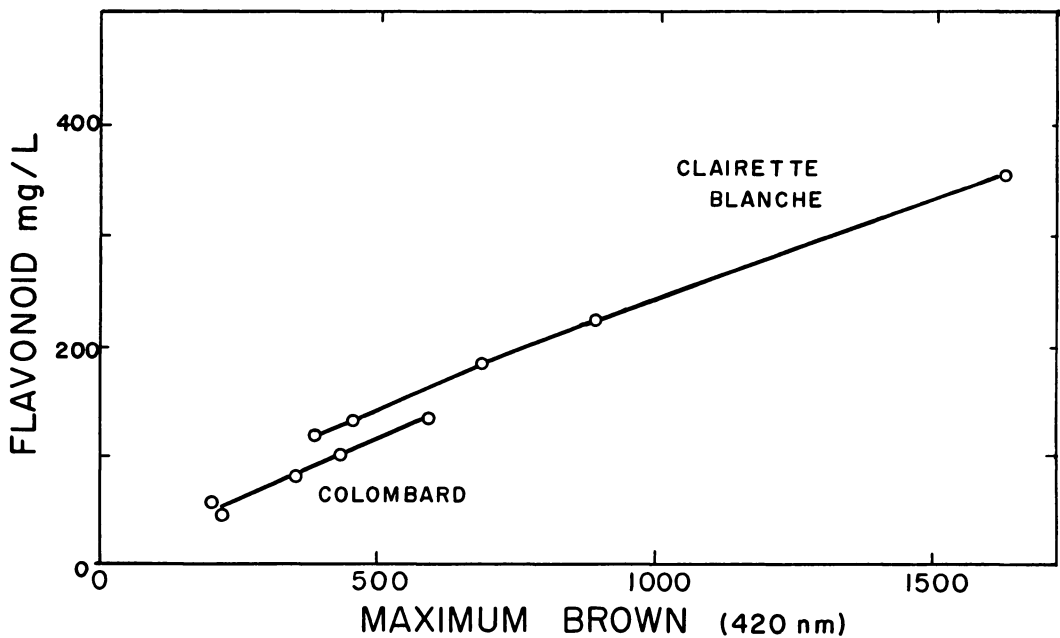
Figure 1.   Nonenzymic maximum browning capacity of white wines in proportion to their flavonoid content, which is in turn proportional to the pomace extraction during their fermentation over 0-5 days.   These two grape varieties were the extremes, others fell between and nearly parallel to the curves shown (*21*).

During autoxidation of caffeic acid, addition of thiols including cysteine and glutathione delayed the consumption of the caffeic acid (22) and, as long as sufficient thiol was present, only golden colors resulted. As soon as the thiol was depleted, amber to muddy brown developed. In the presence of excess cysteine, 2-, 5-, and 2,5-cysteinyl thio-ethers of caffeic acid were produced.

Regenerative Polymerization Verified and Specific Products Identified. Oxygen consumption rose from 3.4 atoms per mole for caffeic acid alone to 5.5 with cysteine and 8.5 with glutathione (22). Neither thiol took up O_2 alone under the test conditions. Similarly, phloroglucinol alone took up no oxygen, but raised caffeic acid's consumption to 4.9 atoms of O_2 per mole. These actions are considered verification of regenerative polymerization (12). In the process of addition to caffeic quinone, the thio-ether or phloroglucinol adduct reverts the caffeic moiety to the hydroquinone form. This new hydroquinone is available for reoxidation and contribution to the total of oxygen consumed. Furthermore, owing to the electron-supplying nature of nucleophiles, the new hydroquinone would have a lower oxidation-reduction potential, be oxidized preferentially, and therefore spare caffeic acid (or other progenitor diphenol).

The products of alkaline oxidation of gallic acid have been formulated as shown in Figure 2 (14). According to kinetic numerical analysis most of the gallic acid is oxidized and dimerized to the hexahydroxydiphenic acid precursor of ellagic acid. It and the portion of gallic acid oxidized without dimerizing are further oxidized to the ring-opened acids shown. Thus, the 4.9 atoms of oxygen consumed lie between the 4 atoms/mole of original gallic acid required to produce the open-ring monomer plus a mole of hydrogen peroxide and the 5 per gallic acid equivalent to produce the open-ring dimer. That this type of ring opening is possible was verified by alkaline oxidation in air of phenanthroquinone to *o*-diphenic acid (23). That the oxidation of gallic acid produces the dimer ellagic acid is well known and has implications for the *in situ* production of ellagitannins from gallotannins.

The products of caffeic acid autoxidation are numerous. The structures of six of them have been determined as dimers (Figure 3) plus at least two trimers (16). Note that, among six different caffeicins, E retains both hydroquinone rings from the two constituent caffeic acid units while the others retain one. Caffeicin E retains neither of the propenoic side chains; the others retain one. Cheynier et al. (24) have shown products of enzymic oxidation of caffeic acid are different, but their general nature appears similar.

Enzymic oxidation. PPO has been shown to occur in latent forms activated by contact with acid (25). Bruising of grapes would expose plastid PPO of the cytoplasm to acid from the vacuole, but a four-fold activation required also the exposure to oxygen (26). Activation by oxygen has also been reported by Lerner, et al. (2) and may have a relationship with PPO functioning as a mobilization against plant injury leading to wound closure and suberization.
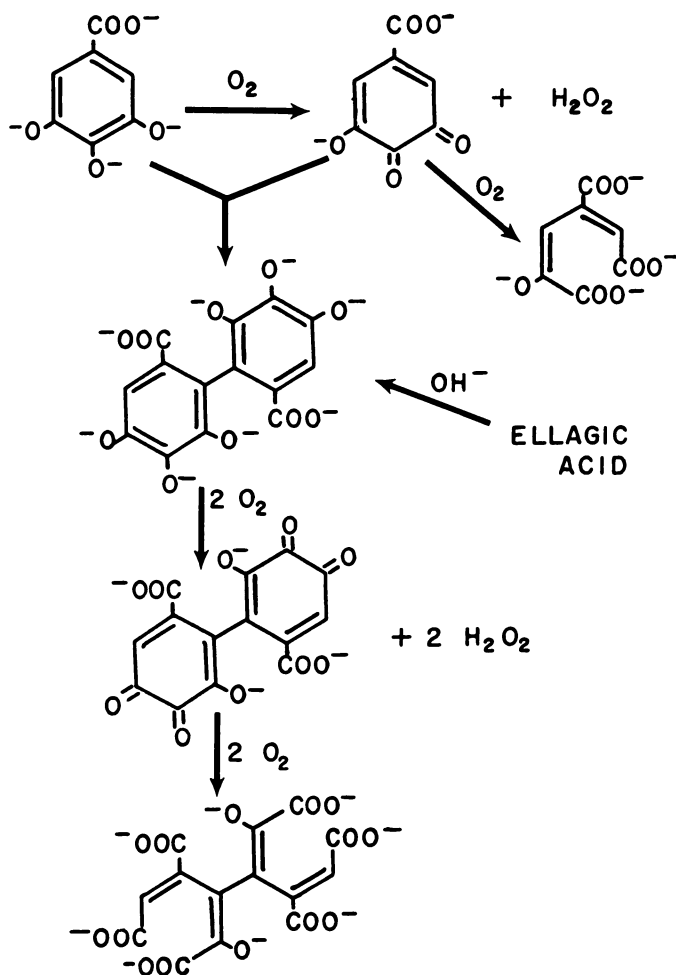
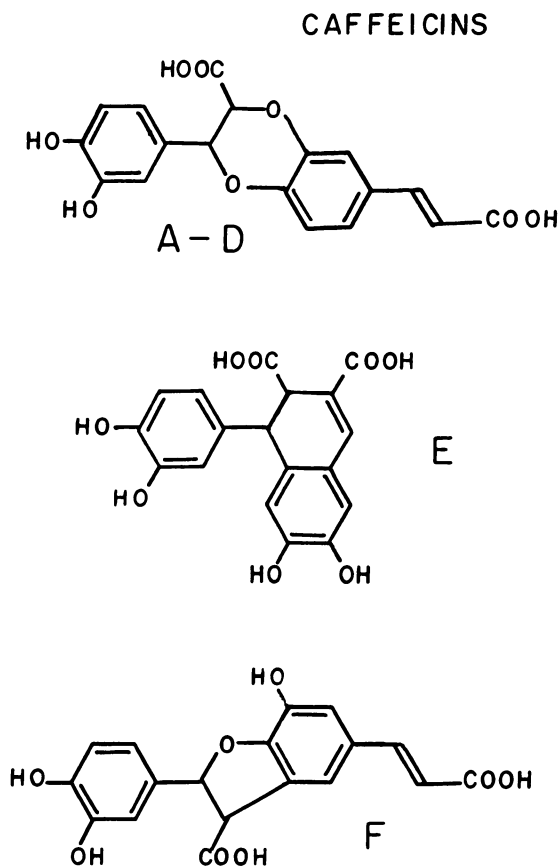Figure 2. Products of alkaline autoxidation of gallic acid (*14*).

CAFFEICINS

A – D

E

F

Figure 3. Products of oxidation of caffeic acid at elevated pH (relative to wine) (*16*).

The reaction that causes much of the early precipitation of PPO from musts and wines appears to be nonspecific complexing with tannins, since a large part of the activity can be solubilized and recovered by adjusting the suspension to pH 10. This would produce phenolates from the tannin's phenolic groups and break hydrogen bonding (27).

Grapes, somewhat variably by variety, are not only high in caftaric (caffeoyl tartaric) acid, but also in glutathione. The combination, when PPO oxidation occurs as in commercial juice production, leads to disappearance of more or less of the caftaric acid and appearance of "grape reaction product" (or glutathione reaction product) GRP (28, 29). Browning tendency depends on enzyme activity, substrate kinds and amounts, and browning inhibitor content (ascorbic acid and glutathione, at least). It is no longer surprising that it has been difficult to categorize grape varieties by chemical means with regard to relative browning tendency, rather than by winery reputation.

GRP, based on the fact that it remains unchanged in the presence of active grape PPO (29), is not a substrate for that enzyme. It is oxidizable by coupled reduction of caffeic quinone, thereby adding to total oxygen uptake via regenerated caffeic acid and demonstrating GRP's lower oxidation-reduction potential. Its structure was determined to be 2-S-glutathionyl caffeic acid (30). Hydrolysis, first of the tartaric acid unit and later of portions of the peptide, leads to a total of seven new phenolic fragments, illustrating the complexity of studying grape must and wine oxidation products. With older wines, 2-S-glutathionyl caffeic acid rises as a result of hydrolysis of GRP, 2-S-cysteinyl caffeic acid eventually falls, presumably from oxidation, and GRP falls from both causes. Although a product of either PPO or autoxidation of caftaric acid in the presence of glutathione, GRP is colorless and its production forestalls browning. In raisins, for reasons attributed to compartmentalization but not understood, GRP does not normally form during sun-drying and this is seen as a reason they are so brown (31).

Six compounds tested having a free SH group, including H_2S, enhanced retention of caftaric acid in the presence of grape PPO and each gave a new HPLC peak (29). That with glutathione and the glutathione-coutaric acid combination matched GRP. The only sulfhydryl compound that neither spared caftaric acid nor gave a new peak was 1,4-dithiothreitol. In unpublished work, a separable peak believed to represent sulfonic acid ring substitution was produced by autoxidation of caffeic acid with SO_2 present, but only at a pH above 5.0, suggesting that the reactive form was the sulfite ion. As already noted the same type of products were produced between quinones and sulfhydryl compounds whether the oxidation was by PPO or without it at higher pH. However, without enzyme, no significant oxygen uptake was noted in experiments to be presented her at pH 5, in the time allotted (arbitrarily 150 minutes maximum) with any of the phenolic substrates. Furthermore, with or without PPO but no phenol, the sulfhydryl compounds took up no oxygen. Neither did the enzyme preparations alone. Phloroglucinol, with active PPO, took up no measurable oxygen in 80 minutes and oxidized glutathione (GSSG) produced no effect during phenol oxidation.

Experimental Details of the Reactions of PPO. It is somewhat difficult to study these reactions because O_2 level, enzyme concentration, substrate phenols, and the nature and concentration of the sulfhydryls present all interact, even when other conditions are constant. Some of the findings to be presented here have been anticipated by previous work with other techniques, but these results add confirmation. The Warburg technique was chosen to focus on oxygen consumption and possible CO_2 generation under proven circumstances. Most other recent studies have used HPLC and focused on phenolic reactants. Phenolic substrates were chosen to represent the major types present in musts and wines; caffeic (o-diphenol), p-coumaric (monophenol), and gallic (vicinal triphenol) acids, d-catechin (flavonoid), plus phloroglucinol as the equivalent of a "bare" A-ring of a flavonoid. The conditions were as constant as possible to prevent as many extraneous effects as possible. Although high concentrations of enzyme were used, activity levels affected the results in ways that will become clear in further discussion.

Limited experimentation was done with oxygen instead of air. Much of the effect of oxygen over air was similar to the effect of higher enzyme concentration as might be anticipated. The faster action tended to obscure rather than illuminate the reaction, given that the Warburg technique is not well adapted to studying very fast reactions (32). Only experiments with air will be reported. One bath temperature (22.0 °C) was used, one (high) shaking speed, pH 5.0 only (chosen to be near reported optimum and nearer high pH autoxidation studies), and usually a single phenolic substrate per trial. The sulfhydryl compounds studied included cysteine, glutathione and dithiothreitol. The results with cysteine were very similar to those with glutathione at an equal molar excess, but slightly less active (averaging about 90%), but apparently differed a little depending on substrate and perhaps enzyme preparation. Cysteine was also more difficult to work with (solubility, pH effects of salts, etc.). Only the results with glutathione will be shown, plus a limited number of tests with dithiothreitol (DTT).

Glutathione is Not Acting as a Reductant. Figure 4 shows that, without glutathione, oxygen uptake by caffeic acid in the presence of a moderate level of mushroom tyrosinase was low and slowed greatly from 0.1 O_2/mole phenol. Under the same conditions with glutathione added, oxygen uptake was much faster, but reached a maximum of 0.5 O_2 per phenolic molecule as the caffeic acid present was exhausted. With ascorbic acid, however, the oxidation continued very rapidly with the same amount of caffeic acid to several moles of O_2 per mole of phenol. With only ascorbic acid and no phenol, relatively little oxygen was consumed as also shown in Figure 4. Ascorbic acid is well known to reduce quinones, caffeic quinone here, back to their hydroquinone form, which then are reoxidized by PPO.

This is one form of coupled oxidation with the net effect of oxidizing ascorbic acid instead of the phenol until ascorbic acid is depleted. Glutathione (GSH), cysteine and similar compounds, are not acting by this means as shown by the fact of approaching the theoretical limit expected, 0.5 μmole O_2/μmole ϕOH. If GSH were acting as a reductant, it, like ascorbic acid, would cause recycling of the
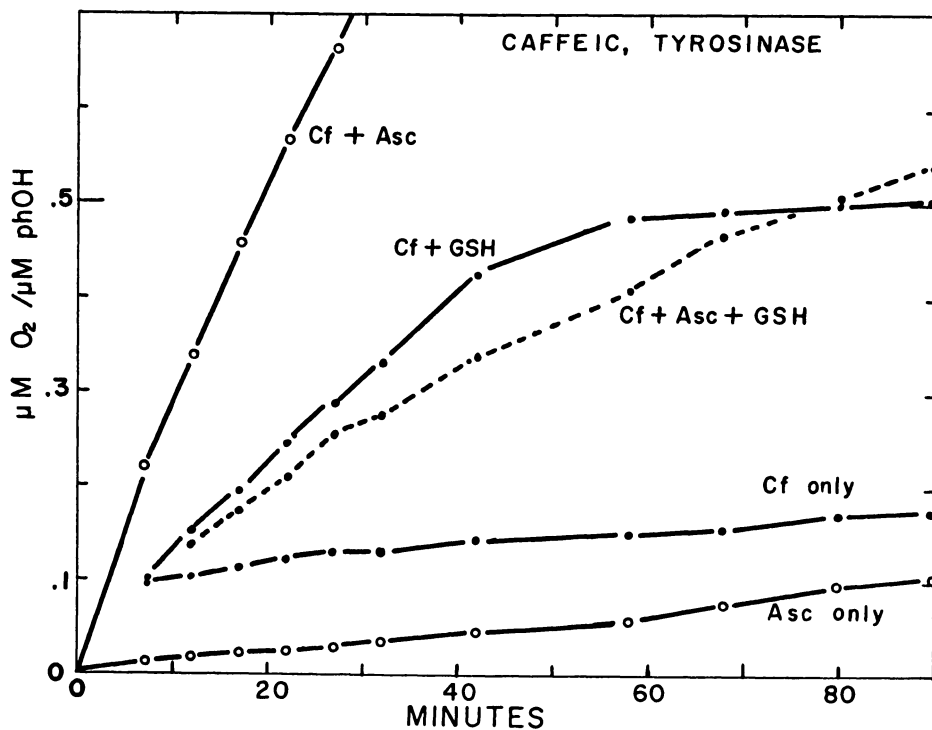
Figure 4. Oxygen uptake by caffeic acid in the presence of mushroom tyrosinase and the effects of glutathione and ascorbic acid at high levels.

reduced phenol and multiply the O_2 uptake. Furthermore, since the GSH is competing to rapidly remove the quinones, it greatly lowers (Figure 4) the effect of co-present ascorbic acid. *Vice versa*, ascorbic acid lowers the production of GRP.

Relative Concentrations and Enzyme Purity Make a Difference. Figure 5 shows the oxidation of caffeic acid with and without glutathione by grape acetone powder PPO (G) and that from mushrooms (T). Note that the uptake by the mushroom tyrosinase in the presence os GSH was very rapid. The shaking rate and the O_2 transfer rate are shown to be adequate for fast reaction, and the differences must, therefore, represent reaction differences. Of the two enzyme samples, the tyrosinase is more active than the grape acetone powder in the presence of GSH, but the reverse without GSH. This suggests that the GSH has two effects. One is to convert the caffeic quinone to the GRP equivalent, 2-S-glutathionyl caffeic acid (CRP), a non-substrate, and thus terminate the reaction upon exhaustion of the caffeic acid at 0.5 μmole O_2/mole ϕOH. The second is to prevent destruction of the enzyme caused by its reaction with the quinones.

Golan-Goldhirsh and Whitaker (*33*) showed that this quinone inactivation involved about 85 molecules of the quinone per enzyme subunit. This certainly suggests a rather nonspecific reaction and that competing proteins could help protect the enzyme's activity. The smaller difference between the activities with and without GSH for the more impure grape acetone powder suggests that the entrained protein impurities help protect the PPO activity, compared to the purer mushroom tyrosinase. Since a high number of quinones are involved in inactivating the enzyme, less GSH should be required for enzyme protection than for complete suppression of other reactions to the 0.5 O_2/phenol level. If this reasoning is correct, addition of a nonspecific protein should protect the purer enzyme. Addition of bovine serum albumin did just that, Figure 5. This addition not only protected the enzyme to give higher total O_2 uptake, but also gave a very dark brown solution whereas GSH and other sulfhydryls prevent browning. Note also that the purer enzyme compared to the less pure or protein-added sample (Figure 5) carried the total oxidation to its maximum level faster. It suggests that the O_2/phenol ratios above 0.5 as in the two highest curves (Figure 5) can be caused by so rapid production of quinone that some escapes conjugation with GSH and is reduced for reoxidation by the enzyme through coupled chemical oxidation of self-condensed dimers or previously conjugated 2-S-glutathionyl caffeic acid. Alternatively, if other protein is tieing up the quinone, the PPO activity can be prolonged to allow similar effects via coupled oxidation of hydroquinones generated by linkage of the caffeic quinone to the protein via sulfhydryl or amine linkages.

Glutathione Does Not Inhibit PPO. It has been often said that cysteine and other sulfhydryl compounds inhibit PPO. This appears to be true if browning is the measure of PPO activity. In no instance was there appreciable browning in the presence of high levels of sulfhydryls relative to the content of phenolic substrate. However, glutathione does not appear to affect the enzyme directly and oxygen
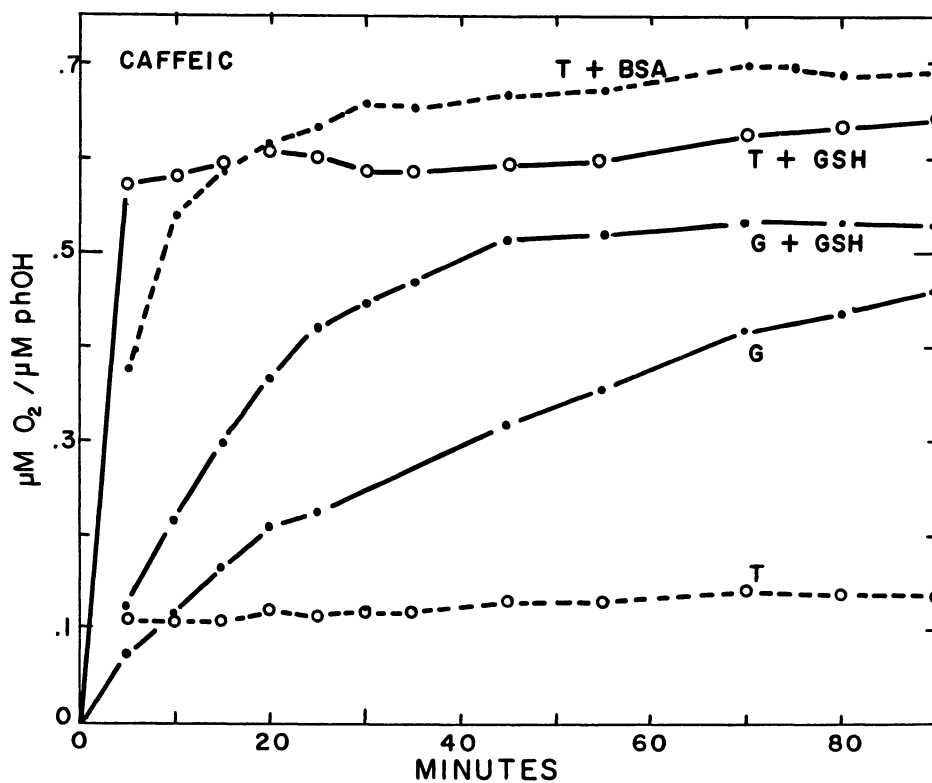
Figure 5. Oxygen uptake by caffeic acid in the presence of Grenache PPO acetone powder (G) and relatively pure mushroom PPO (T), with and without added (5x phenol moles) glutathione and the activity preservation effect of protein impurities. BSA = bovine serum albumin.

uptake may be stimulated or inhibited depending upon the particular phenol being oxidized. Figure 6 compares three levels of tyrosinase (mushroom PPO) at a constant level of caffeic acid (about 5 μmoles/mL) and glutathione (about 30 μmoles/mL). As the enzyme is diluted, the portion of the caffeic acid oxidized at maximum decreases. In the presence of glutathione and dilute PPO, all the caffeic acid is oxidized to the quinone as shown by constant 0.5 μmoles O_2/μmoles ϕOH. At the highest enzyme level, glutathione produced very fast oxygen uptake and to a slightly higher level per mole of phenol. This slightly higher level is attributed, as already described, to some caffeic quinone escaping glutathione conjugation long enough to be reduced back to caffeic acid substrate by already formed codimers or glutathione conjugate, the latter substances oxidized by coupled chemistry, not enzyme action.

Note that the effect of glutathione on the PPO action is as predicted. Glutathione preserved action of weaker enzyme amounts to reach complete oxidation to the quinone form and tied up all the quinone formed to terminate the reaction at that stage.

PPO Inactivation Does Not Inhibit Resumption of the Reaction. Figure 7 shows again that higher active enzyme content, in the presence of a high level of GSH, allows some continued oxygen uptake with time and to levels beyond the simple caffeic acid to caffeic quinone transformation. Without the protection afforded by the GSH, the PPO at level one produced limited oxidation of the caffeic acid. However, addition via a second arm of the Warburg flask of either much more enzyme alone or the same additional amount of PPO plus GSH gave immediately renewed oxygen uptake ultimately to about the respective levels achieved by originally high levels of either enzyme or GSH with enzyme. These data show that there was not some inhibiting condition, but that the PPO had been inactivated. The unreacted caffeic acid remained and then did react with new enzyme to about the same final degree.

Glutathione Concentration Affects the Course of the Reaction. Figure 8 shows, with grape acetone powder and caffeic acid, results concordant with the previous interpretations. At a GSH level of about 6x that of the phenol, all the quinone was trapped and oxygen consumption leveled off at 0.5 O_2 per phenol. As GSH was decreased, all else constant, the uptake of oxygen increased in rate and reached a higher total for reasons already explained. The reaction by enzyme alone was slower (Figure 8), but reached a higher than theoretical oxygen consumption. This is explained by a lower concentration of GSH protecting the enzyme and by, in the absence of GSH, greater polymerization of the caffeic quinone. It is significant that only the sample without any GSH was brown in this and other similar experiments.

The effect of 1,4-dithiothreitol was much different than GSH at similar molar concentrations. It seriously inhibited, particularly at first, the uptake of oxygen by caffeic acid with PPO (Figure 8). No browning occurred.
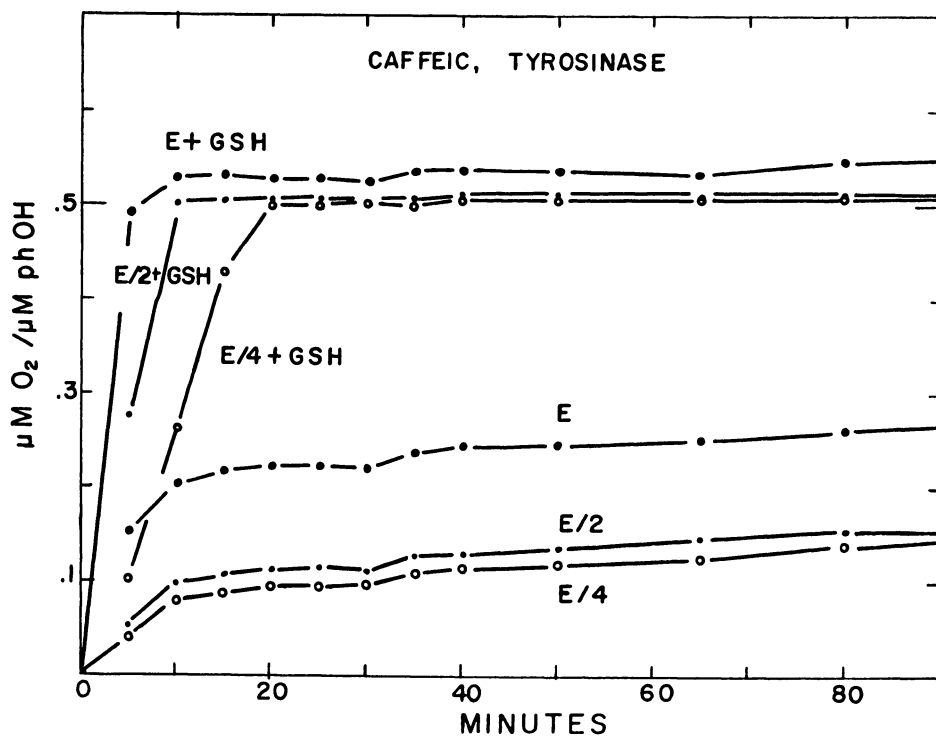
Figure 6. Tyrosinase (mushroom PPO) concentration effects with and without glutathione at a high level (5x phenol moles) on oxygen uptake by caffeic acid. E = 2000 units/mL; E/2 = 1000 units/mL; E/4 = 500 units/mL.
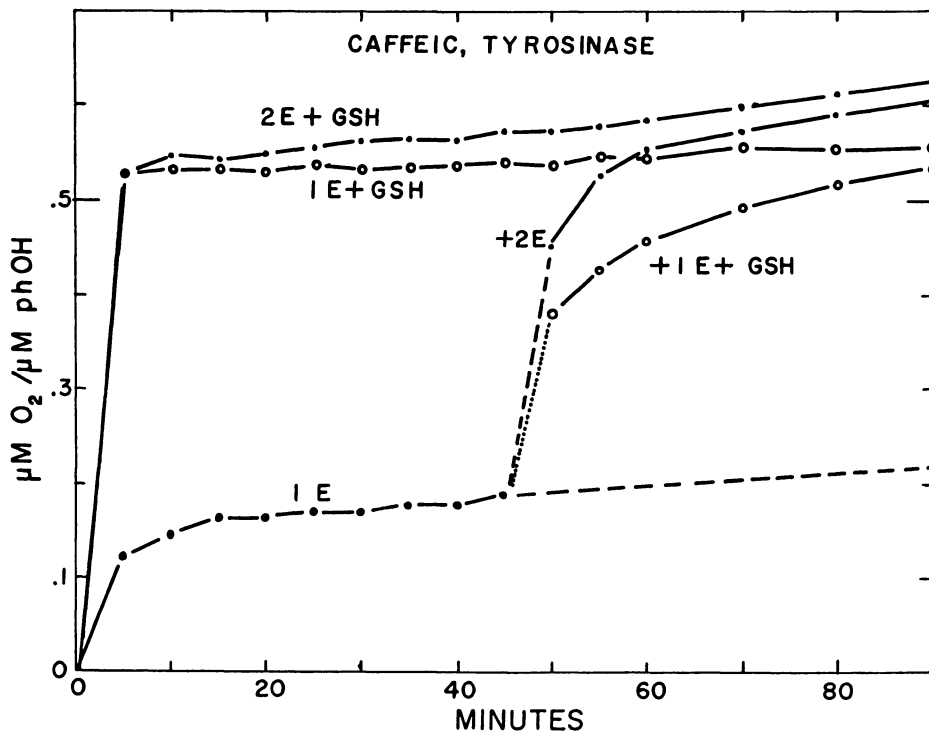
Figure 7.   Effect of adding new active enzyme on a stalled caffeic acid oxidation and comparison with the same conditions with high levels of PPO (mushroom tyrosinase) or added glutathione.
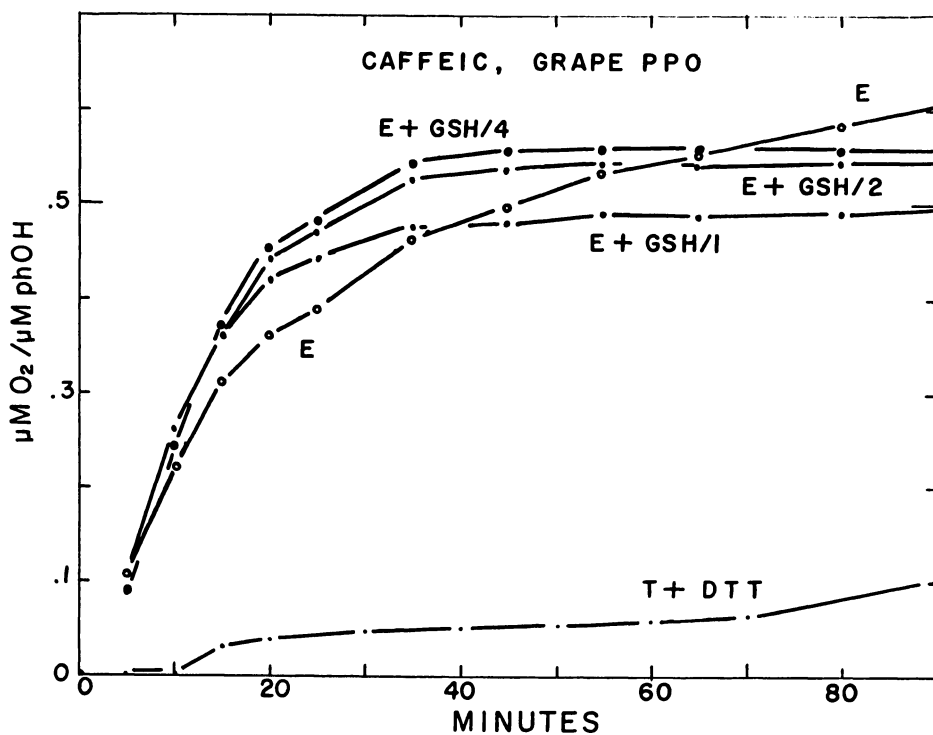
Figure 8. The effect on oxygen uptake of caffeic acid by the action of grape PPO of addition of glutathione at about 0 (E), 1.5 (GSH/4), 3 (GSH/2), and 6 (GSH/1) times the caffeic acid molarity. The oxygen uptake with 1,4-dithiothreitol on the same level of caffeic acid but with mushroom tyrosinase (T) is also shown.

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Monophenol Oxidation. The mushroom preparation and grape acetone powders had only weak and lagging activity toward *p*-coumaric acid. The data in Figure 9 are from studies with the purified grape PPO. This enzyme preparation reacted quickly with coumaric acid and took up oxygen to and beyond the theoretical level of 1.0 O_2/phenol. In another experiment with a higher enzyme level, the rate of uptake of oxygen slowed earlier and nearer the 1.0 ratio, but still continued upward more slowly than in Figure 9. The solutions became quite brown; polymerization was certainly occurring. In direct contract to the effect with caffeic acid, however, glutathione drastically inhibited the reaction. Dithiothreitol also had an inhibiting effect, but less than GSH especially immediately. Again, this is opposite to comparable results with caffeic acid. In limited tests, caffeic acid and coumaric acid appeared to mutually inhibit each other's O_2 uptake reaction with PPO, at least the total uptake was less than the sum of the two separately.

Current thinking places the catecholase and cresolase activities at the same catalytic site. It is speculative of course, but these data suggest that the differences between these actions may lie in the handling of the second oxygen atom. Quantitative oxidation of caffeic acid to a terminal level of 0.5 O_2/phenol in the presence of GSH as quinone trapping agent requires that the second oxygen atom be held on the enzyme until a second caffeic acid molecule consumes it. Either there must be a brief interval of an active form of one oxygen as part of the enzyme or two molecules of phenol must be attacked simultaneously at the active site. The first alternative has generally been involved (*3*) and for caffeic acid it may be unimportant. However, as a way of explaining cresolase activity and how it differs from catecholse, it seems worth consideration. The O_2 apparently binds first followed by the phenol (*3*). Monophenols are reported to have greater affinity than diphenols for the active site (*34*).

Since it takes two atoms of oxygen to convert coumaric acid to caffeic quinone, there is no "residual" atom of oxygen to act on the next molecule, perhaps explaining the slower early and increasing intermediate rate of reaction with pure coumaric acid shown in Figure 9. At high levels of coumaric relative to caffeic acid, oxidation of the caffeic acid would be inhibited because of the high affinity and slow reaction of the monophenol. As monophenol is converted to caffeic quinone and polymerization occurs, some caffeic hydroquinone can result from coupled oxidation-reduction. Perhaps this can explain the autocatalytic rise shown in Figure 9 and from this point on provide PPO with the residual activated oxygen atom to then oxidize coumaric acid more readily. Glutathione would suppress rather than activate the system by preventing caffeic acid quinone's coupled reduction back to free caffeic acid as a substrate for the PPO. To account for its different behavior, dithiothreitol might be directly affecting the PPO's active site, perhaps by affecting any active residual oxidation capacity of the enzyme.

Gallic Acid Oxidation. In contrast to the other substrates, gallic acid gives off considerable carbon dioxide as it is oxidized by PPO. Figure 10 shows typical data. GSH lowered the oxygen consumption to near the half mole needed to
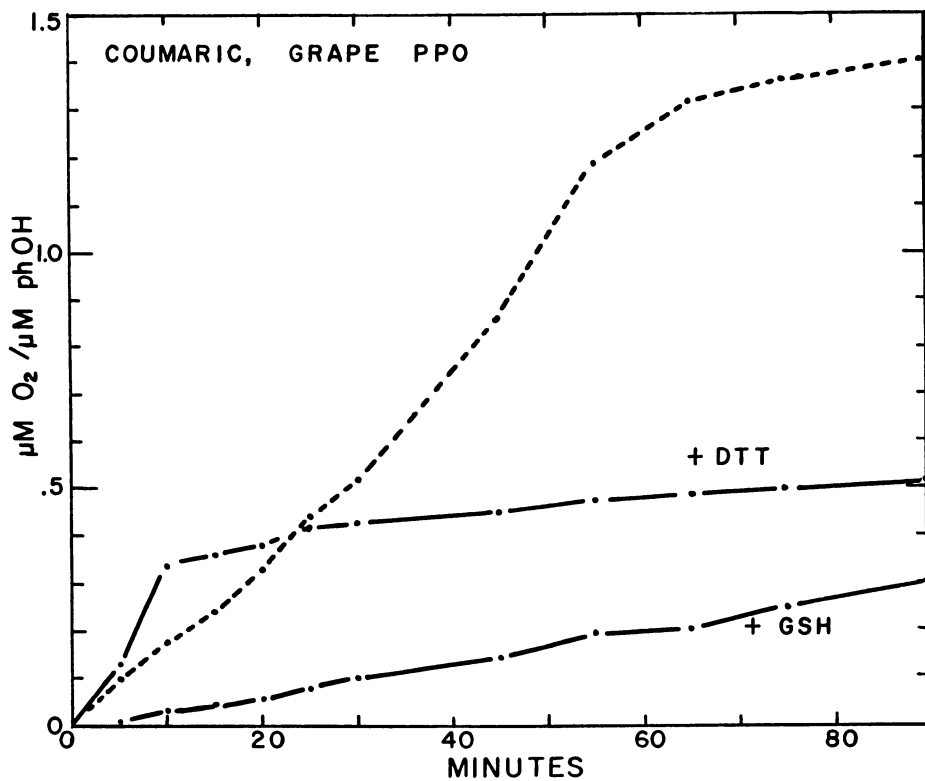
Figure 9. Oxygen uptake by *p*-coumaric acid catalyzed by enriched grape PPO and the effects of glutathione and dithiothreitol.
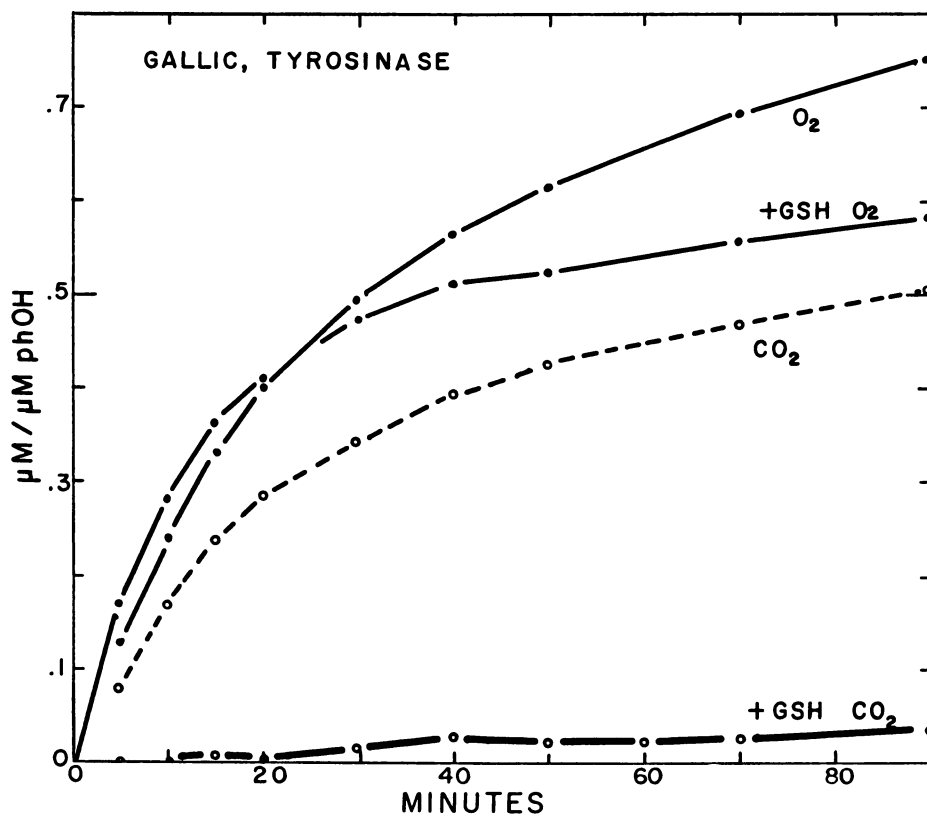
Figure 10. Oxygen uptake and carbon dioxide emission by gallic acid catalyzed by tyrosinase (mushroom PPO) and the effects of glutathione and dithiothreitol.

produce the quinone and almost eliminated the production of CO_2. The CO_2/O_2 ratio was nearly constant over time in this set at about 0.7 in the absence of GSH. This ratio appeared somewhat subject to shift as concentration ratios were changed. It is believed that the GSH, as before, is trapping the gallic quinone and minimizing dimerization.

It is proposed that there are two mechanisms of dimerization. Dimerization to the ellagic acid precursor hexahydroxydiphenic acid is one, but would not produce CO_2. This is the mechanism used to explain alkaline oxidation of gallic acid (*14*). Of course, CO_2 would not be released from alkaline solution and ring oxidation. The second dimerization which appears to account for the CO_2 is the production of carboxypurpurogallin (Figure 11). This is analogous to the reaction important in tea, producing theaflavins, and has been reported with PPO oxidation of gallic acid (*35*). The sample solutions without GSH were very highly colored as the purpurogallin, but not other obvious products, would produce. The proposed reaction would produce one molecule of CO_2 for each gallic acid unit entering the "dimer," one from the carboxyl and the other from the sixth ring carbon and its hydroxyl. Two gallic acid units would consume 1.5 moles of O_2, if this were the exclusive reaction. At 2 CO_2 per 1.5 O_2 the ratio would be 1.33. Since 0.7-0.8 was found, it appears a bit over half of the reaction goes the purpurogallin route, the rest being "ellagic" and others not yielding CO_2. Since alkaline oxidation of gallic acid produces browning and the ellagic acid derivatives *per se* are not expected to be colored, this purpurogallin-type of dimerization and pigmentation probably also occurs n alkaline oxidation.

Oxidation of wines (acidic, nonenzymic) produces CO_2 (*10*) and this or similar reactions must be the source. It would be predicted that further study manipulating the GSH ratio and including catechins would be useful to elucidate the role of gallic acid in wine oxidation. Perhaps theaflavins, which are considered so important in tea color, flavor, "briskness," and quality also have a role in wines.

Catechin Oxidation. As an example of oxidation of a flavonoid important in wine, the data in Figure 12 are offered. Enriched Thompson Seedless grape PPO was used. Oxygen uptake was rapid in the presence of excess GSH to about 0.5 moles O_2 per mole of *d*-catechin and then continued slowly. It was much higher and gave orange-brown, poorly soluble color if GSH was not used. The results are interpreted as showing that in the presence of glutathione to trap the quinone, polymerization is largely prevented and only sufficient oxygen to produce the quinone is used at first. Again one might be tempted, incorrectly, to say the enzyme was inhibited by GSH. No color, again, is produced under a high level of GSH. Dithiothreitol also prevented color development, but severely depressed oxygen uptake, particularly at first.

Oxidation of Caffeic Acid with Phloroglucinol. The polymerization of catechin as it is oxidized by PPO would appear to be simulated by oxidizing caffeic acid and phloroglucinol as a pair. The catechin's B-ring furnishes the quinone as

$$C_{14}H_{12}O_{10} \; (+ \; 1.5 \; O_2) \longrightarrow C_{12}H_8O_7 \; (+ \; 2\,CO_2 + 2\,H_2O)$$
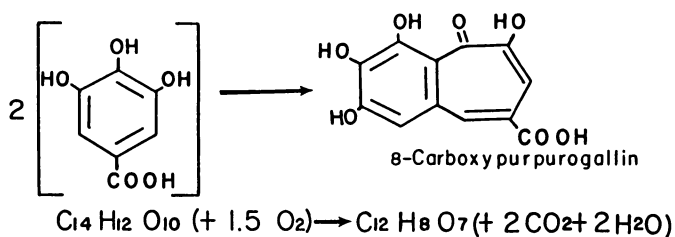
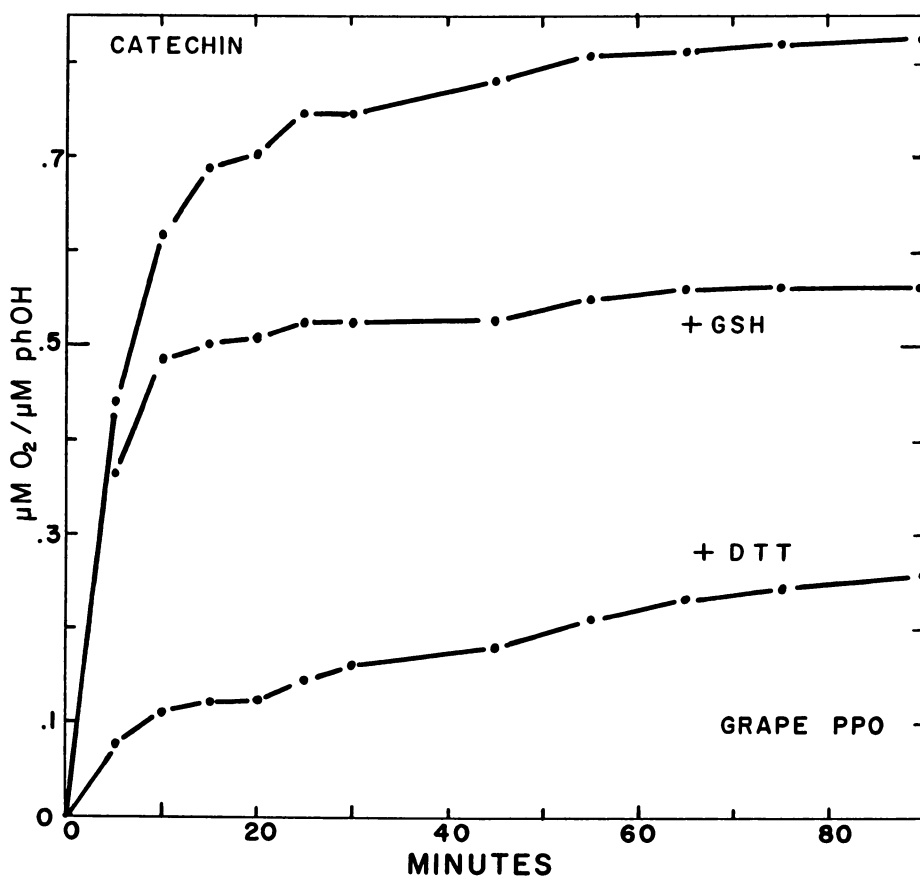Figure 11. Oxidation of gallic acid to carboxypurpurogallin.

Figure 12. Oxygen uptake by *d*-catechin catalyzed by enriched grape PPO and the effects of glutathione and dithiothreitol.

does caffeic acid. The A-ring of the flavonoid is a substituted phloroglucinol. It has already been noted that phloroglucinol alone is not oxidized by PPO, so the calculations of Figure 13 are based on the caffeic acid content alone. As in the other instances, GSH prevented polymerization and the result was much the same whether or not phloroglucinol was present. In the absence of GSH caffeic acid plus phloroglucinol took up considerably more oxygen and gave more intense and more orange color at the end than did plain caffeic acid.

These findings seem very consistent with expectations from the explanations given earlier. We are brought full circle to explain why white wine browning capacity is proportional to their flavonoid content. *Vitis vinifera* wine grapes have similar, roughly 50-300 mg/L, contents of caffeic derivatives and impart that similarity to white wines made standard ways. The flavonoid content is more variable for several reasons, especially degree of solid grape tissue extraction in winemaking. Although caffeic acid is more readily oxidized, it gives less brown color than catechins because of their relative effects in polymerization. Figure 14 illustrates one probable type of caffeic-catechin dimer. Such a dimer, when oxidized, produces extended multiple-ring conjugation of double bonds and much higher intensity of color. For example benzoquinone gives a molar extinction of only 20 at 434 nm whereas diphenoquinone has about 69,000 at 398 nm (*36*). A family of such products would have many individual maxima and together result in wide amber-brown absorbance. Furthermore, for reasons mentioned earlier plus the usually lower oxidation reduction potentials of any 1,4-diphenol analogs produced (like in Figure 14), the products tend to be more easily oxidized than the original reactants.

Conclusions

1. Glutathione inhibits browning, but does not inhibit PPO. Depending on the specific phenol and other conditions, it may stimulate, limit or inhibit oxygen uptake.

2. Glutathione and other sulfhydryl compounds except 1,4-dithiothreitol appear to act on phenol oxidation similarly by trapping quinones and thus competing with further reactions dependent on quinones. Polymerization is particularly dependent on the quinones and limited or prevented by GSH. They are not reductants for PPO quinones in the manner of ascorbic acid.

3. Glutathione prevents destruction of the PPO enzyme by the quinones the enzyme produces. Activity with impure enzyme or enzyme plus extraneous protein is partly spared and is less needful of GSH for preservation of activity.

4. Careful study of the reactions using glutathione as tool and oxygen uptake (and CO_2 generation) have elucidated the course of oxidation by PPO of caffeic, coumaric, gallic acids, caffeic acid plus phloroglucinol, and catechin. The case with phloroglucinol is particularly instructive in that incorporation of an otherwise inoxidizable molecule produces an oxidizable structure leading to great increase in the visible brown absorbance.
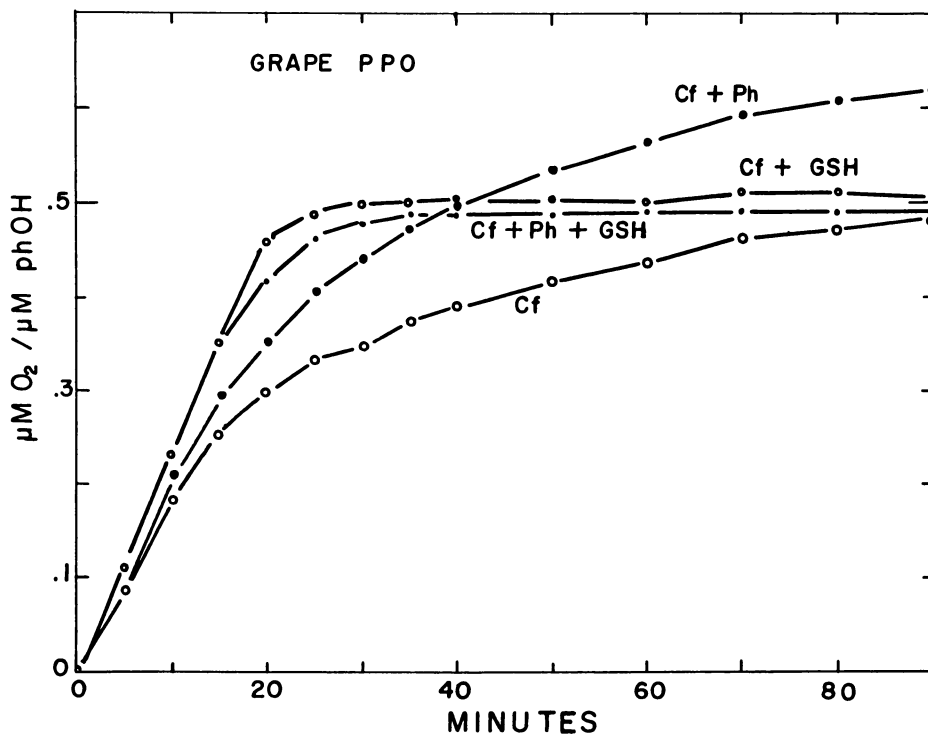
Figure 13. Oxygen uptake by caffeic acid in the presence of phloroglucinol catalyzed by grape PPO acetone powder and the effect of glutathione addition at a high level.
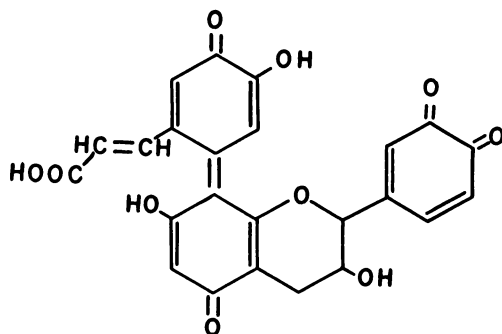

Figure 14. Example of a caffeic-catechin dimer oxidation product.

5. There are four different types of coupled reaction whereby oxidation of vicinal diphenols can cause further oxidation: a). In autoxidation (but not by PPO) hydrogen peroxide is produced resulting in more O_2 consumption and additional oxidation. b). Quinones may be reduced (as by ascorbic acid) resulting in phenol preservation until the reductant is consumed. c). Regenerative dimerization epitomized by the GRP or phloroglucinol reaction regenerates a hydroquinone structure which can be (autoxidatively if not enzymically) oxidized in turn. d). Regenerated dimers and similar products are expected to be more readily oxidized so that they can reduce (contribute hydrogen to) original quinones back to their hydroquinone, enzyme substrate form.

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Chapter 4

Prevention of Enzymatic Browning in Fruits and Vegetables

A Review of Principles and Practice

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Enzymatic browning of fruits and vegetables has not ceased to be a problem for processors, although the underlying basic reactions have been known for a long time. One of the difficulties in selecting the mode of browning prevention consists in the necessity of complying with food safety regulations, while at the same time taking into account the marketability of the product as affected by taste and flavor, texture, etc. The ever increasing amount of literature accumulated on the topic shows great progress in establishing the phenolic composition of foods, enzyme purification and the introduction of new browning inhibitors. This review gives an account on the latest achievements in the prevention of browning along with some of the author's earlier results.

In the early 1980's this author had her first opportunity to review the literature on the polyphenol oxidase (PPO)-catalysed transformation of endogenous phenols in raw food material to - in most cases undesirable - brown pigments (1). Since that time the interest in the topic has not slackened. On the contrary, the field of research has become even broader. Beside enzymic browning of fruits and vegetables, which is still of interest, an increasing number of papers have been devoted to enzymatic discoloration of cereals, oilseeds and sugar cane (2-6) as well as to commodities of animal origin (seafood) (7,8). The latter are of particular interest as, e.g. lobster PPO is present in a latent form in the tissues (7-9), a phenomenon rarely encountered in the plant kingdom. This review tries to give an overview on the prevention of enzymatic browning in fruits and vegetables mainly based on the literature of the 1990's.

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Principles of the Prevention of Enzymatic Browning

The principles of browning prevention have not changed with time and are essentially the same as those applying to the inhibition of any tissue enzyme, i.e.:

> a/ inhibition/inactivation of the enzyme
> b/ elimination/transformaton of the substrate(s)
> c/ combination of a/ and b/.

In the light of recent findings it is, however, not easy to classify an inhibitor or an inhibitory process as belonging exclusively to one of the three categories. Many inhibitors act both on the enzyme and on its substrates. For all the three cases mainly substances or procedures compatible with food safety and marketability requirements shall be dealt with here.

Prevention does not necessarily consist of post-harvest treatment only. Much can be done to reduce browning occurring during storage or processing by selecting cultivars of slight browning tendency (*10*) and by appropriate agricultural techniques (*11*). Studies into the changes in browning tendency, PPO activity and phenolic composition and concentration during ripening (*12, 13*) might be equally helpful to the grower and the processor, although harvest times can hardly be adjusted to the optimum values of these parameters only.

A vast amount of data has been accumulated during the past decade on the characteristics of PPO in different commodities (*14-23*). As methods of enzyme extraction and purification improved simultaneously, findings often are not in agreement with those published earlier for the same commodity. The same applies for the research into the phenolic composition of foods (*24-26*). Here the outstanding importance of HPLC should be stressed. From the aspect of enzymatic browning and its inhibition research into the fate of phenolic substrates during PPO-catalysed oxidation might be of outstanding importance (*27,28*).

Enzyme inhibition can be reversible or irreversible. The latter is often achieved by physical (heat) treatment, while chemicals might act in one or the other way.

Prevention of Enzymatic Browning with Chemicals

Recent Uses of Traditional Browning Inhibitors. Most recent work on the prevention of enzymatic browning is aimed at replacing sulfite (*29, 30*). Sulfur dioxide, sulfites, bisulfates and metabisulfates inhibit enzymic and nonenzymic browning (*31*) and are effective against microbial infection. However, owing to their harmful effect on health their use has been restricted or banned altogether in several countries. It seems difficult to substitute them in vinification (*32-34*). [Some success has been achieved, though, with ascorbic acid or ascorbic acid containing mixtures of chemicals (*34*)]. As shown with metabisulfite, this class of inhibitors act a/ on the quinones formed by PPO-catalysed oxidation of *o*-dihydroxy phenols and b/ on the enzyme itself by irreversibly binding to the "met" and "oxy" forms of binuclear copper at its active site (*35*).

Some recent uses of classical browning inhibitors other than sulfite are summarized in Table I.

Table I. Recent Uses of "Classical" Browning Inhibitors in Fruits and Vegetables

Commodity	Inhibitor(s)	Concentration	Remark	Reference
Apple (slices)	Ascorbic acid	10 (g/l)	Heating (60-70°C, 15 min) or decreasing of pH promoted inhibition	*36*
Apple (cubes) (Golden Delicious)	Ascorbic acid + citric acid	10 AA+2 CA (g/l)	Dip (5 min)	*37*
Apple (cubes) (Golden Delicious)	Ascorbic acid + NaCl	10 AA + 0.5 NaCl (g/l)	Dip (5 min)	*37*
Apple; potato	Ascorbic acid or ascorbic acid phosphate		Infiltration; AAP was hydrolysed by endogenous acid phosphatase	*38*
Potato	Ascorbic acid + citric acid + sodium acid pyrophosphate + CaCl$_2$ +AAP (Mg-salt) + AAP$_3$ (Na-salt)	40 + or 25 + 10 + 10 + 10 + 10 + 2 2 + - 16 + - 15 (g/l)	Effective in extending storage life of abrasion-, lye- or high pressure steam-peeled potatoes after digestion and removal of digested surface prior to applying 5-min dip	*40*
Garlic (chopped)	Citric acid	10 (g/l)	Effective during storage at 4°C	*41*
Avocado	L-cysteine	0.32 mM	100% inhibition	*42*
Banana	L-cysteine	5.0 mM	100% inhibition	*42*

AA: ascorbic acid; AAP: ascorbic acid phosphate; AAP$_3$: ascorbic acid triphosphate; CA: citric acid.

From the data tabulated it can be seen that ascorbic acid is a browning inhibitor still much dealt with. Its action can be enhanced by citric acid and NaCl in concentrations that are ineffective in themselves or even increase enzyme activity. (It is interesting that an increase in activity was noted also for ascorbic acid alone up to the concentration given in the Table) (*38*).

Ascorbic acid phosphate (AAP) might act as an ascorbic acid "reservoir", when the browning tendency of the product is not too strong. Otherwise the AA released by endogenous ascorbic acid phosphatase might be soon depleted. A reduction of the inhibitor solution pH to 2 inhibits ascorbic acid phosphatase and makes the effect of AAP safer. Reduced pH and the use of ascorbic acid-2-triphosphate provide for a gradual release of ascorbic acid (39). The question arises here to what extent lowering of the pH contributes to the inhibition of browning and if this pH is compatible with the sensory and technological properties of the given commodity.

Another group of "classical" browning inhibitors are sulfur-containing amino acids and peptides or alcohols with L-cysteine as prototype. The action of cysteine is complex. It forms addition compounds with phenolic substrates. The structure of some of these compounds is now well established, e.g. 5-S-cysteinyl-3,4-dihydroxytoluene is formed from 4-methylcatechol and 2-S-cysteinylchlorogenic acid from chlorogenic acid (43). Cysteine also forms adducts with quinones. The fate of the quinones and the adducts (and the efficiency of browning inhibition by cysteine) was found to depend on the ratio thiol:phenol and also on the pH. Cysteine-quinone adducts proved to be competitive inhibitors of PPO. They enter enzymatic as well as non-enzymatic oxidation reactions with the quinones, whereby phenols are regenerated. These may undergo enzymatic oxidation causing color formation. In order to prevent discoloration, the relative cysteine concentration must be sufficient to transform all the substrate into colorless adducts (44, 45). N-acetyl-L-cysteine and reduced glutathione were found to be even more potent browning inhibitors than L-cysteine (for apples and potatoes) (46), while dithiothreitol, a reversible inhibitor of PPO was much more efficient than glutathione (for mushrooms) (47). The efficiency of cysteine (and also of aromatic amino acids) and the type of inhibition were found to depend, apart from the nature of the inhibitor, also on the method used for determining activity (polarographic or spectrophotometric) (48, 49).

Some Earlier Results with Traditional Inhibitors from the Author's Laboratory. Some of the author's own work with inhibitors used to prevent browning of apples and peaches is shown in Tables II and III. (Vámos-Vigyázó and Gajzágó, Central Food Research Institute, Budapest, unpublished data). The discoloration was measured by a reflectance method (50, 51) using slices of Starking and Golden Delicious apples as well as Elberta and Ford peaches treated and not treated with inhibitors, respectively. The wavelengths used for reflectance measurements were 540 nm for apples, 470 nm for the white-fleshed peach cultivar Elberta and 580 nm for the yellow-fleshed peach cultivar Ford. Fruit slices were immersed at room temperature (22-24 °C) into inhibitor solutions of various compositions and concentrations for various times. The progress of discoloration was read from the scale of the spectrocolorimeter "Spekol" (Zeiss, Iena, Germany). Readings were performed at least up to 10 min, during the first 2 min every 30 s and later every 60 s. From the linear parts of the saturation curves obtained the "initial browning rate" (BA) of the samples was calculated by linear regression. The number of replicates was 4-7. Mean BA values and standard deviations were calculated. The BA-

values of inhibitor-treated samples were subtracted from the respective values of the untreated controls, related to the control values and expressed as % inhibition. If no discoloration occurred up to 30 min, inhibition was considered to be 100%. The statistical evaluation of the results was performed by Student's t-test (comparing the BA-s of the inhibited with the respective control sample). The results obtained with apples are summarized in Table II.

Table II. Inhibition of the Discoloration of Apple Slices by Dipping into Various Inhibitor Solutions

Apple cultivar	Inhibitor and concentration (%)	Dipping time (min)	Inhibition (%)
Starking	CA (2)	1	0*
		3	57 ± 10
		5	57 ± 23
Starking	CA (4)	1	60 ± 20
		3	80 ± 18
		5	100
Starking	CA (6)	1	73 ± 17
		3	90 ± 13**
		5	100
Starking	AA (1)	1	90 ± 7
		3	98 ± 3**
		5	100
Starking	AA (2)	1	95 ± 13**
		3	99 ± 4**
		5	100
Starking	AA (1) + CaCl$_2$ (0.1), pH 8.1	3	100
Starking	CnA (0.0075)	1	0
		3	78 ± 8
		5	80 ± 8
Starking	CnA (0.015)	1	0
		3	80 ± 10
		5	83 ± 8
Golden Delicious	CnA (0.0075)	1	5 ± 30
		3	84 ± 8
		5	100
Golden Delicious	CnA (0.015)	1	5 ± 30
		3	100
		5	100

CA: citric acid; AA: ascorbic acid; CnA: cinnamic acid;
* statistically non-significant increase; ** not significantly different from 100%.

With Starking apple slices the shortest dipping times and the lowest inhibitor concentrations yielding complete inhibition were 5 min in 4% (0.2 M) citric acid, and 5 min in 1% ascorbic acid (0.057 M), respectively. (Some of the other values were not significantly different from 100 inhibition either). Dipping time could be reduced to 3 min by adding 0.1% (9 mM) $CaCl_2$ to the above ascorbic acid solution. $CaCl_2$ is known to have a beneficial effect on apple flesh firmness. Cinnamic acid could be applied only at very low concentrations (0.5 and 1 mM) as it imparted an off-taste to the fruit, described by the panelists as "metallic". Complete inhibition of the browning of Starking apple slices could not be achieved with either concentration. A prolongation of the immersion time from 3 to 5 min had no significant effect on the efficiency of this inhibitor. With Golden Delicious, the cultivar less susceptible to browning, complete inhibition could be achieved by applying 0.5 mM for 5 or 1 mM cinnamic acid for 3 min.

The results obtained with peach slices are shown in Table III.

Table III. Inhibition of the Browning of Peach Slices by Dipping into Various Inhibitor Solutions

Peach cultivar	Inhibitor and concentration (%)	Dipping time min	Relative Inhibition %
E	AA (1)	1	85 ± 33*
E	AA (3)	1	100
F		1	100
E	CA (1)	1	30 ± 6
E	CA (3)	1	39 ± 12
E		2	42 ± 13
E	CA (5)	2	71 ± 19
E	Sucrose (30)	2	64 ± 8
F		1	82 ± 21*
E	Sucrose (50)	2	62 ± 10
E	Sucrose (30) + CA (3)	2	100
E	Sucrose (50) + CA (3)	2	100
E	NaHSO3 (0.1)	2	100

E: Elberta; F: Ford: AA: ascorbic acid; CA: citric acid
*: not significantly different from 100% inhibition.

Complete inhibition of browning could be achieved with a 1-min dip in 3% ascorbic acid for both cultivars tested. Citric acid was only tried with the cultivar Elberta and did not yield complete inactivation with any of the concentrations and dipping times tried. No complete inhibition could be achieved either with sucrose solutions alone. However, both combinations of sucrose with citric acid gave full protection against browning. The joint action of these two inhibitors seems to be additive. Finally,

complete inhibition of browning of Elberta peaches could be achieved with a 0.1% solution of sodium bisulfite.

Recently Developed Inhibitors of Enzymatic Browning. The past years have witnessed a boom in the development of new browning inhibitors. Part of them are now available on the market. Some of them are listed in Table IV along with their field of application tried so far.

     Aromatic Compounds. Aromatic carboxylic acids and substituted phenols (*29, 52*) have long been in use as inhibitors of PPO, mainly for kinetic studies of inhibition, but partly also for preventing browning with more or less success. 4-Hexylresorcinol (4HR) is one of the recently discovered, patented (*53*) and approved browning inhibitors of this class. It was first reported to inhibit shrimp black-spotting caused by PPO (*54*). 4HR was much more effective on the purified mushroom enzyme than on a crude extract and did not inhibit laccase (*55*). Apple slices (in syrup) required only 1/5 the concentration of 4HR of that necessary for achieving the same result with sulfite (*56*).

     Tropolone (2-hydroxy-2,4,6-cycloheptatrien-1-one) is a copper chelator slowly binding to the "oxy" form of the enzyme (*58*). It was found to be a substrate of horseradish peroxidase in the presence of H_2O_2 and is, therefore, assumed to be helpful in distinguishing this enzyme from PPO (*59, 60*).

     Kojic acid [5-hydroxy-2-(hydroxymethyl)-γ-pyrone] interacts with o-quinone formation from o-diphenols by decreasing O_2 uptake by the enzyme. It inhibits also the monophenolase activity of PPO (*62*).

     Glucosidated Substrates. (+)-Catechin 3'-O-α-D-glucopyranoside obtained from (+)-catechin by different enzyme-catalysed reactions (with cyclodextrin glucanotransferase, E.C.2.4.1.19 and soluble starch, and with a bacterial sucrose phosphorylase, E.C.2.4.1.7, respectively), proved to be a strong inhibitor of mushroom PPO (*63, 64*). As other PPO-substrates can be transformed by glucosidation to inhibitors as well (*64*), this group of compounds might be of interest for future research.

     Proteolytic Enzymes. The possibility of an enzyme, itself a protein, being attacked and inactivated by proteolytic enzymes is more than obvious. In spite of this, protease preparations have not been systematically tried for a long time as inhibitors of PPO and enzymatic browning. The observation that the contact with kiwi slices or puree inhibits enzymatic browning of otherwise susceptible commodities, drew the attention to this group of compounds. [Kiwi fruit is known to contain a highly active protease (actinidine, *65, 66*)]. Of the proteolytic enzymes tested so far mainly three plant proteases (ficin from figs, papain from papaya and bromelain from pineapple) proved to be effective. All the three proteases are sulfhydryl enzymes of broad specificity (*66*).

Table IV. Some Recently Developed and/or Tried Browning Inhibitors

Name of inhibitor	Chemical nature	Field of application	Remark	Reference
4-Hexyl-resorcinol	substituted phenol	mushroom, apples, etc.	90% inhibition achieved with 100 μM (crude mushroom PPO); delay of onset of browning obtained with 200 μM at 25 °C (apple slices in syrup)	55, 56
Tropolone	cycloheptatriene derivative	mushroom and grape PPO	causes biphasic inhibition at up to 30 μM concentration; both initial and constant rate of reaction depend on inhibitor concentration	57, 58
Kojic acid	γ-pyrone derivative	mushroom, apple, potato, shrimp, spiny lobster	especially efficient at inhibiting enzymatic L-DOPA oxidation	61, 62
(+)-Catechin 3'-O-α-D-gluco-pyranoside		mushroom		63, 64
Proteases	ficin, papain, bromelain (sulfhydryl enzymes)	apple and potato slices	the relative efficiency of the enzymes depends on treatment temperature and on the commodity	66
Carrageenans	sulfated polysaccharides	apple juice and dice	synergistic effect with citric acid; long-lasting inhibition	67, 68
Maltodextrins	oligosaccharides	ground apples	K^+-ions enhance inhibition	69
Honey	Peptide of M_w 600	apples, grape juice		70
Carbon monoxide		mushroom PPO	reversible inhibition of catecholase activity	71
Hypochlorites	Na- and Ca-salts	apple, potato slices	dips containing low concentrations (17.5-140 ppm) of inhibitor	72

Carbohydrates. A number of carbohydrate derivatives were found to be effective in preventing enzymatic browning of foods. Carrageenans, a group of naturally occurring sulfated polysaccharides, but also amylose sulfate and xylan sulfate were reported to inhibit, in low concentrations (less than 0.5%), browning of unpasteurized apple juice and diced apples. The inhibitory effect could be synergistically enhanced by citric acid (0.5%) (*67, 68*).

The inhibitory effect of maltodextrin (DE 10) was enhanced by K^+ and assumed, therefore, to be related to pyruvate kinase in apples (*69*).

Peptides. PPO activity and browning were found to be inhibited by honey. Experiments with model solutions showed PPO inhibition to be non-competitive with (-)-epicatechin as substrate and enzyme activity could be progressively inhibited with time by preincubation in solutions of honey (*70*). The inhibitory effect was due to a peptide of MW 600.

Carbon Monoxide. CO gas atmosphere was found to inhibit mushroom PPO reversibly, whereby it prevented self-inactivation of the enzyme (*71*). The latter feature of this inhibitor might be useful in studies on PPO action. However, CO - as a substance harmful to human health - does not seem to be of any practical use in preventing browning of food materials.

Hypochlorite. Sodium and calcium hypochlorites in low concentrations (down to 17.5 ppm) were reported to inhibit enzymic browning of green beans, apples and potatoes. These compounds obviously act on the enzyme protein (*72*). It is, however, improbable that this potent disinfectant should ever be approved for use in foods.

Miscellaneous Browning Inhibitors. A number of browning inhibitors were found by researchers by observation or trial-and-error methods. The chemical nature of these inhibitors is mostly unknown.

Good results were achieved with pineapple juice in treating apple rings. The juice was treated in different ways, whereby the best results were achieved with a cation exchanged fraction of the original juice. This fraction contained ascorbic acid (0.1 mg/ml), phenolics (0.41 mg/ml) and amino acids (formol value: 0.64 meq/100 ml) and ensured 100% inhibition for at least 12 h. Pineapple juice was effective with fresh apple rings kept in air or vacuum-packed as well as with dried apples. The inhibitor in pineapple juice was found to be a neutral, low-molecular substance (*73*).

A patent has been recently granted for an inhibitor obtained by repeated filtration of a fig latex suspension. The inhibitor is claimed not to contain any fig protease (ficin) and to inhibit enzymic and non-enzymic browning, e.g. of mushrooms, wine and shrimps. An MW < 5000 was established for its active principle (*74*).

Acetone soluble fractions (ASF) prepared from various fruits (plums, peaches, apples, pears and grapes) were found to act on the PPO activity of these fruits as measured with chlorogenic acid in a selective way. For example, ASF from grapes inhibited the enzyme from peaches, apples and grapes: ASF from plums inhibited PPO

from peaches and apples, but accelerated the reaction catalysed by the enzyme from pears, plums and grapes, while ASF from peaches and apples showed only a stimulating action (75).

Maillard reaction products obtained by heating a solution containing glucose and glycine were found to inhibit PPO and also peroxidase activity (76).

Stabilization of Fruit Juices Against Enzymatic Browning. The inhibition of the enzymatic oxidation of endogenous phenols in fruit juices represents a special problem. Non-enzymic oxidation of the phenols in the juice using an air-flow (bubbling) with subsequent removal of the colored macromolecular products by filtration has long been practised by juice manufacturers for obtaining clear and stable products. Recent research has led to the development of a fungal laccase preparation (by fermentation using the white-rot fungus *Trametes/Polyporus versicolor*, 77). The enzyme immobilized on agarose-based activated matrices was reported to lend itself to the removal of phenolics from white grape musts and wines. The immobilized enzyme-reactor could be reused at least 8 times, and showed practically no losses of activity when properly stored (78).

Another method of browning control is based on the addition of chitosan to apple or pear juice prior to filtration through diatomaceous earth. For the treatment of McIntosh apples about 200 ppm chitosan proved efficient, whereas Bartlett and Bosc pears required 1000 ppm. Chitosan proved to be inefficient with very ripe pears and could not be used if the juices were to be centrifuged (79).

A recent patent reports on trapping phenols of raw fruit and vegetable juices using soluble or insoluble cyclodextrins. The latter could be used also as column fillings (80).

Traditional and Recently Developed Physical Methods for Inhibitng Enzymatic Browning

Reports on the use of traditional methods of browning prevention by heat treatment can still be found in the literature.

Blanching. Water blanching was used to prevent enzymatic darkening in frozen sweet potatoes. Treatment at 100 °C for 3 min or at 94 °C for 5 min gave satisfactory protection against darkening without reducing the phenol levels (81).
Blanching in boiling water was used also for preserving the green color in dried pepper. Longer boiling times (15 min) resulted in better color retention for berries. Microwave heating alone was insufficient and did not yield berries of lighter color as compared to sun-drying. Microwave heating combined with blanching was found to be most efficient for enzyme inactivation and permitted reduced treatment times (82).

Ultrafiltration. Enzymic browning of fluids can be reduced by ultrafiltration (UF). A study of wine treatment by UF at different molecular weight cut-offs between 100,000

and 30,000, before or after fermentation showed the results to be cultivar-dependent. Browning during storage was found to be dependent also on the headspace in the bottles (*83*).

Sonication. Recent studies have been aimed at combining heat treatment with ultrasonic waves in order to reduce the heat resistance of mushroom PPO.   The simultaneous application of heat and ultrasonic waves had a synergistic effect on enzyme inactivation. The efficiency of the procedure was reported to increase with increasing amplitudes of the ultrasonic waves. This manifested itself in the decrease of D values (decimal reduction times at constant temperature). The process named manothermosonication needs, however, special and probably expensive equipment and might be of use, in the first place, with commodities that are damaged by drastic heat treatment (*84*).

Supercritical carbon dioxide. Supercritical carbon dioxide (SC-CO_2) treatment was also tried for inactivating PPO from potato peel, fresh Florida spiny lobster and fresh brown shrimp. The purified enzyme preparations dissolved in a pH 5.3 buffer showed but slight losses of activity (5% for potato peel PPO) when heated at 43 °C for 30 min. Treatment of the enzymes with high-pressure (58 atm) CO_2 caused a dramatic loss of activity: after 1 min, the residual activity of potato PPO, the most resistant of the three preparations, was only 45%. However, 30 min were required for an activity loss of 91%. The pH of the potato PPO solution was reduced from 6.1 to 4.1. The treatment caused changes in the isoenzyme composition of all the three PPO-s and compositional changes in secondary structure. These were slightest for the most resistant potato enzyme. The SC-CO_2-treated potato-enzyme regained 28% of the original activity in the first 2 weeks of 6 weeks of frozen storage. This activity then gradually decreased as time progressed. The pH of the enzyme solution returned to its original value (*85*).

With the exception of ultrafiltration, (which is applicable only to fluids) all the procedures dealt with in this last section also involve heat treatments. This restricts their application to products consumed in the cooked, stewed, fried, etc. state. Moreover, most of them require sophisticated equipment. It seems, especially with fruits, that inhibition with chemicals will play the primary role in the prevention of enzymatic browning also, at least, in the near future. However, as alien additives are considered ever more undesirable in food for health reasons (justified or exaggerated) the search for other methods will, most probably, go on. As the "appropriate agrotechnics" mentioned in the introduction also involve the use of chemicals, these might be banned sooner or later as well. Cultivar selection might remain promising in the future, too, and great hopes might be set on the genetic modification of PPO in various commodities. Research in this direction is in progress in several countries with promising results (see the respective papers in this volume).

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Chapter 5

Tyrosinase: Molecular and Active-Site Structure

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Tyrosinase, a widely distributed copper-containing monooxygenase, is a key enzyme in the biosynthesis of melanins and other polyphenolic compounds. It catalyzes both the hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the oxidation of *o*-diphenols to *o*-quinones (catecholase activity). In many plants, the enzyme is responsible for the undesirable browning reaction, occurring particularly during the processing of fruits and vegetables. Tyrosinase has been isolated from a variety of sources, but pigment contamination and the occurrence of multiple forms have frequently hampered its characterization. Progress in the last decade has, however, allowed the construction of a rather detailed picture of the molecular structure and the reactivity of tyrosinase. In particular, the interaction of the unique binuclear copper complex in this enzyme with O_2, substrates and inhibitors is now well understood at the molecular level. Moreover, comparison of the cloned DNA sequences of tyrosinases from many different species (bacteria, fungi, plants and mammals) revealed two conserved histidine-rich regions, involved in the binding of the active site copper.

Tyrosinase is a copper-containing monooxygenase which catalyzes the *o*-hydroxylation of monophenols (equation 1) and the oxidation of *o*-diphenols to *o*-quinones (equation 2).

1)

2) $2 \quad$ (benzene ring with R at top, OH at bottom positions) $\quad + O_2 \longrightarrow 2 \quad$ (benzene ring with R at top, two =O at bottom) $\quad + 2 H_2O$
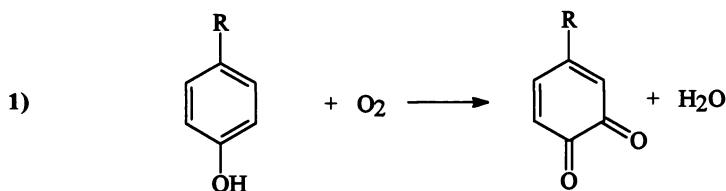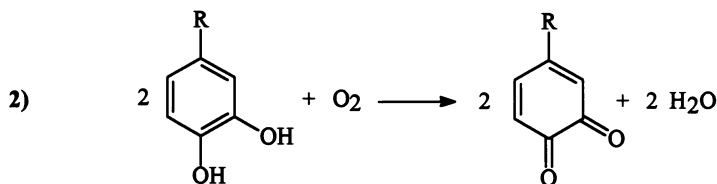
The two enzymatic activities are commonly referred to as **cresolase** or **monophenolase** activity and **catecholase** or **diphenolase** activity, respectively (*1*). The two-electron donor required in the hydroxylation reaction is the *o*-diphenol which is generated internally from the monophenol substrate.

Tyrosinase is widely distributed in microorganisms (bacteria and fungi), plants and animals, where it is involved in the biosynthesis of melanins and other polyphenolic compounds (*1*). In mammals, L-tyrosine is the initial substrate in the pathway leading to the final products of black-brown eumelanins, red-yellow pheomelanins or a mixture of pheo- and eumelanins (*2*). In microorganisms and plants a large number of structurally different monophenols, diphenols and polyphenols serve as substrates for tyrosinase. As many plants are rich in polyphenols, the name polyphenoloxidase has been frequently used for this enzyme (*3*).

Tyrosinase has been isolated from a variety of sources, but pigment contamination and the occurrence of multiple forms have frequently hampered its characterization. The pertinent molecular and chemical properties of well characterized tyrosinases are displayed in Table I.

Table I. Molecular and Chemical Properties of Tyrosinases

Species	M_r (kD)	Subunit M_r (kD)	% Cu	Reference
Microorganisms				
Streptomyces glaucescens	29	29	0.43	4
Agaricus bisporus	120	13.4 (L)[a]	0.19	5
		43 (H)[a]		
Neurospora crassa	120	46	0.29	6
Plants				
Solanum tuberosum	290	36	0.20	7,8
Beta vulgaris	40	40	0.31	9
Animals				
Rana pipiens	200	54	0.15	10
Homo sapiens	66.7	66.7	0.20	11

a) This tyrosinase consists of two different subunits: L (light) and H (heavy)

Depending on the source of the enzyme, the M_r's vary between 29,000 and 200,000 with subunit M_r's from 29,000 to 67,000. Until now, the enzyme from the bacterium *Streptomyces glaucescens* was found to have the smallest functional unit with one copper pair per polypeptide chain of 29,000. Tyrosinases from microorganisms and plants are generally present as soluble proteins; in contrast those from mammals are membrane-associated with specific organelles (melanosomes) in cells termed melanocytes (*12*).

Amino Acid Sequences of Tyrosinases

Tyrosinases are generally present in low concentrations in all organisms studied so far. Furthermore, they are difficult to obtain in pure form due to pigment contamination and the occurrence of multiple forms. It is therefore not surprising that up to date the enzyme from only one species (*Neurospora crassa*) has been sequenced by classical protein chemical means (*13*). With the advent of recombinant DNA technology, however, numerous amino acid sequences have become available, recently (*14, 15-24*). These include tyrosinases from the bacterium *Streptomyces glaucescens* (*15*) and *Streptomyces antibioticus* (*16*), from several plant species such as tomato (*17, 18*), broad bean (*19*) and potato (*20*) as well as from mouse (*14, 21*) and man (*22-24*). All tyrosinase molecules sequenced so far are single-chain proteins with calculated M_r's of 30,900 (273 amino acid residues) for *S. glaucescens* and *S. antibioticus*, 46,000 (407 amino acid residues) for *N. crassa*, 68,000 (606 amino acid residues) for the broad bean *Vicia faba*, 66,300 (587 amino acid residues) for tomato and 67,000 (588 amino acid residues) for potato, 5,900 (480 amino acid residues) and 58,550 (515 amino acid residues) for mouse and 58,000 (515 amino acid residues) and 6,600 (548 amino acid residues) for man. Amino acid sequence comparison of the different tyrosinases shows that they are structurally related. The sequence identity is 24% between bacterial and fungal tyrosinase and 26% between mouse and *N. crassa* tyrosinase. A comparison of the four types of tyrosinase (bacterial, fungal plant, and mammalian) reveals a sequence identity of only 8.7%. However, there are two stretches in the molecules which have values of more than 3% suggesting that they may play an important role in the active-site structure.

Posttranslational Modification of Tyrosinases

Tyrosinases from eucaryotic organisms are subject to substantial post-translational modifications. Thus, tyrosinase from *N. crassa* was found to be synthesized as a precursor with a very large C-terminal extension of 200 amino acids (*24*). It has been suggested, that this extension could be involved in the activation of a protyrosinase by limited proteolysis as shown in Figure 1.

The cloning of several tyrosinase genes from plant demonstrated that the enzyme is synthesized as precursor with an N-terminal extension of ca 10 kD (Figure 1). It has been suggested that this extension serves as a transit peptide which post-translationally directs the protein to the chloroplast envelope (*17-20*). Limited proteolytic cleavage then leads to the correct size of the mature enzyme.

Mammalian tyrosinases (mouse and man) are synthesized as proforms containing a typical signal peptide at the amino-terminal ends (*14, 21-24*). This peptide is required for the translocation of the molecule into the melanosomes, the sites of melanin biosynthesis. In addition, mammalian tyrosinases are glycosylated (5-6 putative glycosylation sites) and are rather rich in cysteine. From 17 residues, 10 are clustered within the first 100 amino acid residues and 5 are found in the middle of the molecule. In the form of disulfides, these residues contribute significantly to the unusually high resistance of mammalian tyrosinases towards digestion by proteases.

Copper-binding Regions of Tyrosinase and Hemocyanin

Our understanding of tyrosinase structure has been greatly enhanced by solution of the X-ray crystal structure of hemocyanin from the spiny lobster *Panulirus interruptus* (*28*). As shown in Figure 2, the two copper ions, designated as CuA and CuB, are each coordinated by three histidines, which originate from four α-helices. Two of the ligands of CuA and CuB come from successive turns of helix 2.1 and 2.5, respectively. The third ligand of each copper is provided by the remaining 2 helices.

Sequence comparison in the CuB region with different tyrosinases and a molluscan hemocyanin from *Octopus dofleini* (*26*) shows a highly conserved region of 56 amino acids (Figure 1). The invariant and isofunctional residues comprise the three histidines known to be ligands to CuB in *P. interruptus* hemocyanin (*28*). As shown in Figure 3a, two of these are separated by three amino acids whereas the third one is located 37 residues towards the C-terminus. The flanking sequences of the third histidine residue show a high degree of sequence similarity and are characterized by numerous large aliphatic and aromatic residues. The remarkable sequence homology of this 56 residue region strongly suggest that these three histidine residues constitute the CuB ligands in all six proteins quoted in Figure 1 and 3a. This conjecture is supported both by active-site directed inactivation of *N. crassa* tyrosinase (*29*) and site-specific mutagenesis experiments (*30, 31*) of *S. glaucescens* tyrosinase. From these studies, the third histidine (His306) of *N. crassa* tyrosinase was found to be specifically destroyed in a mechanism-based inactivation process with the concomitant loss of one copper (*29*). Substitution of His189 by Asn, His193 by Gln, and His215 by Gln through site-specific mutagenesis of *S. glaucescens* tyrosinase resulted in inactive enzymes. In the case of His189 and His193, the substitution led to enzyme species retaining only one copper per molecule. In contrast, no copper was lost, when His215 was replaced (*30, 31*).

In contrast to the situation of CuB, the sequence comparison doesn't reveal a corresponding common structure for CuA. The arrangement of the three histidines in a helical pair found also for CuA in *P. interruptus* hemocyanin is not found in the four tyrosinases and *O. dofleini* hemocyanin. In *N. crassa* tyrosinase His188 and His193 have been implicated as copper ligands by photochemical oxidation (*33*). This segment shows some resemblence to the Cu-A site of arthropodan hemocyanins, although the two histidines are separated by four rather than three amino acid residues. Surprisingly, these histidine residues are absent in *S. glaucescens* and mouse tyrosinase as well as in molluscan hemocyanins. The tyrosinases and molluscan hemocyanins, however share a region with a rather high degree of sequence similarity (Figure 1 and 3b). Furthermore,

Cu A   Cu B

Ty S. g.

Ty N. c.

Ty L. e.

Ty M. m.

Hc O. d. e

Hc P. i. a
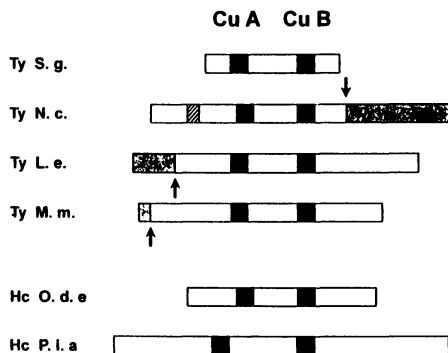
Figure 1.   Schematic representation of the polypeptide chains of tyrosinase from *Streptomyces glaucescens* (*15*), *Neurospora crassa* (*13, 25*), *Lycopersicon esculentum* (*17*), *Mus musculus* (*14*), and hemocyanin from *Octopus dofleini* e (*26*) and *Panulirus interruptus* (*27*). The solid bars indicate the regions of the CuA and CuB sites. The alignment of the six polypeptide chains is based on the conserved regions of the CuB site. The hatched bar represents a non-functional, ancient CuA site in *N. crassa* tyrosinase. The shaded areas indicate extensions which are post-translationally removed by limited proteolysis (site of cleavage is shown by an arrow).

His-198

His-194 Cu_A His-348

Cu_B

His-344

His-224

His-384
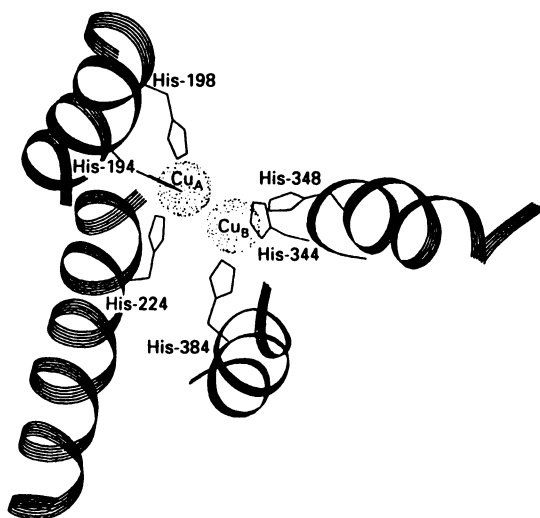
Figure 2.   Active-site structure of hemocyanin from the spiny lobster *P. interruptus*. The copper ions are ligated by 6 histidines which are provided by 4 helices of the second domain. (Reproduced with permission from ref. 32. Copyright 1992. Biochemical Society and Portland Press.)

```
                        +     +                              +
Ty S.g.     184    LHNRVHV   9   MSPNDPLFWLHHAYVDRLWAEWQ
                                                 ♦
Ty N.c.     272    VHNEIHD   9   VSAFDPLFWLHHVNVDRLWSIWQ

Ty L.e.     196    PHTPVHI  18   SAGLDPIFYCHHANVDRMWNEWK

Ty m.M.     344    LHNALHI  10   GSANDPIFLLHHAFVDSIFDQWL

Hc O.d. e 173    AHNAIHS  14   YAAYDPIFYLHHSNVDRLWVIWQ

Hc P.i. a 346    LHNTAHV  21   TATKDPSFFRLHKYMDNIFKKHT
                    *     *                            *
```

↑

Figure 3a.   Alignment of amino acid sequences around the CuB site in Ty's and a molluscan and an arthropodan Hc. Histidine residues identified as copper ligands in *Panulirus interruptus* hemocyanin (*28*) are marked with an asterisk. + indicates histidines changed to asparagine or glutamine, respectively by site-directed mutagenesis of *Streptomyces glaucescens* tyrosinase (*30, 31*). The diamond represents a histidine which is destroyed by active-site inactivation of *Neurospora crassa* tyrosinase (*29*). Arrows indicate amino acid residues which were deleted to obtain optimal alignment of the sequences. Identical residues are printed in bold type, conservative substitutions in italics. The following proteins are compared: Ty S.g. (tyrosinase from *Streptomyces glaucescens*)[49], Ty N.c. (tyrosinase from *Neurospora crassa*, ref. 13, 25), Ty L.e. (tyrosinase from *Lycopersicon esculentum*, ref. 17) and Ty M.m. (tyrosinase from *Mus musculus*, ref. 14), and hemocyanin O.d. e (hemocyanin from *Octopus dofleini*, ref. 26) and Hc. P.i. a (hemocyanin from *Panulirus interruptus* subunit a, ref. 27).

```
                        +                  +           +
Ty S.g.    30    YDEFVTTHN  10   TGHRSPSFLPWHRRYLLEFERALQ

Ty N.c.    59    YYQVAGIHG  24   CTHSSILFITWHRPYLALYEQALY

Ty L.e.   172    FKQQANIHC  14   QVHFSWLFFPFHRWYLYFYERILG

Ty M.m.   155    YDLFVWMHY  16   FAHEAPGFLPWHRLFLLLWEQEIR

Hc O.d. e  39    FEAIASFHA  14   CLHGMATFPHWHRLYVVQFEQALL
```

↑

3b.   Alignment of amino acid sequences around the putative CuA site in tyrosinases and a molluscan hemocyanin (*26*). Cys94 is covalently linked to His96 in *N. crassa* tyrosinase (see Figure 1). The same code is used as in Figure 3a.

.ch005

this stretch contains several invariant histidine residues. Replacement of His37 by Gln, His53 by Gln and His 62 by Asn via site-directed mutagenesis of *S. glaucescens* tyrosinase led to inactivation of the enzyme with the concomitant loss of one copper per molecule (*30, 31*). It is therefore likely, that the same histidines also serve as ligands to CuA in plant and mammalian tyrosinases and molluscan hemocyanins.

Structure of the Copper Active Site

A structural similarity between the copper active sites of tyrosinase and hemocyanin was recognized as early as 1938 (*7*). However, major progress in understanding their structural details was only made in the course of the last decade. First direct evidence for the presence of a binuclear copper center in the two proteins was provided by EPR spectroscopical studies (*34*). In the mean time, a wealth of structural information about the binuclear copper centers has been gained by various spectroscopic methods. A range of derivatives have been prepared and extensively studied (*35, 36*), leading to a detailed model of the active site copper complex.

Three different functional states of tyrosinase can be distinguished: **met, deoxy** and **oxy**. In a 2e⁻ step mettyrosinase is converted to the deoxy form, which binds molecular oxygen reversibly. The interrelationship of the three forms and their active site structural models are shown on Figure 4. All three forms are EPR non-detectable. In the following the key physicochemical properties of these forms will be outlined.

Mettyrosinase. The binuclear copper site of mettyrosinase consists of two tetragonal Cu(II) ions bridged by one or two ligands (Figure 4). The bridge, most likely formed by hydroxo ions (OH⁻) accounts for the diamagnetic state of this form by providing an effective pathway for superexchange between the two Cu(II) ions (*35, 36*). The bicupric nature of mettyrosinase is also consistent with the absorption and circular dichroism features attributable to Cu(II) d-d transitions (Figure 5). EXAFS studies indicated a Cu-Cu distance of 3.39 Å for *N. crassa* tyrosinase (*37*).

Oxytyrosinase. The oxy form can be obtained by treatment of mettyrosinase with reducing agents (Figure 4) in the presence of molecular oxygen. Besides hydrogen peroxide, a number of different reducing agents such as hydroxylamine, ascorbic acid, sodium dithionite, sodium borohydride and *o*-diphenols have been used.

The highly unusual and unique spectral features of oxytyrosinase have been studied in great detail by many different techniques. Thus, Resonance Raman spectroscopy has shown that in oxytyrosinase (*38*), dioxygen is bound as peroxide to the binuclear site (Figure 4). These data imply that the two copper ions are in the cupric oxidation state. Like the met form, oxytyrosinase is EPR non-detectable and shows strong antiferromagnetic coupling between the two Cu(II) ions as demonstrated by magnetic susceptibility studies (*39*). The Cu-Cu distance measured by EXAFS spectroscopy was found to be 3.63 Å, a value slightly larger than the one for mettyrosinase (*37*).

The absorption and circular dichroism spectra of oxytyrosinase (Figure 5) are characterized by a prominent band at 345 nm ($\epsilon \sim 17'200$ M⁻¹cm⁻¹) and several bands in the visible region. These absorption features are absent in the met form and hence have
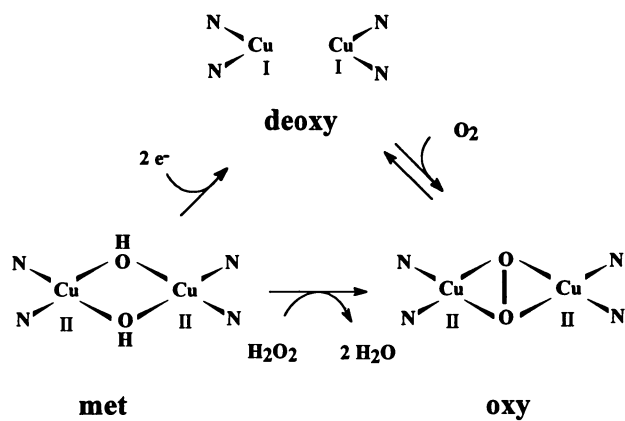
Figure 4.   Interrelation of the three functional states of tyrosinase
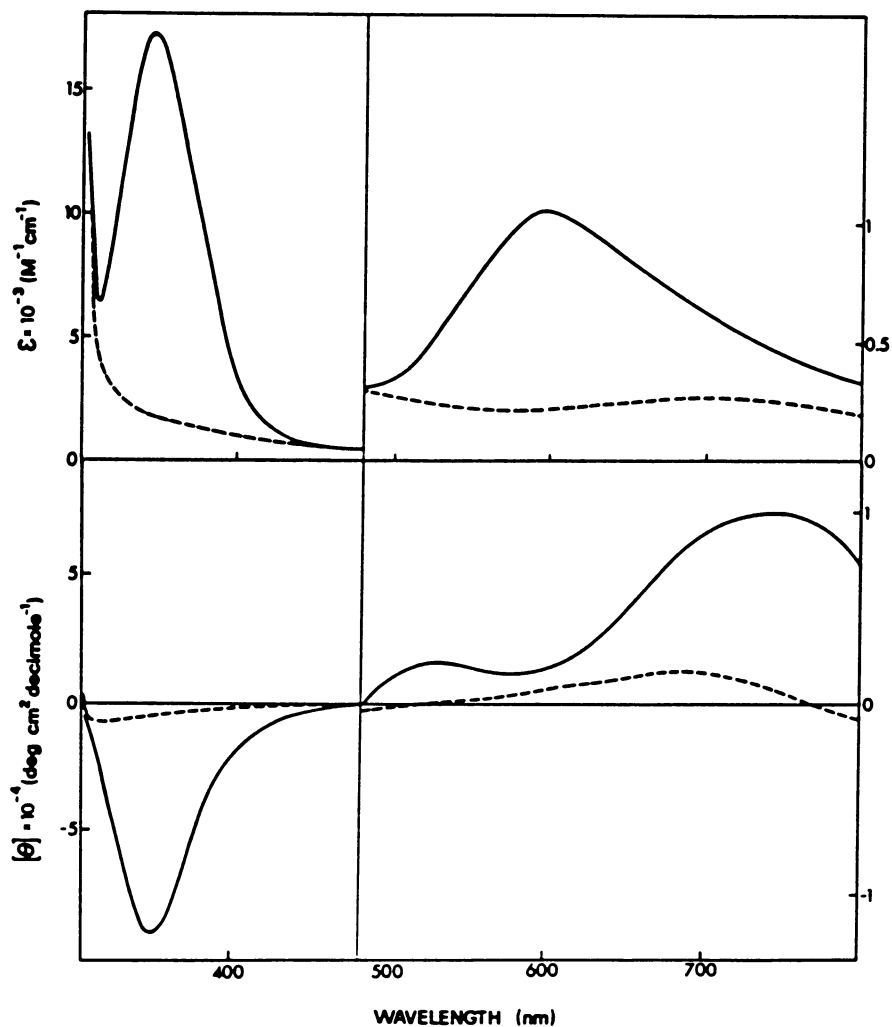
Figure 5.   Absorption and circular dichroism spectra of *N. crassa* oxy (—)
and mettyrosinase (----). Adapted from ref. 40.

been assigned as peroxide-to-Cu(II) charge transfer transitions (*35, 36, 40*).

From a recent X-ray structural analysis of *Limulus polyphemus* oxyhemocyanin a μ-η^2:η^2 side-on peroxo coordination has been found (*41*). In this study a Cu-Cu distance of ca. 3.6 Å was found, in good agreement with the values obtained by EXAFS spectroscopy (*42*). It is very likely, that the same side-on peroxo complex is also present in oxytyrosinase.

Deoxytyrosinase. In contrast to met- and oxytyrosinase the deoxy form is devoid of spectral features in the visible region. Hence little information on the electronic and geometric structure is available. Consistent with the lack of an EPR signal, deoxytyrosinase was assigned a bicuprous Cu(I) Cu(I) structure (*35, 36*).

Besides reacting with molecular oxygen reversibly, deoxytyrosinase also binds one carbon monoxide per molecule (*43*). The CO-complex absorbs at 300 nm and, on irradiation at 295 nm, emits an intense luminescence (λ_{max} = 550 nm).

Half-mettyrosinase. When metttyrosinase is treated with an excess of sodium nitrite and ascorbic acid, an EPR-detectable half-met form can be obtained (*40*). This half-met-NO_2^- derivative shows an EPR signal typical for a mononuclear Cu(II) ion with signal intensity accounting for ca. 50% of the total copper (Figure 6a). By ligand substitution of half-met-NO_2^- tyrosinase a series of new half-met have been generated (*40*).

Ligand Interaction with Oxy- and Half-mettyrosinase

Although the binuclear copper active sites of tyrosinase and hemocyanin are structurally very similar, distinct differences between the copper centers of the two proteins are apparent from peroxide displacement studies with different ligands. Both azide and the competitive tyrosinase inhibitor L-mimosine have been shown to displace the bound peroxide in an associative displacement mechanism leading to the met-azide and the met-L-mimosine derivatives, respectively (*40*). By following the absorption band at 345 nm of oxytyrosinase and 340 nm of oxyhemocyanin (*40, 44*), pseudo-first order rate constants for the two ligands were determined. The values obtained unambiguously demonstrated that the tyrosinase active site is much more accessible to large ligands such as organic substrates and inhibitors than the one of hemocyanin. Tyrosinase can therefore be viewed as a hemocyanin with an exposed binuclear active site. This idea is consistent with the results of the three-dimensional structure of *P. interruptus* hemocyanin, showing a highly buried copper site (*28*).

When half-met-NO_2^- tyrosinase is treated with L-mimosine, a new half-met complex is formed with very unusual EPR properties (*44*). The EPR spectrum of half-met-L-mimosine tyrosinase (Figure 6b) is characterized by a large rhombic distortion and a fourline splitting pattern in the perpendicular region. These spectral features related to a significant distortion of the Cu(II) site from a tetragonal toward a trigonal bipyramidal geometry. As will be discussed below, this change of geometry is an important step in the hydroxylation pathway of tyrosinase.
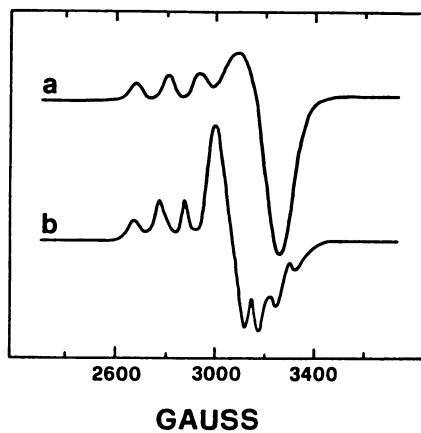
Figure 6.   EPR spectra of half-met-NO_2^- (a) and half-met-L-mimosine (b) tyrosinase from *N. crassa*. Adapted from ref. 44 and 47.

Reaction Mechanism of Tyrosinase

Based on the information of the electronic and geometric structure of the binuclear copper complex and on the results how different anions and organic ligands interact with the site, the following reaction mechanism of tyrosinase was proposed (*44*).
In Figure 7, both the hydroxylation reaction pathway (**cresolase activity**) and the oxidation reaction pathway (**catecholase activity**) are shown.

The catecholase activity involves the oxidation of two molecules of *o*-diphenol to two molecules of *o*-quinone with the concomitant 4e⁻ reduction of molecular oxygen to two molecules of water. For this reaction, the binuclear copper site in met- and oxytyrosinase is geometrically correct for axial coordination of both orthophenolic oxygens to the Cu(II) ions with a Cu-Cu distance of 3.4 - 3.6 Å. This would allow for an efficient 2e⁻ transfer from the *o*-diphenol substrate to the binuclear site. As shown in Figure 7 A, the binding of an *o*-diphenol to mettyrosinase and the subsequent reduction of the bicupric cluster leads to deoxytyrosinase which yields the oxy form upon binding of molecular oxygen. The binding of a second *o*-diphenol to oxytyrosinase and the subsequent reduction of the bound peroxide to water closes the catecholase cycle. The involvement of oxytyrosinase as a catalytic intermediate during the oxidation reaction of *o*-diphenol has been documented by stopped-flow kinetic measurement (*45*). In a recent study, using rapid scanning photometric techniques, the formation of oxytyrosinase as a transient species has been directly confirmed. The overall rates for the two half reactions (met → oxy; oxy → met) were found to be very similar (*46*).

For the cresolase activity (Figure 7 B), axial coordination of a monophenol to one copper of the binuclear site of oxytyrosinase leads to the formation of a ternary $(Cu^{II})_2O_2^{2-}$-substrate complex. Rearrangement of this complex through a trigonal bipyramidal intermediate would then be followed by *ortho*-hydroxylation of the monophenol. As pointed out above, the protein substrate pocket makes a significant contribution in positioning the monophenol and stabilizing the substrate copper complex in a geometry suitable for hydroxylation. The substrate-induced rearrangement leads to an asymmetrically coordinated peroxide, which could attack the monophenol substrate either directly or via an initial O-O bond cleavage process. As depicted in Figure 7 B, the hydroxylation is followed by the formation of an equatorially bound hydroxyde and an *o*-diphenol. Intramolecular electron transfer then leads to the release of an *o*-quinone and water. The concomitantly formed deoxytyrosinase is finally regenerated to the oxy form.

From kinetic investigations, it was found that the oxidation of *o*-diphenols to *o*-quinones by tyrosinase has less geometric and electronic requirements than the hydroxylation of monophenols (*47*). Monophenols with bulky substituents were hydroxylated very poorly by tyrosinase, indicating steric hindrance of the substrate to undergo an axial-to-equatorial rearrangement. In contrast, variation of the substituents of *o*-diphenol substrates had only little influence on their oxidation rates.

Substrates and Inhibitors

Substrates.   Monophenols and *o*-diphenols have been considered as the exclusive
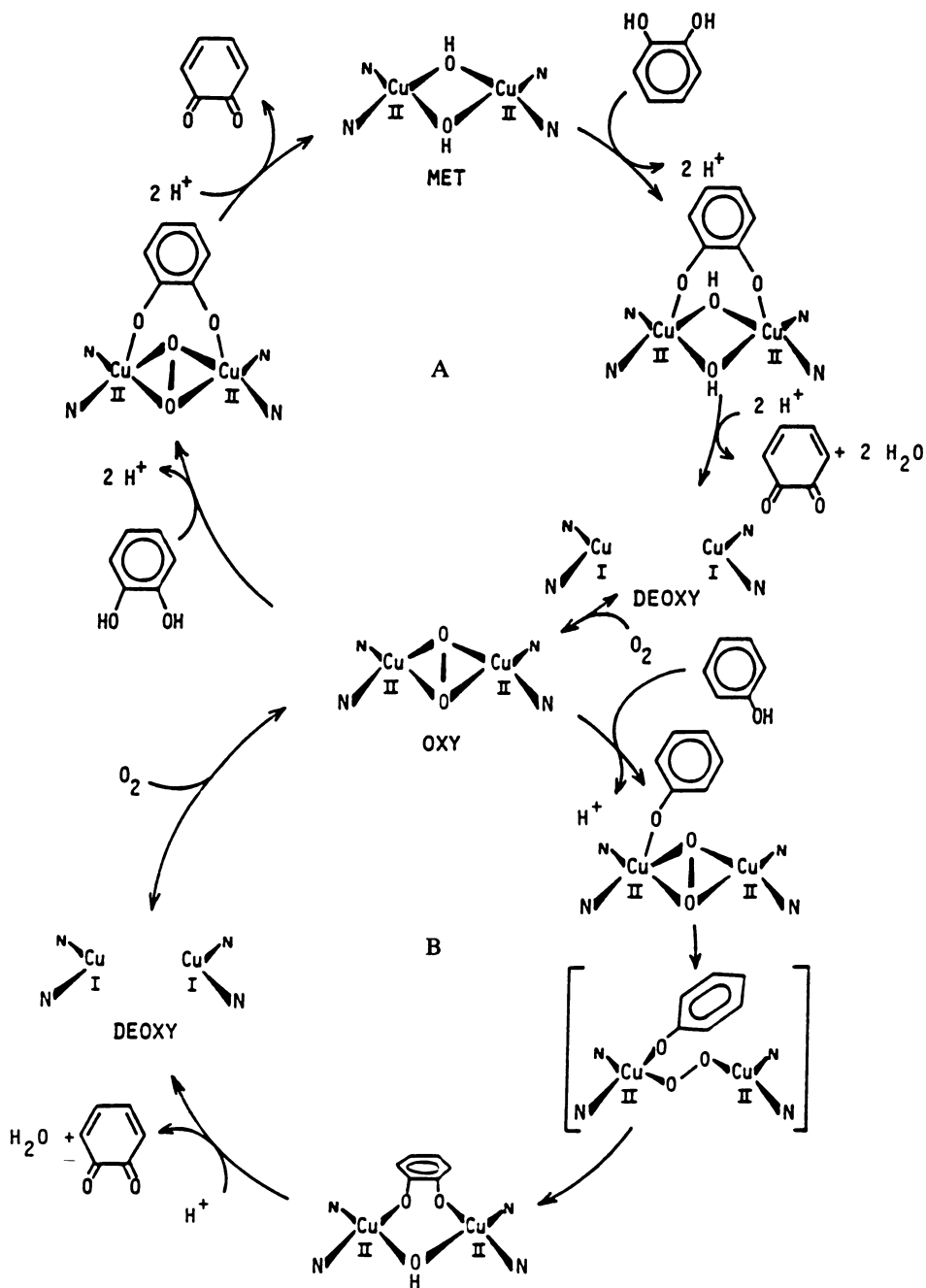
Figure 7.　Mechanistic scheme of the catecholase (A) and the cresolase (B) activity of tyrosinase. Adapted from ref. 47.

substrates of tyrosinase for a long time. In a recent study, the reaction of tyrosinase was studied with two new classes of substrates: aromatic amines and *o*-aminophenols, structural analogues of monophenols and *o*-diphenols, respectively (*48*). They were found to undergo the same catalytic reactions (*ortho* hydroxylation and oxidation), as documented by product analysis and kinetic studies. In the presence of tyrosinase, arylamines are converted to *o*-aminophenols (hydroxylation) and subsequently oxidized to *o*-quinoneimines (Equation 3). The resulting *o*-quinoneimines are isolated as quinone anils or phenoxazones.
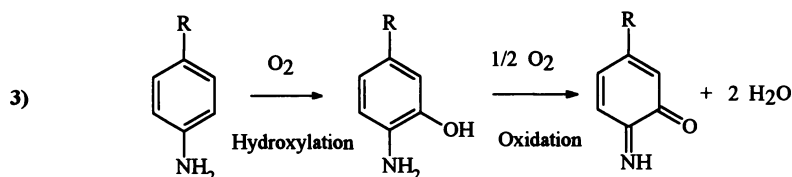
As an example, 2-amino-3-hydroxybenzoic acid is converted to cinnabarinic acid, a well-known phenoxazone (Scheme 1), while p-amino-toluene gives rise to the formation of 5-amino-2-methyl-1,4-benzoquinone 1-(4-methyl-anil) (Scheme 2).
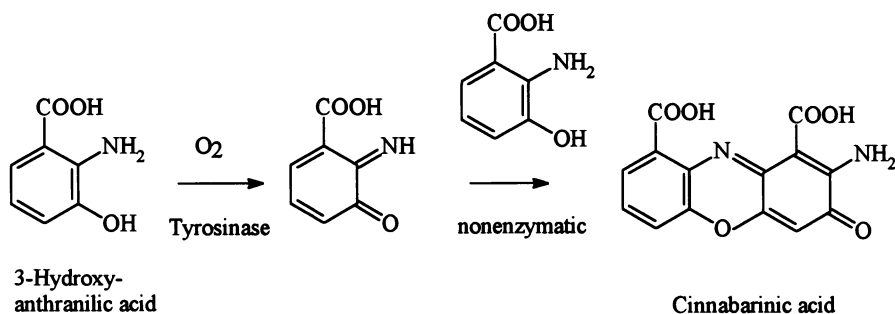
Scheme 1 Formation of cinnabarinic acid from 3-hydroxyanthranilic acid
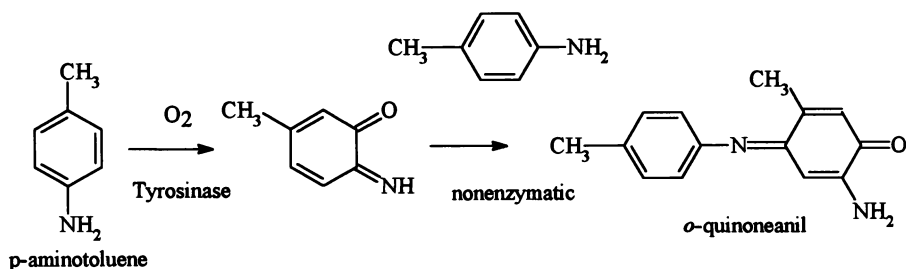
Scheme 2 Formation of *o*-quinoneanil from p-aminotoluene

Kinetic studies using an oxygen electrode showed that arylamines and the corresponding monophenols exhibit similar Michaelis constants (K_m = 0.11-0.49 mM). In contrast, the reaction rates observed for aromatic amines are relatively slow (k_{cat} = 1 - 3 min^{-1}) as compared to monophenols (1320 - 6960 min^{-1}). The oxidation rates, k_{cat} for o-aminophenols were found to be similar to the value for 2,3-dihydroxybenzoic acid (0.2 - 0.8 s^{-1}). The large differences observed for the hydroxylation rates between monophenols and arylamines have been ascribed to markedly different pK_A values of the two classes of molecules. As can be seen in Figure 7 B, the coordination of a monophenol (or arylamine) to one of the two Cu(II) ions requires a deprotonation step of the hydroxy (or amino) group of the substrate. Hence, the known difference of 10 - 15 pH units could easily explain the sharp decrease in the reaction rates of arylamines as compared to monophenols.

Inhibitors.   A large number of different compounds are known to inhibit tyrosinase activity (*35, 36, 49*). To the classical types of tyrosinase inhibitors belong small ligands such as cyanide, carbon monoxide, azide, fluoride, acetate, which coordinate to either Cu(I) or Cu(II). In addition, the enzyme is strongly inhibited by sulfur containing compounds (*49*) such as H_2S, 2-mercaptoethanol, L-cysteine, diethyldithiocarbamate, phenylthiourea, etc. As was shown in a kinetic and spectroscopic study, one mercaptan molecule reduces in a first step one of the two Cu(II) ions of mettyrosinase followed by the binding of a second mercaptan molecule to yield a half-met-mercaptan derivative (*49*). A third class of tyrosinase inhibitors are organic substrate analogs like L-mimosine, benzoic acid, benzhydroxamic acid, pyridones, etc. Chemical and spectroscopic studies have shown that these compounds bind to the binuclear copper active site in the half-reduced (half-mettyrosinase) or oxidized (mettyrosinase) state (*40, 44*).

During the oxidation of catechol to o-quinone, tyrosinase was found to undergo a peculiar, irreversible inactivation (*50*). The kinetic data of the process (first-order kinetics) are consistent with the idea that the inactivation occurs concomitantly with the oxidation of catechol to o-quinone in the second half reaction of the catecholase activity (see Figure 7 A and Scheme 3). Depending of the source of the enzyme k_{cat}/k_{inh} ratios of 5 x 10^3 (tyrosinase from *N. crassa*, ref. 29) and 17.5 x 10^4 (tyrosinase from *A. bisporus*, ref. 51) were found.

$$(Cu^{II})_2\, O_2^{-2} \quad + \quad C \;\rightleftharpoons\; (Cu^{II})_2\, O_2^{-2}\, C \;\rightarrow\; [X] \;\xrightarrow{k_{cat}}\; (Cu^{II})_2 \;+\; Q$$

     oxy   ternary complex   met

$$[X] \;\xrightarrow{k_{inh}}\; E^{*}$$
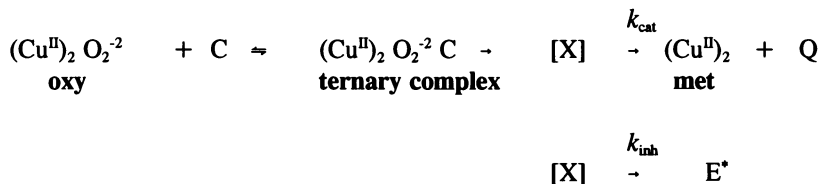
(C = catechol, Q = o-quinone, [X] = unidentified intermediate, E^{*} = inactivated enzyme, k_{cat} = catalytic rate constant, k_{inh} = inactivation rate constant)

Scheme 3   Reaction inactivation of tyrosinase in the presence of catechol

Initially, this inactivation was postulated to arise from an active-site directed attack of an *o*-quinone molecule on a nucleophilic group in proximity to the active center (*50*). Subsequently it was shown, that the inactivation is the result of an active-site specific destruction of histidine 306 in *N. crassa* tyrosinase with the concomitant loss of the binding of one copper (*29*). Protein-chemical studies with *in vivo* labeled [2,5-^{3}H] and [5-^{3}H] histidine tyrosinase strongly argued for an attack at position 2 of the imidazole nucleus. On mechanistic and chemical grounds, the highly reactive hydroxyl radical OH appears to be the most likely species to modify histidine residue 306. Since addition of hydroxyl radical trapping agents had no effect on the extent of the inactivation, a copper-bound rather than a free radical may be responsible for the destruction of histidine.

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Chapter 6

Differentiation of Fungal Tyrosinases and Laccases Using Selective Inhibitors and Substrates

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Fungal tyrosinases and laccases can utilize similar substrates and can be difficult to differentiate. In **Agaricus bisporus**, both enzymes can use dopa, p-cresol, and diaminobenzidine as substrates, albeit at different rates. Tolidine can be used as a selective substrate to differentiate **Agaricus** laccase from **Agaricus** tyrosinase. Oxidation of this substrate, however, did not appear to be inhibited by cetyltrimethylammonium bromide, a common laccase inhibitor. N-hydroxylglycine appeared to be a selective inhibitor of laccase since it did not inhibit tyrosinase activity. Tropolone, salicylhydroxamic acid, and 4-hexylresorcinol were more effective inhibitors of tyrosinase at low concentrations than cinnamic acid or 2,3-naphthalenediol. Similar patterns of substrate and inhibitor preferences were noted in other mushroom species such as Oyster, Enoki, and Shiitake mushrooms.

Many varieties of cultivated mushrooms are available for commercial use and consumption. Some of these varieties include the common cultivated button mushroom, either as a white or brown (Crimini) strain, as well as Oyster, Shiitake, Padi Straw, Portabella and Enoki mushrooms. Browning of the common cultivated mushroom, **Agaricus bisporus**, can occur after mechanical damage, infection, bruising and desiccation to mushroom tissues. However, browning of the cream colored Oyster and Enoki mushrooms is not as apparent. The potential for browning is dependent on several factors, one of which is the presence of enzymes involved in browning reactions. These enzymes act upon endogeneous phenolic compounds to eventually generate discoloration within the tissue. Browning of food products, such as mushrooms, not only decreases consumer appeal but can also decrease the nutritive value of the product.

Several enzymes capable of oxidizing phenolic compounds can be found in fungi. Three of these enzymes - laccase, peroxidase, and tyrosinase (polyphenol oxidase) - may be present in a given fungus and may display considerable overlap in their substrate specificity (*1-7*). For example, all three enzymes have been reported to be present in **Agaricus bisporus** (*4,9-10*). Differentiating between these three enzymes can be difficult if they are present at varying concentrations and if they can utilize similar substrates. If they do show a similar substrate preference, they can sometimes be differentiated with judicious use of selective

inhibitors. Although there are selective inhibitors of tyrosinases, there are relatively few specific inhibitors of fungal laccases.

Tyrosinase activity in fungi has been demonstrated using tyrosine, dopa, p-cresol, and 4-methyl catechol as substrates (9). Some fungal laccases, however, can use either tyrosine, dopa or p-cresol as substrates (8). Substrates for assaying fungal laccases have included p-quinol, quaiacol, syringaldazine, toluquinol, p-cresol and tolidine (8,10-16). In particular, quaiacol, tolidine, diaminobenzidine, and phenylenediamine derivatives have been used to locate and monitor intracellular and extracellular **Agaricus** laccase (9,10,13). Peroxidase can also oxidize many of the above substrates in the presence of hydrogen peroxide (4,17). Thus, it is sometimes difficult to distinguish between the three enzymes based soley on the use of a particular substrate.

Selective inhibition of one or more of these enzymes has been achieved in the presence of multiple types of phenol oxidases. For example, inhibition of tyrosinase, but not laccase, has been reported using tropolone, salicylhydroxamic acid (SHAM), 4-hexylresorcinol (4HR), cinnamic acids, naphthalenediol, and phenylhydrazine (12,16-21). Three of these inhibitors - tropolone, SHAM, and 4-hexylresorcinol - can be used at relatively low concentrations to differentiate tyrosinase from laccase. While selective inhibitors of tyrosinase have been identified, few specific inhibitors of laccase have been reported. For example, cetyltrimethylammonium bromide (CTAB) has been reported to be a selective inhibitor of laccase but not tyrosinase; however, this may vary according to the fungal source (12). N-hydroxylglycine has also been reported to be selective inhibitor of **Coriolus versicolor** laccase and incapable of inhibiting mushroom tyrosinase, plant polyphenol oxidase or **Penicillium sclerotium** pyrogallol oxidase (22). The effects of hydroxylglycine on other fungal laccases are not known.

Experimental

Materials. **Agaricus bisporus** tyrosinase and **Pyricularia oryzae** laccase were obtained from Sigma Chemical Co.(St. Louis, MO). An extracellular **Agaricus bisporus** laccase was a gift from R. Kerrigan (Sylvan Foods, Worthington, PA). **Agaricus** (common button and Crimini), Enoki, Oyster, and Shiitake mushrooms were obtained from local groceries. N-hydroxylglycine was synthesized by R. Kjonaas according to the method of Murao et al. (22). All other chemicals were of reagent grade.

Enzyme extracts. Extracts from whole fresh mushrooms or mushrooms stored at - 80 °C were prepared as described previously (9,19). All samples were stored frozen in small aliquots and centrifuged before use. Protein content was determined as reported earlier (9,19).

Tyrosinase assays. Tyrosinase activity was measured spectrophotometrically as described earlier (9,19) using either 1 mM tyrosine, 5 mM L-dopa, or 5 mM p-cresol as substrates. Increases in absorbance were monitored at 280 nm for tyrosine, 475 nm for dopa, and 410 nm for p-cresol. Slopes were measured from the linear portion of the absorbance time curve and one unit of activity was defined as a change of 1.0 absorbance unit per min. When indicated, salicylhydroxamic acid (SHAM), tropolone, 4-hexylresorcinol (4HR), cinnamic acid, or 2,3-naphthalenediol were added to the assay as tyrosinase inhibitors.

Laccase assays. Laccase activity was also measured spectrophotometrically as described (23) using either 5 mM p-cresol, 5 mM p-phenylenediamine, 1 mM 3,3-diaminobenzidine, 20 uM syringaldazine, 2 mM toluquinol, or 2 mM tolidine as

substrates. Increases in absorbance were monitored at 410 nm for p-cresol, 3,3-diaminobenzidine, and p-phenylenediamine, 525 nm for syringaldazine, 280 nm for toluquinol, and 630 nm for tolidine. Units of enzyme activity were defined as above. When indicated, cetyltrimethylammonium bromide (CTAB) or N-hydroxylglycine were added to the assays as laccase inhibitors.

Electrophoresis. Native polyacrylamide gel electrophoresis was carried out using preparative mini-gels (8%) as described previously (*9,24*). Crude extracts were mixed with bromophenol blue and glycerol and layered along the entire stacking gel surface. When electrophoresis was terminated, the gels were incubated for 10 min in 0.1 M sodium phosphate buffer (pH 6.0) for tyrosinase and 0.1 M sodium acetate buffer (pH 4.8) for laccase detection. At the end of 10 min, one cm wide strips were cut out of the gels and placed in the appropriate buffer, plus or minus the inhibitor, for 10 min. After this second incubation, the gel strips were placed in buffer containing the substrate, plus or minus the inhibitor, for color development and localization of enzyme activity. Photographs of zymograms were made using Kodak EDF duplicating film.

Results and Discussion

Crude extracts of **Agaricus bisporus** mushrooms, which contain tyrosinase and laccase activity, can oxidize a variety of phenolic compounds. Using dopa and p-cresol as substrates for tyrosinase and p-cresol, p-phenylenediamine, and syringaldazine as substrates for laccase, specific inhibitors were able to distinguish between these oxidative activities (Table I).

Table I.   Differential Inhibition of Phenol Oxidase Activities in Crude Extracts of **Agaricus bisporus**

Substrate	Inhibitor					
	none	cinnamic acid (500 uM)	naptha-lenediol (5 mM)	SHAM (50 uM)	tropo-lone (100 uM)	CTAB (300 uM)
dopa	1.8	1.47	0.56	0	0	nd
p-cresol	0.029	0.023	0.022	0.022	0.022	nd
p-phenyl-enediamine	0.094	0.09	0.086	0.084	0.09	0.057
syringald-azine	0.045	nd	0.043	0.042	nd	0

Adapted from reference (*9*). Numbers represent specific acitivity in units/mg. nd indicates not determined.

At low concentrations SHAM and tropolone were very effective in inhibiting dopa oxidase or tyrosinase activity. This is in accord with reports by Kahn (*18*) and Walker (*12*). In contrast, cinnamic acid and 2,3-naphthalenediol required much higher concentrations for partial inhibition. All of these tyrosinase inhibitors showed partial inhibition when p-cresol was used as a substrate.

Conversely, none of the tyrosinase inhibitors showed much inhibition when p-phenylenediamine was used as a substrate, indicating that this substrate may be used by a laccase. CTAB, on the other hand, showed a significant amount of inhibition when p-phenylenediamine and syringaldazine were used as a substrates. These results indicated that crude **Agaricus** extracts contained at least two oxidative enzymes, tyrosinase and laccase, and that both can use similar and dissimilar substrates. This substantiates previous reports on the inhibition of SHAM to differentiate tyrosinase from laccase and the ability of SHAM to inhibit both mono and diphenolase activities of tyrosinase (*9,12,24*).

Interestingly, commercial **Agaricus** tyrosinase preparations can also oxidize a number of phenolic compounds. In some of these preparations tyrosinase activity could be detected using tyrosine, dopa, and p-cresol as substrates while laccase activity was detected using p-phenylenediamine, 3,3-diaminobenzidine and toluquinol as substrates (Table II).

Table II.   Inhibition and Differentiation of Tyrosinase and Laccase by SHAM and 4-Hexylresorcinol

Substrate	**Agaricus** tyrosinase			**Pyricularia** laccase		
	none	SHAM	4HR	none	SHAM	4HR
Tyrosinase						
tyrosine	100	0	4	--	--	--
dopa	100	5	14	100	115	92
p-cresol	100	0	61	--	--	--
Laccase						
p-phenylenediamine	100	101	92	100	100	92
3,3-diaminobenzidine	100	95	106	100	98	107
toluquinol	100	106	102	100	96	96

Adapted from reference (*24*). Numbers represent percent of control specific acitivity. (-) desiginates an activity to low to be measured accurately. SHAM and 4HR concentrations were 100 uM.

SHAM effectively blocked tyrosinase activity but not laccase activity in these preparations. In addition, 4-HR was used as an inhibitor to determine if this compound could inhibit tyrosinase but not laccase. Although the inhibition by 4-HR was not as great as SHAM, 4-HR is more soluble and can be used to differentiate tyrosinase from laccase using a variety of substrates.

A commercial preparation of **Pyricularia** laccase was used to determine if SHAM and 4-HR could affect other types of laccase (Table II). **Pyricularia** laccase apparently lacked a detectable tyrosinase activity but could still use dopa as a substrate. SHAM and 4-HR showed little, if any, inhibition of this laccase using toluquinol, diaminobenzidine, or phenylenediamine as substrates.

Differential identification of tyrosinase and laccase was attempted in common button and Crimini (both **Agaricus** strains) mushrooms using a variety of substrates. In conjunction with these substrates, SHAM was used to inhibit tyrosinase and CTAB was used as a potential laccase inhibitor (*16*). Tyrosinase activity was severely inhibited with SHAM using p-cresol, dopa or tyrosine as substrates in both **Agaricus** strains (Table III, data not shown).

Table III. Differention of Tyrosinase and Laccase in Agaricus and Crimini Mushrooms using SHAM and CTAB

| | Agaricus | | | Crimini | | |
	none	SHAM	CTAB	none	SHAM	CTAB
Tyrosinase						
dopa	100	0	112	100	3	86
tyrosine	100	0	150	100	1	81
p-cresol	100	0	148	100	2	150
Laccase						
p-cresol	100	0	101	100	7	232
diamino-benzidine	100	93	nd	100	80	nd
phenylene-diamine	100	75	100	100	105	107
syring-aldazine	100	100	50	100	130	69
toluquinol	100	91	25	100	86	210

Adapted from reference (*23*). Numbers represent percent of control specific activity. nd indicates not determined. SHAM and CTAB concentrations were 100 uM and 1 mM respectively.

CTAB showed little inhibition and apparent activation was noted with some substrates. Laccase activity, when assessed using diaminobenzidine, phenylenediamine, syringaldazine or toluquinol as substrates, was relatively unaffected by SHAM. In contrast, utilization of p-cresol in both **Agaricus** and Crimini extracts was inhibited by SHAM. Except for syringaldazine, little inhibition of laccase activity was noted when CTAB was used as a laccase inhibitor. This suggests that CTAB may not be a preferential laccase inhibitor as reported earlier (*16*) or that CTAB inhibition may be dependent on the type of substrate employed.

Oyster and Shiitake extracts showed low levels of activity using p-cresol, dopa or tyrosine as indicators of tyrosinase activity (Table IV, data not shown) . These results are in agreement with Oddson (*25*) and Bano and Rajarathnam (*26*) who found PPO activity to be much lower in **Plerotus florida** than **Agaricus.** They also agree with those of Marr et al. (*6*) who classified Oyster mushroom into a high laccase low tyrosinase group. Laccase activity was detected in both extracts but was not inhibited by SHAM to a large extent. CTAB also had little effect on laccase in these two varieties except for when syringaldazine, p-cresol, and toluquinol were used as substrates for laccase in Oyster extracts.

Tolidine has been used by Kerrigan et al. (*13*) and Choi et al. (*27*) to assay for laccase and locate laccase isoforms from mushroom cultures after electrophoretic separation. To determine if tolidine could be a useful and a selective substrate for laccase, but nonselective for tyrosinase, a spectrophotometric assay was developed which monitored the increase in absorbance due to product formation at 630 nm. A blue colored product, presumably a meriquinoid blue dye, was produced by the action of laccase on tolidine. A similar assay was reported by Hanke and Ebert (*28*) and Mohr et al. (*17*) for the oxidation of tolidine by peroxidase. As seen in Table V, oxidation of tolidine occured in extracts of five different varieties of mushrooms.

Table IV. Differentiation of Tyrosinase and Laccase in Oyster and Shiitake
Mushrooms using SHAM and CTAB

	Oyster			Shiitake		
	none	SHAM	CTAB	none	SHAM	CTAB
Tyrosinase						
dopa	--	--	--	--	--	--
tyrosine	100	0	0	--	--	--
p-cresol	100	0	0	--	--	--
Laccase						
p-cresol	100	123	18	100	100	200
dimino-benzidine	100	80	nd	100	90	nd
phenylene-diamine	100	105	89	100	86	77
syring-aldazine	100	123	50	--	--	--
toluquinol	100	42	27	100	100	200

Adapted from reference (23). Numbers represent percent of control specific
acitvity. (-) designates a value low to be determined accurately. nd means not
determined. SHAM and CTAB concentrations were 100 uM and 1 mM
respectively.

Table V.   Differentiation of Tyrosinase and Laccase using Dopa and Tolidine as
Substrates and SHAM N-hydroxylglycine as Inhibitors

Source	Dopa			Tolidine		
	-	SHAM	HG	-	SHAM	HG
Agaricus	100	1	93	100	81	88
Crimini	100	1	90	100	94	87
Enoki	0	0	0	100	100	1
Oyster	100	48	44	100	91	19
Shiitake	100	10	89	100	66	28

Numbers represent percent of control specific activity. SHAM and HG
concentrations were 200 uM each.

The highest level of tolidine oxidation was found in Shiitake (0.67 units/mg) followed by Oyster (0.4 units/mg) and Enoki (0.007 units/mg) extracts. The lowest level of tolidine oxidative activity was in **Agaricus** (0.0015 units/mg) and Crimini (.0015 units/mg) extracts. SHAM was relatively ineffective in inhibiting laccase activity using tolidine as a substrate, suggesting that tyrosinase cannot use tolidine as a substrate. In contrast, hydroxylglycine was very effective in blocking laccase activity in Shiitake, Oyster, and Enoki mushrooms but not as effective in **Agaricus** or Crimini mushrooms.

The highest level of dopa oxidative activity was in Crimini (1.1 units/mg) extracts, followed by **Agaricus** (0.1 units/mg), Shiitake (0.04 units/mg) and Oyster (0.01 units/mg) (Table V). Very little tyrosinase activity was present in Enoki extracts. Tyrosinase activity was severely inhibited by SHAM in **Agaricus**, Crimini, Oyster and Shiitake extracts. Hydroxylglycine had little effect on tyrosinase activity except in Oyster extracts in which there was a significant percent inhibition.

Differentiation of tyrosinase and laccase was also determined by electrophoretic separation of isozymes and selective inhibiton of isoforms by SHAM and HG. An extracellular filtrate of **Agaricus** laccase showed two major isozymes when separated by PAGE and stained with DAB (Fig. 1 a). These results are similar to those reported previously (4,13,24). SHAM, 4-HR, and tropolone showed no inhibition of staining or the number of observed bands. In fact, the presence of tropolone appeared to enhance staining intensity. CTAB also showed no inhibition of enzyme staining. No other oxidative enzyme, such as peroxidase, appeared to be present when hydrogen peroxide was added to the staining medium.

Tolidine was compared to DAB for use as a selective substrate in the presence and absence of SHAM and HG (Fig. 1 b). Tolidine gave a stronger signal in a much shorter staining time compared to DAB (data not shown). SHAM had no effect on DAB oxidation but did show an apparent decrease in the coloration of tolidine staining. In fact, inclusion of SHAM caused the blue color to assume a blue-green hue instead of a bright blue color. Hydroxylglycine inhibited oxidation of both DAB and tolidine by laccase.

We also examined the usefulness of tolidine, SHAM, and HG to differentiate tyrosinase from laccase in commercial tyrosinase preparations. As seen in Fig. 1 c, a single band of tyrosinase was observed and this band showed no staining with tolidine. SHAM blocked this dopa oxidase activity while HG appeared to decrease the intensity of dopa staining. Two apparent laccase isoforms were noted when stained with DAB and tolidine, and neither of these forms showed tyrosinase activity or staining with dopa. SHAM increased the staining intensity of DAB while HG inhibited the staining intensity. In contrast, SHAM decreased the intensity of tolidine staining while HG completely inhibited this reaction. These results suggest that some inhibitors may be reacting with the product or intermediates in the enzyme catalyzed reactions of tyrosinase and laccase.

Conclusions

Depending on the mushroom species, it may be relatively easy to differentiate tyrosinase from laccase using selective substrates. In other species, selective inhibitors, in conjunction with selective substrates, can be used to differentiate tyrosinase from laccase. Tolidine appears to be useful as a substrate for colorimetric determination of laccase activity and for differentiating laccase from tyrosinase and peroxidase. Hydroxylglycine appears to be a specific inhibitor of laccase when used with certain substrates and may be of general utility in distinguishing tyrosinase from laccase.
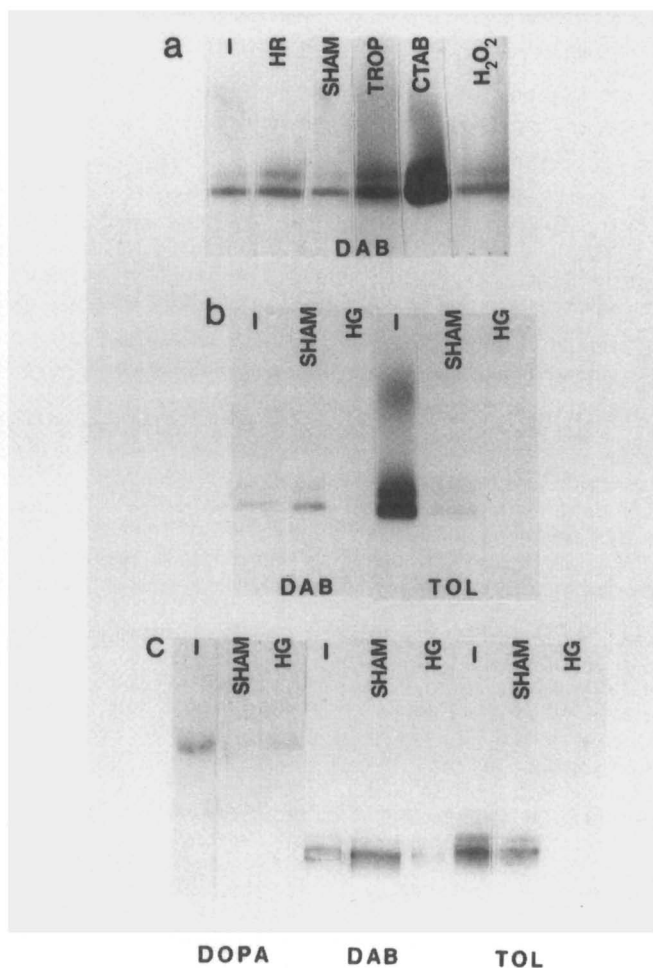
Figure 1. Localization of tyrosinase and laccase after native electrophoresis. (a) Extracellular **Agaricus** laccase was subjected to native electrophoresis as described in the Methods section and stained for activity using diaminobenzidine (DAB) in the presence and absence of 200 uM 4-hexylresorcinol (4HR), 200 uM salicylhydroxamic acid (SHAM), 200 uM tropolone (TROP), 5 mM CTAB and 0.01% hydrogen peroxide. (b) Extracellular **Agaricus** laccase was subjected to native electrophoresis and stained for laccase activity using DAB and tolidine (TOL) in the presence and absence of 500 uM SHAM and 500 uM hydroxylglycine (HG). (c) Commercial tyrosinase was subjected to native electrophoresis and stained for tyrosinase activity using DOPA and for laccase activity using DAB and TOL. Staining was carried out in the presence and absence of SHAM and HG as above.

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Chapter 7

Fruit Polyphenol Oxidases

New Data on an Old Problem

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Many studies have been conducted on fruit polyphenoloxidases (PPO)
and their involvement in browning but several unanswered questions still
remain, e.g. enzyme latency, physical characteristics and structure
-function relationships. Apple pulp PPO is associated with thylakoid
membranes. Several forms were detected but the most important was a
64 kD monomer which, when folded by internal disulphide bonds,
forms an active protein with an apparent molecular weight of 42 kD and
optimal pH of 4.5-5 (inside thylakoid pH). This protein can be
proteolysed into a 42 kD monomer that forms a 27 kD apparent
molecular weight protein after folding. This latter protein is active over a
broad pH range. Its resistance to proteolysis enabled development of an
effective purification method. A highly specific polyclonal serum capable
of detecting all proteolysed / nonproteolysed and folded / unfolded PPO
forms was obtained. All of these different forms are present in apple
pulp. The overall results are discussed in the light of recent reports.

The organoleptic and biochemical characteristics of fruits may be profoundly modified
by the appearance of brown pigments whose color is superposed on the natural
colors. With a few exceptions (medlars, prunes, raisins, dates, figs, etc.), these
changes are considered as degradation products and lower fruit quality both visually
and with regard to taste and nutritional characteristics. Some browning may be
nonenzymatic, resulting from the Maillard reaction when mixtures of amino acids and
carbohydrates are heated. However, most browning, and particularly rapidly-
occurring types, are caused by enzymatic oxidation of phenolic compounds under the
effect of polyphenol oxidase *(1-5)*.

Three different situations may cause browning in fruits: first, in a few cases,
physiological evolution related to maturation (e.g., in dates); second, some disorders
which may occur during cold storage; and third, various technological processes
which involve wounding, crushing, extraction etc. Many of these problems have been
known for a long time *(6)*, and are still topical, they have even become more
important with the increasing use of mechanical equipment in the harvesting and
packaging of fruits and the recent marketing of new, ready-prepared plant products.

There is a common feature in all cases: contact between phenolic compounds with predominantly vacuolar localization and oxidative enzymes located in the cytoplasm *(7, 8)*. The cellular decompartimentation is needed (in the presence of oxygen) to trigger browning.

Although browning is generally considered to be harmful, with the above-mentioned exceptions, browning products may contribute to resistance to some stresses. The appearance of browning is thus one of the first signs of a response to wounding or fungal attack and the *o*-quinones formed appear to possess more effective antimicrobial properties than the original compounds and participate in resistance mechanisms *(9)*.

The tendency of a fruit to turn brown results from the action of a number of parameters which are naturally involved in limiting browning and its intensity *(6, 10-12)*. All of these parameters vary with the age of the fruit, its physiological state and health, and also in relation to the variety and treatments to which it is subjected. In the past ten years, there has been ample progress in understanding the mechanisms of enzymatic browning, particularly through model system studies of coupled reactions between several phenolic compounds, quinones and other compounds *(13-16)*. On the other hand, new methods to control browning have been found since the use of sulfites was discouraged and banned in many cases *(10, 12, 17)*.

There are few new answers to the question of PPO function and expression in fruits *(4)*, despite intense study on biochemical and physiological aspects *(18)*. Recently, several genes encoding PPO have been isolated and characterized from other plant organs *(19-22)*. Such studies with fruits are necessary to evaluate changes in expression of PPO during growth, maturation and post-harvest treatment, or to modulate PPO levels by antisense RNA approaches and generate transgenic plants with fruits possessing altered PPO expression. The purpose of the present investigation is firstly to summarize the state of our present knowledge of fruit PPO and, secondly, to report some results on the significance of the multiple forms of apple PPO.

Present Knowledge of Fruit PPOs

Most PPOs involved in fruit browning are catecholoxidases *(4, 6)* also known as *o*-diphenoloxidases (EC 1.10.3.1), except in peach and apricot where the simultaneous presence of *o*- and *p*-diphenoloxidases (EC. 1.10.3.2) has been reported *(23, 24)*. Nevertheless, laccase was only detected in a limited number of peach varieties. Both enzymes are also found in mango fruit; catecholoxidase was located in the flesh, whereas laccase was present in the secretory ducts from cut pedicel *(25)*. Cresolase activity (EC 1.14.18.1) has been detected in several fruits, but this activity is often lost during the purification steps and its role is controversial *(4, 6)*.

Presently, the overall results indicate high heterogeneity in the expression of fruit PPO concerning enzymatic activity (optimum pH, latency, specificity, etc.), number of isoforms and apparent molecular mass. This heterogeneity may be due to differential genomic expression of the species, the physiological stage of the fruit, or to the nature of the tissues studied. Furthermore, artefacts may appear during the extraction and purification processes.

Tissular and Subcellular Localization. PPO activity has been detected in all parts of fruits including the peel, flesh, and cortex *(6, 8)*.When 12 apple cultivars were analyzed, the activity was mainly concentrated in the peel or the cortex, although the levels in the cortex were higher than the peel in seven cultivars, similar in four others and lower in one cultivar *(26)*. Recently, immunostaining of the nitrocellulose tissue prints of apple fruit cross sections, suggested that chlorogenic acid oxidase is not uniformaly distributed, but is mainly localized at the core in apple fruit and then

near the skin *(27)*. PPO localization corresponds to the distribution of browning of the same apple section.

Since it is a membrane-bound enzyme, the use of detergents (Triton X-100, X-114, SDS, etc.), acetone powder or limited digestion with proteolytic enzymes are required in most cases, to obtain maximum fruit PPO activity. The intracellular localization of fruit PPO has been shown in chloroplasts, particularly on the inner face of thylakoids *(28)*. In some cases, PPO activity has also been obtained from mitochondria in olive and apple *(28, 29)*, microbodies in avocado *(30)*, or partly associated with the cell wall in banana *(31)*. Nevertheless, these data should be confirmed using modern cytolocalization techniques. Many authors report the presence of PPO soluble activity, and the extent of its binding to membranes appears to vary depending on the tissue, species and the stage of development. In unripe olive, PPO is thus tightly bound to the chloroplast, whereas in the ripe fruit it is essentially soluble *(32)*. Practically all of the activity was found to be soluble when olive fruit was deeply coloured by anthocyanins. It is suggested that, at this stage, chloroplast membranes are disrupted and their lamellar structure disintegrates, thus facilitating liberation of the enzyme. Similar increased solubilization has been reported for other fruits *(6, 8)* and was also observed to increase with age in tissue cultures of apple fruit *(33)*.

Changes in PPO Expression during Growth, Maturation and Storage. Many studies on PPO have been conducted on fruits at commercial maturity because of the economic potential of this product. However, PPO activity is at its lowest point during this physiological stage. In fact, PPO activity is generally more important in young green fruits than in ripe fruits *(6, 8)*. Changes are not the same for the different forms of PPO. As previously mentioned, there is generally a change from bound to more soluble enzyme forms during maturation, but activity of the latter form is always lower than observed in young fruits (about 17% in apple and 4 to 5% in grape). Variations in PPO activity differ according to fruit species. In peach, the activity in the particulate fraction increases during maturation, while it falls considerably at picking and becomes lower than the soluble activity. In avocado, more and more PPO activity is associated with the particulate fraction, relative to the cytosolic fraction, as the fruit ripens. In apple tissue cultures, PPO activity may be compared to that reported for intact apples, with low initial activity which increases and then falls again with time.

Fruit browning occurs frequently during storage. Wound or bruising, caused by handling at picking and storage, freezing, cold storage and thawing, cause marked deterioration in fruit color and texture. At a commercial level, the development of browning and sensitivity to bruising are among the factors that limit the storage life of fruits. Various defects caused by cold (chilling injury) can be accompanied by fungal attack during cold storage and are responsible for discoloration or browning in stored fruits. Fruit preservation methods other than cold (e.g. controlled atmosphere, gamma irradiation and drying) may also cause browning and discoloration. In all cases, physicochemical modifications of membranes cause subcellular decompartmentation, leading to enzyme-substrate contact and browning. The changes that occur in PPO activity during storage are not clear *(6)*: an increase is sometimes noticed, but the activity often remains almost constant. With γ-irradiation, there is a good correlation between browning and PPO activity. In banana and mango, PPO activity was found to increase in irradiated fruit suggesting that this increase was possibly due to conformational changes, activation of latent enzyme or *de novo* synthesis.

Biochemical Characteristics. Inactive or latent PPO forms have been frequently reported in plants and require activation. In many cases, PPO activity was fully detected in the presence of SDS *(34, 35)*, which is particularly interesting since few enzymes are known to be activated by this detergent. In fruits, PPO latency has been

reported in mango *(25)*, avocado *(36)* and grape. In the latter fruit, a latent PPO form has been isolated by temperature-induced phase separation in Triton X-114, and the most effective activators were trypsin and CTAB *(37)*. However, this latency has not been reported in grape by other author *(38)*.These differences do not seem to be related to the cultivar but rather to the pH level used in the tests to measure PPO activity. Moreover, in apple, we found that the SDS effect was pH-dependent and there is not as much evidence of PPO latency in fruits as there is in leaves.

Very different pH optima have been reported for fruit PPO activity ranging from acidic pH *(3.5)* in grape *(38)* to neutral levels in kiwi *(39)*. In some cases, two different pH optima have been observed in the same species (e.g. 4 or 6.5-7.5 for eggplant *(40, 41)*), and the same extract, as in apple *(29)* and cherry *(42)*.

There is also inter- and intra-species diversity in the number of PPO isoforms and their molecular masses. In apple fruit, previous studies have described one form of 46 kD *(26)* or 26 kD *(43)*, or several forms ranging from 24 to 134 kD *(44, 45)*. This may be related to genetic differences, stage of development, storage at cold or freezing temperatures, or the tissue studied. Nevertheless, many of these forms are thought to be artefactual due to partial release from membranes, cross-linking reactions, or partial denaturation with phenols that appear during PPO extraction and purification *(4)*. In pear, anion exchange resins were particularly effective in removing phenolics, did not adsorb PPO and reduced the electrophoretically separable bands of PPO activity from 11 to 3 *(46)*. Until 1992, only apparent molecular masses were determined for fruit PPO isoforms. In most cases, purification to homogeneity was not obtained and the absence of antibodies made it impossible to obtain the correct molecular mass from fully denatured extracts. Fortunately, recent studies on grape *(47)* and apple *(27, 48)* with anti-PPO have clearly shown that the main native enzyme has a molecular mass of around 60000. This is similar to masses obtained recently from broan bean *(49, 50)*, and corresponds to known genes which are represented by approximately 2 kb transcripts encoding 65-70 kD polypeptides *(19, 20, 47, 50)*.

The specificity of PPO towards its phenolic substrates has been extensively studied and many discrepancies in the apparent Km values appear between species, cultivars and experimental conditions *(5, 6, 10, 12, 18)*. Only a few studies have been devoted to the effect of the second substrate, i.e. oxygen. With apple PPO, it was recently shown that the reaction is an ordered mechanism whereby oxygen is the first substrate to bind to the enzyme. An equation has also been drawn up from model systems to predict initial oxygen uptake from the relative concentrations and kinetic parameters of each individual phenolic substrate *(12)*.

Current molecular studies by many research teams worldwide should soon provide answers to questions surrounding this heterogeneity and the possible physiological significance. The first step in this research is to fully characterize any existing relations between observed enzymatic forms. Our investigations in apple using newly available PPO antibodies and one outstanding property of these enzymes, i.e. their resistance to proteolysis, have been focused on this objective.

Study of Apple Polyphenoloxidase Polymorphism

Apple fruit is an economically important fruit with the production of several processed products including crude or fermented juice, stewed apples, apple sauce and slices. During postharvest processing, enzymatic browning is the main factor responsible for losses due to visual and nutritional depreciation of the final product *(12)*. There have been recent studies on the enzymatic mechanism and oxidation of model solutions of apple PPO *(16, 51)*. Good purification of apple PPO constitutes the first essential step, although it is still difficult. We proposed a new approach to extract and purify apple *(Pyrus malus* cv Granny Smith) pulp PPO based on SDS-proteinase K digestion *(48)*. Our results which highlighted the proteolysis phenomenon could

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explain the inconsistent results sometimes obtained with the same fruit. Moreover, this proteolysis, as explained later, can purify enough PPO to raise anti-PPO antibodies and study the relationships between multiple forms of apple PPO.

Apple PPO Extraction. Several problems arise during extraction of PPO from fruits. First, fruits having low-levels of proteins, large quantities are required for enzyme extraction. For example, the most recent apple PPO purification was conducted from 42 kg of apples to obtain a 470-fold purification, a figure never reached before (52). Secondly, fruits are a rich source of phenolic compounds that react with proteins, which may lead to changes and inactivation of the extracted enzyme. A third difficulty appears when extracting membrane-bound enzymes such as PPO, the detergent required to solubilize them, also solubilizes chlorophylls and highly interfers with the classical Bradford procedure to measure protein content. Extraction of apple pulp PPO from plastidic membranes, using Triton X-114 as detergent (37), allowed us to overcome these difficulties (Figure 1).

Apple pulp was shown to contain active chloroplasts, and it was possible to get a greenish thylakoid membrane pellet from pulp (Figure 1). Colocalization of PPO with succino-dehydrogenase (SDH, a mitochondrial enzyme) and chlorophyll (chloroplastic marker), on a Percoll gradient, demonstrated that the fraction containing most of the SDH did not exhibit any PPO activity, whereas chlorophyll and PPO activity showed similar variations in the gradient (53). Apple pulp PPO is thus localized in chloroplasts, not in mitochondria.

TX-114 was used instead of the common TX-100 in the extraction buffer because this non-ionic detergent has a cloud point at about 20°C, which is compatible with isolation of native proteins (54). At room temperature, a Triton solution turns turbid and centrifugation induces phase partitioning with an upper detergent-depleted phase and a lower detergent-enriched phase. Membrane proteins, phenolic compounds and chlorophyll were discarded with the lower phase since PPO was recovered in the upper phase with hydrophilic proteins. This surprizing PPO partitioning has already been noted in other plants (55, 56), and suggests that PPO, although membrane bound, is a hydrophilic protein with only a short hydrophobic anchor. Phase-partitoning with TX-114 quickly results in a 5-fold purified extract, free of phenol and detergent, and with a 73% yield (Figure 1), which is superior to the 45% yield obtained with the $(NH_4)_2SO_4$ precipitation method (26). We considered this phase-partitioning extract as the native PPO extract.

PPO Purification Based on its Resistance to SDS-Proteinase K Digestion. It was already shown that mouse tyrosinase is resistant to SDS-proteinase K digestion (57). We tested this property on apple PPO and found that, although resistance to proteolysis is not complete, it is much more than with other apple proteins. Indeed, after 3 h proteolysis of the phase partitioning extract at 25°C with 2% SDS and 0.05 U ml^{-1} proteinase K, we recovered 65% PPO activity (48). Proteolysis, thus, appears to be a very rapid and efficient means to purify apple PPO. After passage on a DEAE-cellulose column, to eliminate amino acids and protease, apparent homogeneity was reached as shown by silver staining SDS-PAGE (48). PPO was purified 388-fold with 44% yield (Figure 1).

Apple PPO is both resistant to proteolysis, and, like PPOs from different origins, to SDS. When SDS-PAGE is stained with DOPA, PPO activity can be clearly detected. After proteolysis, the electrophoretic mobility was increased and the DOPA stained band shifted from a 42 kD to a 27 kD band (Figure 2). Part of the main active 42 kD apple PPO was thus eliminated by proteinase K (ca 36% of the apparent molecular mass), and a shorter but still active PPO form was obtained. The active site was functionally conserved in this proteolysed form which is quite resistant to further proteolysis. Enzymatically active intermediate bands were also noted between 42 and
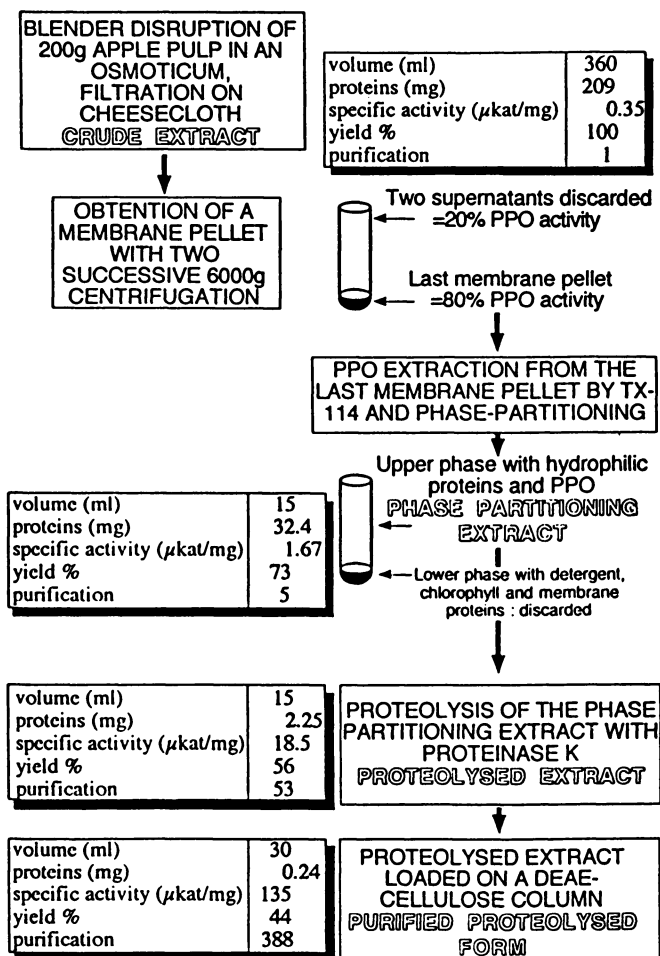
Figure 1. Apple PPO extraction and purification scheme *(48)*.

27 kD which were DOPA stained on SDS-PAGE and allowed for studying the kinetics of mild proteolysis (Figure 2). With further proteolysis, all of these forms gave a stable and more resistant 27-kD band. This *in vitro* experiments showed that in apple extract, the active band with greatest electrophoretic mobilities, resulted from the proteolysis of the main active PPO. When an anti-protease cocktail was used during extraction, the 27 kD proteolysed form was still present but in lower quantities. This proteolysis phenomenon may explain why confusing electrophoretic patterns were often obtained. Indeed more or less intense accidental proteolysis may lead to several forms present at the same time, as already reported in the broad bean leaves (58). In the apple model, it is easy to control the degree of proteolysis of the extract, since native and proteolysed forms have very different migrations on DOPA stained SDS-PAGE.

Characterization of Apple PPO Forms. The native and proteolysed PPO extracts exhibited the same Km but showed different behaviours towards pH and SDS (Table I). The native extract presented a relatively sharp optimum pH of between 4 to 4.5, and its activity was completely modified in the presence of SDS, with inhibition at low pH and strong activation at pH above 5 regardlesss of the substrate (Marquès *et al., Plant Physiol. Biochem.*, in press). Note that the level of activity when PPO was activated by SDS at pH above 5 was not higher than that reached at pH 4 without SDS. Thus, apple pulp PPO is not latent, which is different of other fruits (25,36,37) and some leaves (35). Indeed, broad bean leaf PPO (35) has always been described as completely latent and becomes fully active in the presence of SDS. The results with fruits are less consistent and our own data suggest that pH plays an important role, which has rarely been considered. The proteolysed form showed almost the same behaviour towards pH as the main active form in the presence of SDS, i.e. no defined pH from pH 5 to 7.5. However, this form was not affected by SDS. The double pH optima sometimes reported (see before) were certainly the result of partial proteolysis of PPO during extraction since it was difficult to control the degree of proteolysis.

To detect all PPO forms (active and inactive ones), PPO antibodies were raised against the proteolysed purified form (48). The anti-serum obtained was very specific and detected both active and nonactive PPO forms with molecular weights higher than the proteolysed form (Figure 3). This latter form, short but still active, may contain the consensus domain of PPO protein. In nondenatured native extracts, separated by SDS-PAGE, three forms were immunodetected on the corresponding blot. These consisted of the main active 42 kD PPO band, a faint band with 27 kD representing the proteolysed part of the enzyme and a third inactive band, never detected by DOPA-staining, at 63 kD (Figure 3, lanes D and H).

Western blot analysis was also used to study native and proteolysed extracts in complete denaturing conditions. The extracts were reduced and boiled before being run on SDS-PAGE. The gel was then blotted and the blot was revealed with anti-PPO antibodies (Figure 3, lanes A to D). The main active form was shown to be a 64 kD protein, while the proteolysed form was a 42 kD protein (Marquès *et al., Plant Physiol. Biochem.*, in press). We obtained almost the same results as with broad bean, where the native form is a 60 kD protein and the proteolysed form a 45 kD protein (50, 58). Broad bean PPO was long considered to be a 45 kD protein and the 60 kD protein was only found when an anti-protease cocktail was used (49). Since the electrophoretic mobilities of the two apple PPO forms were reduced when denaturation was complete (Figure 3, lanes A to D), both forms are monomers.

Analysis of electrophoretic mobility of native PPO extract in guanidinium chloride (GdmCl)-denaturing conditions showed that a 63 kD protein was obtained in the absence of dithiothreitol (DTT) and a 64 kD protein with DTT. This demonstrates that internal disulphide bonds exist in apple PPO. These strong links in the structure may explain the great resistance of PPO to powerful denaturants such as SDS, urea or
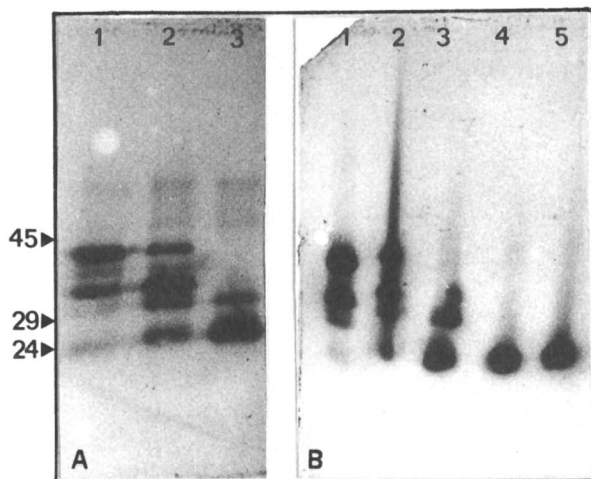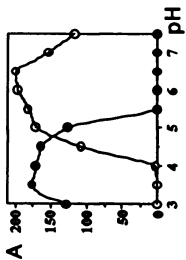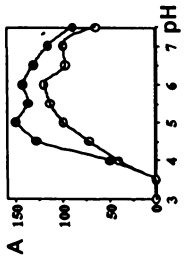
Figure 2. SDS-PAGE stained for PPO activity of gradual SDS-proteinase K digestions of the phase partitioned extract, (A) SDS-PAGE of the digest carried out with a low concentration of proteinase K (0.001 U ml^{-1}) at different incubation times (1: 10 min, 2: 1 h, 3: 4 h), (B) the 42 kD band was previously cut out from a first gel and digested with different proteinase K concentrations (1: 0.002 U ml^{-1}, 2: 0.02 U ml^{-1}, 3: 0.011 U ml^{-1}, 4: 0.2 U ml^{-1}, 5: 2 U ml^{-1}) in the stacking gel for 30 min before rerun. Reprinted from ref 48, Copyright 1994, with kind permission from Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington OX5 1GB, UK.

protease. We suggest, therefore, that the inactive 63 kD form detected by immunoblotting in the native extract (Figure 3, lanes D and H) was a partially unfolded form still containing disulfide bonds, since it was not further denatured by GdmCl. The presence of this enzyme form is not surprizing, since it has been shown, recently, that PPO was routed to the thylakoid lumen in two steps with intermediate forms *(59)*, and that the translocatory competent form entering the thylakoid membranes was reported to be a partially unfolded protein *(60)*. We thus propose that in a native apple extract, the three immunodetected PPO forms are closely related to the main native and active 42 kD band as well as the 27 kD active band as the proteolysed form, and the inactive 63 kD band as the partially unfolded form (Figure 4).

Conclusion. The use of proteolysis with apple PPO extract allowed us to determine that much of the confusion related to electrophoretic patterns, molecular weights and pH optima is related to the degree of proteolysis of the extract. This phenomenon, which is easily controlled in apple extract, may be difficult to evaluate with some other materials and, in this case, anti-protesases must be used to conduct such enzymatic studies. Apple PPO exhibits no latency, which is an original finding with regard to broad bean leaf PPO. Without SDS, optimum activity was found at pH 4, corresponding to the lumen pH of thylakoids where PPO is localized. We found that when extracting PPO, an inactive form was obtained that was probably the

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Table I. Characteristics of Native and Proteolysed PPO extracts

	NATIVE	PROTEOLYSED
K_m		
4MC* (mM)	7	6.8
CA* (μM)	100	99
TBC* (μM)	610	580
pH OPTIMUM		
• without SDS		
○ 3.5 mM SDS		
A=PPO activity in 1 $DO_{410\ nm}$ ml^{-1} min^{-1}, at 30°C Substrat= 4MC* (20 mM)		
MOLECULAR MASSES		
- with partially denaturing conditions	42 kD	27 kD
- with complete denaturing conditions	64 kD	42 kD

a4MC = 4-methylcatechol, CA = chlorogenic acid, TBC = *tert*-butylcatechol.
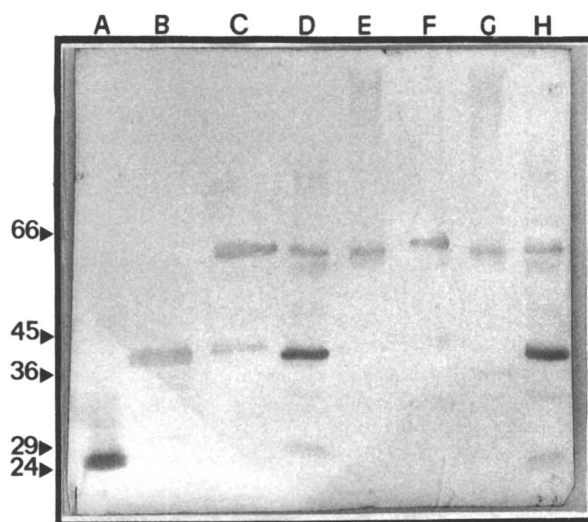
Figure 3. Western blot of SDS-PAGE revealed with anti-PPO antibodies. Lane A: proteolysed extract; lane B: proteolysed extract boiled during 3 min in Laemmli denaturing buffer; lane C: native extract denatured as B; lane D: native extract; lane E: native extract denatured by 6M GdmCl, Tris-HCl pH 8, at 25°C during 2 h; lane F: native extract denatured such as E but with 100 mM DTT added; lane G: same as E; lane H: same as D.

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Native extract PPO forms

PPO forms obtained by different denaturations

64 kD form: completely unfolded form obtained after complete denaturation and reduction of the native form

63 kD form: partially unfolded inactive form proposed to be the translocation-competent form to enter thylakoid membranes

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63 kD form: partially unfolded form still containing disulphide bonds

DTT reduction

42 kD form: folded native form, enzymatically active and representing the main form of the native extract

GdmCl 6M denaturation

Boiling in Laemmli denaturing buffer

CuA
CuB

Proteolysis

27 kD form: folded proteolysed form, enzymatically active

CuA
CuB

Figure 4. Proposed relationships between the different PPO forms of the native extract.

partially unfolded form entering thylakoidal membranes. Further study of this PPO processing in the cell, coupled with post-translational regulation analysis of this enzyme, may lead to an interesting new way to prevent browning in plants. Alternative molecular solutions to this technological problem are to be investigated.

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Chapter 8

Implications of the Phylogenetic Distribution of Polyphenol Oxidase in Plants

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A limited phylogenetic survey of the distribution of polyphenol oxidase (PPO: EC 1.10.3.2) was conducted in order to determine if this enigmatous enzyme is present in all plants groups or in only certain phylogenetic lines. Evidence for presence of the enzyme was gathered via enzyme assays using various recognized substrates and through the use a heterologous DNA sequence from the PPO of *Vicia faba* in DNA hybridization experiments and in polymerase chain reaction amplification of conserved sequence within the gene. Enzyme assays showed PPO activity in members of all eukaryotic groups tested, albeit at low levels in some groups, including green algae, mosses, and gymnosperms. DNA hybridization experiments showed varying degrees of signal strength obtained on the dot blots. This could mean limited homology between *Vicia* probe and target DNA or different copy number in the target DNA. Amplification experiments using primers for a 111 base pair region of the *Vicia* copper A binding region yielded 111 base pair products in all members of all phylogenetic groups tested. These data strongly suggest the presence of active PPO in all photosynthetic organisms more advanced than cyanophytes. This, along with data from studies with PPO-less mutants, implies some essential function of the enzyme in plants.

Polyphenol oxidase (PPO; EC 1.10.3.2), also known as catechol oxidase, phenolase, and *o*-diphenol oxygen oxidoreductase, has no clearly established function (*1-3*). The enzyme is widely distributed in higher plants (*4-7*), and is found in the plastids of all tissues types in species possessing PPO activity (*2*). A number of nuclearly inherited isozymes have been shown to exist in tobacco (*8,9*). Recent investigations at the DNA-level have shown that PPO is represented as a multigene family in broadbean (*10*), tomato (*11*) and potato (*12*). In the Solanaceae, different members

of the family are expressed in various tissue types, with certain forms that are involved in pathogen defense being expressed exclusively in epidermis and type A glandular trichomes on the surface of the leaf (*13-15*). There is certainly evidence that the enzyme plays a part in deterrence of insect and fungal pathogens in many, but not all, species and cell types. This role has been reviewed elsewhere (*16*) and will be addressed by others in this book. There has long been speculation that PPO's have other roles in plants as well. The multigenic nature of the enzyme and differential tissue-specific gene expression implies that there is more than a single function for PPO.

PPO activity has been monitored previously in a number of individual plant species (*7, 17-22*). Also, there have been two more broadly based surveys of PPO published (*4, 5*). That conducted by Cambie and Bocks (*5*) was limited to the gymnosperms, and focused primarily on the family, *Podocarpaceae*. The other covered a wide spectrum of the plant kingdom, including representatives from the green algae, mosses, liverworts, hornworts, ferns, gymnosperms, and angiosperms. In that work, three assay protocols were utilized (spectrophotometric assays, PAGE enzymic activity stains, and cytochemistry) to determine the presence of PPO activity in a given species (*4*). Although PPO was found almost ubiquitously in the angiosperms, a number of groups had no detectable activity. These including most of green algae, the mosses, and essentially all of the gymnosperms tested. The question remained, was the enzyme present but in an inactive form, unable to utilize the substrates used for assay, or simply absent? With the cloning of PPO in a number of species (*10, 11, 13, 23-26*), it is now possible to look for the presence of the PPO gene in these previously examined phylogenetic groups and to address the question as to the presence or absence in groups that previously show no enzyme activity. The survey conducted here, builds upon this previous work (*4*), covering a wider spectrum of the higher plant kingdom, and examines a number of groups that have not been previously assayed. Here, spectrophotometric assays utilizing a group of different PPO substrates have been utilized to verify the presence of PPO enzyme activity in newly assayed and previously recalcitrant species, and genomic DNA has been isolated from many representatives within each group and examined with a heterologous probe for a highly conserved region of the PPO gene from *Vicia faba*. From this, we hoped to gain a more comprehensive and accurate view of the distribution of PPO in the algae, the bryophytes, and higher plants, and to make inferences concerning essentiality of this enigmatous protein.

Experimental

Plant Material. Plant materials were collected locally (Mobile, AL) when possible or obtained from commercial sources. *Monotropa uniflora* was collected in Perry Co., AR. Axenic *Trebouxia erici was* obtained from Ward Biologicals. *Chlorella vulgaris* was a gift from E. Funkhouser (Texas A&M Univ., College Station, TX). *Chlamydomonas reinhardtii* strain cw 92 mt[+] was a gift from J. Salisbury (Mayo Clinic, Rochester, MN). *Chara fibrosa* and *Phaeoceros laevis* were gifts from K.

Renzaglia (E. Tenn. St. Univ., Johnson City, TN). *Anthoceros fusiformis* and *Megoceros aenigmaticus* were gifts from K. Vaughn (USDA, Stoneville, MS). *Notothylas breutelii* was a gift from R. Brown (Univ. of S. LA, Lafayette, LA). *Artemisia vulgaris* was a gift from S. Weller (Purdue Univ., West Lafayette, IN).

PPO Extraction and Assay. Tissue employed for assay varied with the size of the organism. Algae and bryophytes were homogenized as whole plants or large pieces of plants. For vascular plants, frond or leaf tissue was used, with woody midribs removed. Cells or tissues were homogenized at a ratio of 200 mg tissue/1 mL buffer in cold (0-4°C) 0.1 M sodium cacodylate buffer, pH 7.2, containing 0.1% (^{W}/v) sodium dodecyl sulfate using glass, hand-held homogenizers or with a Brinkmann Polytron. The homogenate was then clarified by centrifugation at 12,000 x g for 2 min. In the standard PPO assay, the conversion of DL-dihydroxyphenylalanine (DOPA) to quinone polymers in the supernatant was monitored spectrophotometrically at 490 nm. The assay solution consisted of 1 mL of 5 mg/mL DOPA in 0.1 M sodium cacodylate, pH 7.2, which had been aerated for 5 min prior to assay. To prevent oxidation of the substrate by peroxidase, 280 units of bovine liver catalase in 0.1 mL of water was added. The assay was initiated by addition of 0.05 mL of the plant extract and monitored for 2 minutes at 30°C. Some of the species with very low or no activity in the standard assay were also examined in a modified assay in which samples were incubated in the presence of each of four different commonly used PPO substrates, along with appropriate controls in 96-well plates for 10 h at 22°C with intermittent shaking and hourly absorbance readings taken at 490 nm. These assays were conducted in a volume of 0.2 mL and contained either 5 mg/mL DOPA, 10 mM catechol, 10 mM caffeic acid, or 10 mM chlorogenic acid in 0.1 M sodium cacodylate, pH 7.2. All assays also contained 40 units/mL catalase to prevent peroxidation of the substrate. Assays were started with the addition of 0.01 mL of plant extract (prepared as outlined above). One set of control wells contained additional 1 mM diethyldithiocarbamate as an inhibitor of PPO. A second control set contained plant extract but no additional substrate. Absorbance readings from these two controls were used to correct for non-PPO induced browning and oxidation of substrate that was introduced into the assay along with the plant extract, respectively.

DNA Isolation. A modification of the method of Lodhi *et al.* (*27*) was used for DNA isolation from all plant species. Briefly, this was a follows. Approximately 500 mg fresh weight plant material, similar to that used for PPO activity assays, was frozen and ground to a fine powder in liquid nitrogen. While still frozen, the slurry was transferred to preheated (60°C) extraction buffer containing 5 mL of 100 mM Tris (pH 8 at 20°C), 20 mM Na_2EDTA, 1.4 M NaCl, 2% (^{W}/v) cetyltrimethylammonium bromide, 0.2% (^{V}/v) beta-mercaptoethanol and 50 mg insoluble polyvinylpyrrolidone polymer. The slurry was mixed thoroughly and allowed to incubate for 30 min at 60°C. The sample was then allowed to cool and extracted once with one half volume of chloroform: isoamyl alcohol (24:1). After

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centrifugation at 1,600 x g for 10 min., the upper phase was transferred to clean tube and the nucleic acids were ethanol precipitated through the addition of one half volume of 5 M NaCl and two volumes of 95% ethanol and allowed to stand overnight at -20°C. Nucleic acids were collected by centrifugation at 1,600 x g for 10 min., washed with 76% ethanol, and resuspended in a small volume of 10 mM Tris (pH 8 at 20 °C), 1 mM Na$_2$EDTA. RNA was degraded by treatment with 100 µg/mL RNAse A and 10 U/mL RNAse T1 for 1 h at 37°C. The remaining DNA was quantified using the method of Cesarone *et al.* (*28*) and checked for integrity by agarose gel electrophoresis (*29*).

DNA Hybridizations. One microgram samples of genomic DNA isolated as described above were spotted onto nitrocellulose, allowed to air-dry, and baked at 80°C for 1 hr in a vacuum oven prior to hybridizations. A 798 base pair segment of the cDNA clone for *Vicia faba* PPO covering the area between amino acids 175 to 441 and including the copper A and B regions (*10*) was random primed labelled with α^{32}P-dCTP using a Prime-a-Gene kit (Promega) according to manufacturers instructions. Hybridization were conducted in the presence of 50% formamide, 5x Denhardt's solution, 1% sodium dodecyl sulfate, 2x SSPE (*29*) at 42°C. Blots were washed at low stringency (2x SSPE, 0.1% sodium dodecyl sulfate), and exposed to X-Omat AR film (Kodak) overnight or longer. Hybridization signals were empirically determined and scaled from 0 (no hybridization signal) to 4 (strong hybridization signal). Because it was not know how many copies of the PPO gene would be present in the genomic DNA of each species, it was decided that more quantitative determination of hybridization signal would have little meaning and was not attempted.

Amplification of Conserved Regions of the Copper A Region of PPO. DNA samples from representatives of various phylogenetic groups were chosen from samples isolated as described above. Oligonucleotide primers were synthesized that correspond to the left and right borders of the conserved copper-binding domain (copper A) of the PPO cDNA clone from *Vicia Faba* (*10*). DNA samples (100 ng) from the various species were combined with 100 pmol of each right and left primer and amplified using a Perkin-Elmer AmpliTaq polymerase chain reaction (PCR) kit according to the manufacturers instructions. Thermal cycling conditions were: 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min for 30 cycles followed by 72°C for 5 min. PCR reaction products were analyzed by electrophoresis on a 5% acrylamide gel using a TBE buffer system (*29*), stained with ethidium bromide, and photographed under ultraviolet radiation. Plasmid pBR322 DNA that had been digested with the restriction enzyme MspI was used as molecular mass standards. *Vicia faba* DNA used under these conditions is expected to generate DNA of 111 base pairs.

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Results and Discussion

Ubiquitously found in healthy green plants, there is a form of PPO that appears to be localized exclusively in the chloroplast and is associated with the thylakoid membrane. This is evidenced by immunogold labelling and the fact that cytochemical reaction product accumulates in the lumen of the thylakoid (*4, 30-32*). It has also been demonstrated that the protein precursor of the enzyme contains the targeting sequence necessary for import into the chloroplast and insertion in the thylakoid membrane (*33*). The data that we will report here indicates that PPO may be present in all plant ranging in complexity from the green algae through the angiosperms and gymnosperms (Table I). In light of this distribution, PPO may well serve some indispensible function to the chloroplast. A model for a possibility of this function will be outlined first, providing a conceptual framework to interpret the data.

Although the enzyme takes its name from its ability to catalyze the oxidation of *o*-diphenols, and is most commonly assayed by this activity, this may in fact be a secondary function or have no physiological relevance in many plastid types. One possible function that has been speculated is in the modulation of photosystem I (PSI) reduction of molecular oxygen (the Mehler reaction or pseudocyclic photophosphorylation) (*2, 3, 34, 35*). Normally, photosynthetic energy is utilized in the reduction of intermediates necessary for biosynthetic processes (NADP$^+$, nitrite, etc.), but it has been suggested that under circumstances that limit carbon dioxide availability, destructive, high oxygen concentrations can occur temporarily due to the continued operation of the photosythetic apparatus. A model of a possible scenario where energy can be diverted to dissipation of molecular oxygen via a Mehler-like reaction involving PPO is shown in Figure 1. The mechanism depicted in this figure is different slightly from that outlined for the Mehler reaction as it has been previously presented (*2, 35*), because the electron-donating end of the PSI complex, believed to be involved in Mehler activity, is on the stromal side of the thylakoid membrane, whereas PPO appears to be associated with PSII on the lumenal side of this membrane (*36, 37*). In the scheme shown here, PPO acts to oxidize the reduced quinones (internal to the membrane) generated by PSI activity, thereby preventing molecular oxygen accumulation and regenerating substrate for continued operation of the cycle. It is well known that there are bound quinones associated with PSII (Q_a and Q_b) and are involved in electron transport so the possiblity of other membrane localized quinones can not be ruled out. Additionally, this model requires quinones to be present in only catalytic quantities within the plastid, and thus would be possible even in species that are not known to possess high levels of tissue-quinones. Furthermore, a manganese-containing superoxide dismutase has long been thought to be associated with the thylakoids of cyanobacteria and green algae (*38, 39*). The presence of a similar enzyme in higher plants was reported earlier (*40*), but was later disputed (*41*). If such an enzyme exists, it would allow for hydrogen peroxided that is not particularly reactive in itself (*42*) to cross the membrane for further processing in the stroma. In this model, a role for PPO in an oxygen mediating reaction is possible that is consistent with

Table I. Summary of PPO data concerning enzyme activity and DNA hybridization

Group	Genus/species	Common name or discriptor	PPO Activity[1]	DNA collected	Hyb. Signal (0-4)[2]
Chlorophytes					
(Green algae)					
	Chlorella vulgaris		none	+	
	Chlamydomonas reinhardtii	strain: cw92 mt (+)	trace	+	
	Choleochaete scutata		trace	-	
	Spirogyra grevileana		none	+	1
	Microspora sp.		none	-	
	Stigeoclonium tenue		none	-	
	Trebouxia erici (axenic)		none	-	
	Ulva faciata		none	-	
(other algae)					
	Fucus sp.		trace	+	
	Rhodymenia sp.		trace	+	1
	Laminaria sp.		none	+	0
	Chondrus sp.		low	+	4
	Monostroma sp.		none	+	1
(Charlean algae)					
	Chara fibrosa		none	+	1
	Nitella sp.		low	+	0

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Table 1. (continued) Genus/species	Common name or descriptor	PPO Activity[1]	DNA collected	Hyb. Signal (0-4)[2]
Bryophytes				
(hepatics)				
Conocephalum conicum		low	+	0
Marchantia polymorpha		high	+	1
Riccia fluitans		none	-	
Riccia sp.		none	+	
(anthocerotes)				
Anthoceros fusiformis		low	+	
Megoceros anaegmaticus		low	-	
Phaeoeros laevis		low	-	
(mosses)				
Dicranum sp.		none	+	1
Hypnum sp.		trace	+	
Mnium sp.		low	+	3
Sphagnum sp.		none	+	
Thuidium delicatulum		none	-	
Polypodiophytes				
(ferns)				
Asplenium platyneuron	Ebony spleenwort	low	+	4
Marsilea quadrifolia	Water clover	low	-	
Polystichum acrostichoides	Christmas fern	low	-	
Salvinia sp.		low	+	0.5

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Table 1. (continued)	Genus/species	Common name or discriptor	PPO Activity[1]	DNA collected	Hyb. Signal (0-4)[2]
Lycopodiophytes					
(fern allies)	*Equisetum* sp.	Horsetail	low	+	4
	Isoetes sp.	Quillwort	low	-	
	Lycopodium sp.	Club moss	n.a.	+	3
	Psilotum nudum	Wisk fern	high	+	4
	Selaginella apoda	Spikemoss	mod	+	0
Pinophytes					
(gymnosperms)	*Araucaria excelsa*	Norfolk Island Pine	none	+	1
	Cedrus lebani	Lebanon Cedar	none	-	
	Chamaecyparis thyoides	Atlantic White-cedar	none	-	
	Ginkgo biloba	Ginkgo	none	+	
	Juniperus virginiana	Northern Redcedar	none	+	
	Pinus palustris	Longleaf pine	none	+	
	Pinus strobus	White pine	none	-	
	Pinus virginiana	Virginia pine	none	+	1
	Podocarpus macrophyllus	Buddhist-pine	none	+	1
	Taxodium distichum	Baldcypress	none	-	
	Zamia integrifolia	Coontie	trace	+	

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Table 1. (continued)	Genus/species	Common name or discriptor	PPO Activity[1]	DNA collected	Hyb. Signal (0-4)[2]
Magnoliophytes					
(angiosperms)					
	(aquatic)				
	Elodea densa (*Anacharis densa*)	Brazilian waterweed	v.high	+	
	Cryptocoryne affinis		low	-	
	Echinodorus sp.	Burhead	trace	-	
	Lemna minor	Duckweed	trace	+	
	Ludwigia arcuata Walt.	False loosestrife	low	-	
	Myriophyllum sp.	Parrot feather	n.a.	+	0.5
	Vallisneria sp.	Eel grass	low	+	4
	(terrestrial)				
	Albizia julibrissin	Senstive plant	n.a.	+	1
	Ambrosia artemesifolia	Ragweed	n.a.	+	
	Artemisia vulgaris	Mugwort	v.high	-	
	Borrichia frutescens	Sea Oxeye	n.a.	+	1
	Carya tomentosa	Mockernut hickory	n.a.	+	0.5
	Catalpa bignonioides	Southern Catalpa	n.a.	+	
	Chlorophytum comosium	Spiderplant	n.a.	+	3

Table 1. (continued)

angiosperms (cont)	Genus/species	Common name or descriptor	PPO Activity[1]	DNA collected	Hyb. Signal (0-4)[2]
	Cornus florida	Flowering Dogwood	n.a.	+	
	Cucurbita maxima	Cucumber	trace	-	
	Cyrilla racemiflora	Cyrilla	n.a.	+	3
	Diospyros virginiana	Common Persimmon	n.a.	+	
	Distichlis spicata	Saltgrass	n.a.	+	4
	Juncus sp.	Rush	n.a.	+	0
	Hydrocotyle bonariensis	Pennywort	low	+	0
	Lactuca sativa	Lettuce	v.high	+	
	Lycospersicon esculentum	Tomato	n.a.	+	4
	Magnolia grandiflora	Southern Magnolia	n.a.	+	3
	Magnolia virginiana	Sweetbay Magnolia	v.high	-	
	Monotropa virginiana	Indian Pipes	trace	-	
	Orontium aquaticum (L.)	Golden Club	n.a.	+	
	Plantanus occidentalis	E. Sycamore	n.a.	+	
	Quercus falcata	S. Red Oak	n.a.	+	3
	Quercus virginiana.	Virginia Live Oak	n.a.	+	4
	Robinia pseudoacacia	Black Locust	low	+	

Table 1. (continued) Genus/species	Common name or discriptor	PPO Activity[1]	DNA collected	Hyb. Signal (0-4)[2]
angiosperms (cont)				
Sabatia sp.	Marsh Pink	n.a.	+	1
Sagittaria sp.	Arrowhead	n.a.	+	3
Salicornia biglovii	Glasswort	n.a.	+	0.5
Sapium sebiferum (L.)Roxb.	Tallowtree	n.a.	+	3
Sesuvium sp.	Sea purslane	n.a.	+	0
Spatina alternaflora	SaltmarshCordgrass	n.a.	-	0
Spinacia oleracea	Spinach	low	+	
Thalassia testudinum	Turtle grass	n.a.	+	
Vicia faba	Broadbean	high	+	
Vigna radiata	Mung bean	v.high	-	
Vigna unguiculata	Cowpea	low	-	
Vitus aestivulus	Summer grape	n.a.	+	
Vitus rotundifolia	Muscadine grape	n.a.	+	
Zygocactus sp.	Christmas cactus	none	+	

[1] trace: < 1 unit; low: 2-33; moderate: 34-66; high: 67-100; very high > 100 (1 unit = A_{490} min^{-1} g^{-1} fr.wt.) n.a. (not assayed)

[2] relative hybridization signal one xray film. 0 (no signal) - 4 (dark signal)

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photosystem and membrane topology. In support of this theory, it has been reported that cells that generate little or no molecular oxygen, such as agranal bundle sheath cells of C$_4$ plants (31) and guard cells (43, 44), generally have no PPO. There is an additional benefit to the plant of having an oxygen consuming system localized within the thylakoid. Oxygen, which diffuses easily across biological membranes, becomes an additional problem when it arrives in the stroma because of its competitive inhibition of ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO).
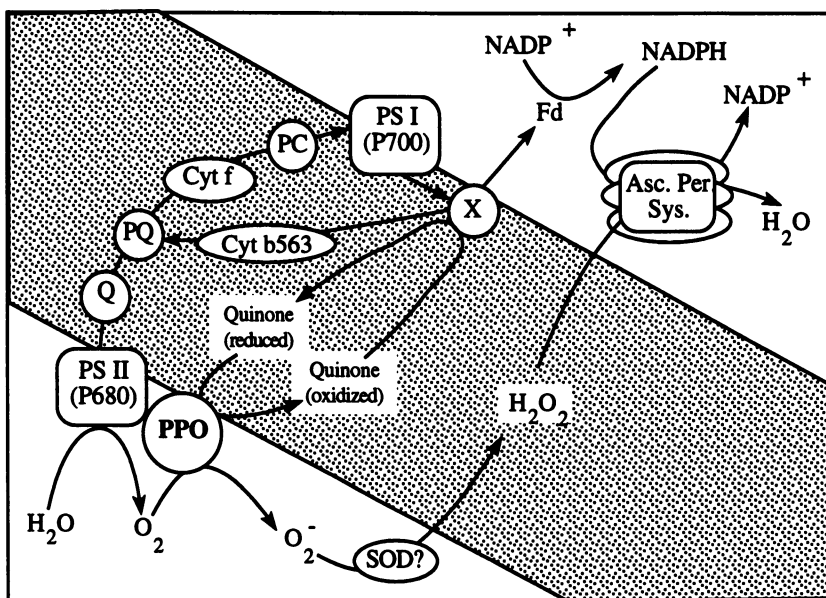
Figure 1. Model for PPO's possible involvement in a Mehler-like reaction for detoxifying oxygen species.

This is only one possible role for PPO and it is entirely possible that it has a number of unrelated functions in normal plant growth and development that utilize its ability to interact in redox reactions with quinones. Whatever its role, some form of PPO seems to be essential for some aspect of plant function. This is evidenced by the fact that there are no reported naturally-occurring or experimentally-generated viable mutants that completely lack all PPOs. In mutants of grape that have sections of the leaves that lack of PPO activity, these sections are lacking in competent photosynthesis and appear pale yellow (25). Also, in plants that have been treated with tentoxin, a cyclic peptide of fungal origin, PPO import into the chloroplast is blocked (33, 45). These plants are photosynthetically incompetent and have this same yellow pigmentation as that seen in the grape mutant. Recently, Steffens'

group (*12*) has expressed PPO-anti-sense mRNA in potato so as to decrease PPO activity to less than 5% of that found in the untransformed variety, and yet the transformed plants seem to be morphologically normal. These data indicate that either the essential form of PPO remained uneffected by the treatment or that very little PPO is necessary for normal plant growth.

If it is true that PPO plays some important role in defense against pathogens and/or oxidative damage within the plastid, it is stands to reason that this enzyme should have wide distribution within the plant kingdom. If, in fact, it is physiologically necessary, it should be present in all green plants. In this case, data presented here is strongly supportive of an important physiological role. DNA sequence corresponding to the copper-binding region (copper A) is highly conserved among those PPOs that have been cloned and sequenced (*10, 11, 13, 24, 26*) and the sequence in this region is shared by no other proteins reported to the genetic databases to date. It is apparent, based upon the results of the PCR experiments, that this 111 base pair sequence is present in all of the phylogenetically dissimilar genera that were examined here (Figure 2). Homology of this region between *Vicia faba* and a phylogenetically distant member of the group, *Chlamydomonas,* was determined by subcloning and sequencing the amplified algal DNA. Sequence analysis of this DNA showed only a single amino acid substitution of an isoleucine for a histidine at position number 211 in the 37 amino acids coded for in the 111 base pair section. This region of DNA could exist as a vestigial remnant of some ancestral gene, but there is a great likelihood that mutations would create changes in the sequence over the hundreds of millions of years since the divergence of these two groups. In fact, the sequence homology reported for the chloroplastically-encoded large subunit of RuBisCO varies by 21% between *Chlamydomonas* and *Lycopersicon,* and the rate of silent substitution in chloroplastic genes is in the order of two to three times lower than that of nuclear encoded genes (of which PPO is a member) (*46*). Based upon the highly conserved nature of this copper-binding region that has been reported here and elsewhere, it would seem that the sequence and length of this region is necessary for function and that the gene encoded in these various species is likely to be functional. The DNA evidence is also supported by the enzyme activity assays reported here. Oxidative activity was found to be present at low levels and with different substrate affinities in groups that showed no activity in the previous survey (the green algae, mosses and gymnosperms). Representatives of these assays are shown in Figure 3. Here catechol was a preferred substrate to DOPA in all cases, but measured activity was very low in each case, relative to the activity monitored in species such as *Vicia faba* or *Lactuca sativa* that reached off-scale absorbance readings in minutes rather than hours (data not shown). The substrates chlorogenic acid and caffeic acid, that are preferred by the trichome form of PPO in potato (*14*) showed essentially no activity with these species that showed very low activity levels. It is tempting to speculate that this low level of activity found in these species is in keeping with the "catalytic" quantities described above that would be involved in a PPO-based "Mehler"-like scenario. Also, this data emphasizes the point that traditional enzyme
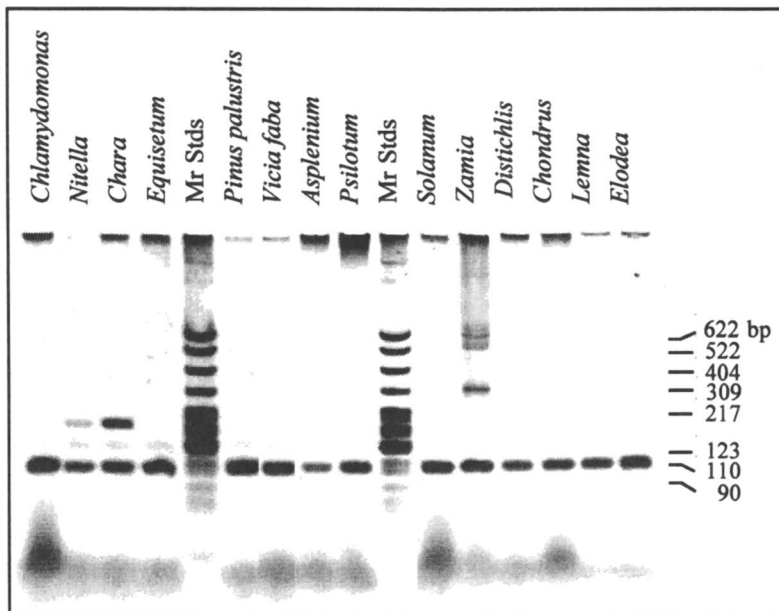
Figure 2. PCR amplification products of genomic DNA corresponding to the 111 bp fragment of the copper-binding region (copper A) of *Vicia faba* PPO. (Mr standards: MspI digested pBR322 DNA)

assays used to monitor PPO may be monitoring a secondary and possibly physiologically irrelevant activity, at least in this ubiquitous form of PPO reported here.

In conclusion, the data presented reinforce the idea that some form of PPO has an essential role in plant growth and/or metabolism. That function is still open to speculation. One possibilty is presented here that encompasses a number of ideas and observations from other published work. Further analysis of the gene encoding the *Chlamydomonas* PPO, its use in anti-sense expression studies, and search for the presence of PPO in the more primitive photosynthetic cyanophytes, will be examined in the future and will hopefully add pieces to the rapidly assembling puzzle of PPO function.

Acknowledgments
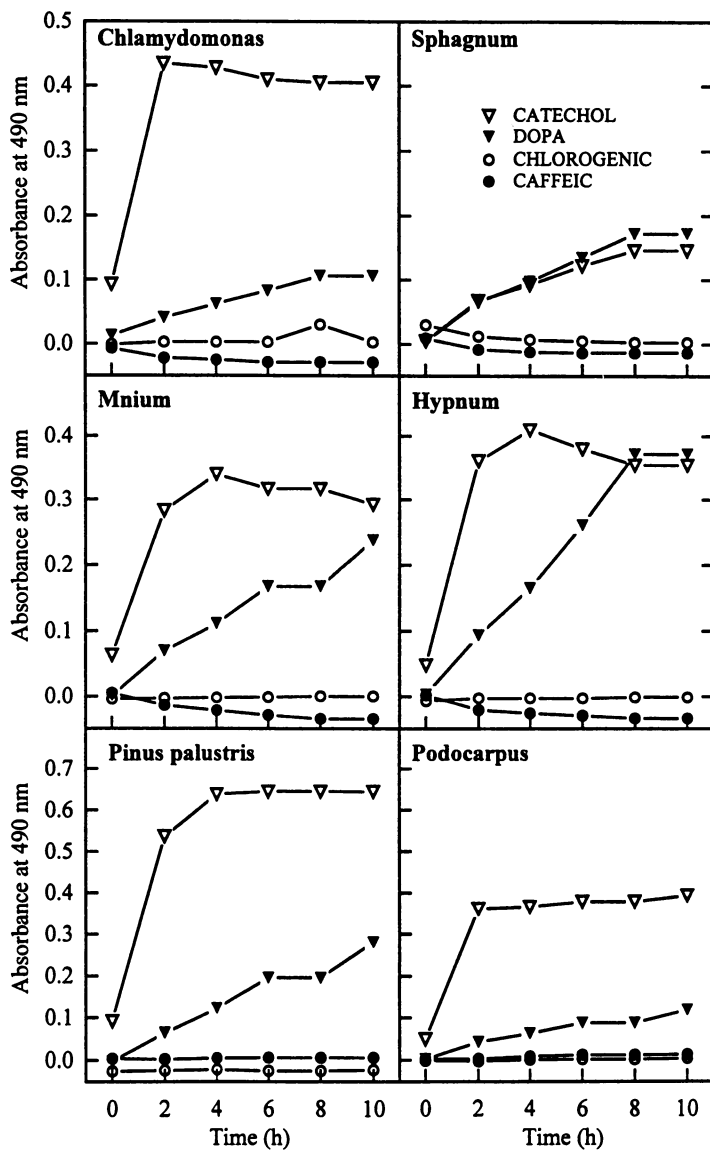
Figure 3. Long-term assays for PPO in species showing little activity in the standard assay.

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Biology and Molecular Biology of Polyphenol Oxidase

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Polyphenol oxidases (E.C. 1.10.3.2) are ubiquitous in the plant kingdom, but little is known of their subcellular localization or physiological significance. Recent work in our and several other laboratories have succeeded in cloning and characterizing the genes encoding polyphenol oxidase from a variety of species. Analysis of the clones indicates a high degree of homology among the PPOs from the investigated species. The data indicate that PPOs are synthesized as precursor proteins having a transit sequence for transport into the chloroplast and lack sequences for specific targeting into the thylakoid membrane. Cloning of the genes for PPO offers the possibility to determine the physiological role of PPO within the chloroplast and to begin to manipulate the levels of PPO within specific organs to improve the characteristics of the desired commodity.

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Biology of Polyphenol Oxidase

Polyphenol oxidases (E.C.1.10.3.2.) are reported to be ubiquitous in higher plants, although no function has been firmly established for the enzyme. Several exhaustive reviews concerning the biology and physiology of the enzyme have been presented recently. This review is not meant to be exhaustive but rather to update recent findings concerning the molecular biology of the enzyme. This information in conjunction with the known biological features of PPO will be of paramount importance to understanding the *in vivo* physiological significance of this intriguing enzyme. Several varied forms of the enzyme are known including a chloroplast localized form and an enzyme which is found in glandular trichomes of *Solanaceous* plants (*1-9*). In the case of the latter, a formal function of plant protection from predation by insects has been ascribed and can be

defended (*7*). In the case of the former many functions have been postulated but, as yet, not proved. Functions attributed to chloroplast PPO include mediation of the Mehler reaction, phenol metabolism, protection from insect and herbivore damage, and a role in host defense, with conflicting data offered in the literature regarding all of these postulated roles (*2, 7*). Much of the difficulty in assigning function to PPO in the higher plant chloroplast is a result of its imprecise subcellular localization. Recent fractionations have now fairly well established that PPO is located in the grana lamellae loosely associated with photosynthetic photosystem II (*10*). Earlier reports that associated PPO with photosystem I (*11*) can now be rather unequivocally ruled out based upon both biochemical fractionation and immunocytochemical localization experiments. There is little compelling reason to believe that PPO functions in the Mehler reaction because of its association with Photosystem II. However, PPO may play a role in a Mehler-like reaction utilizing PSII generated diffusible quinones as depicted in elsewhere in this volume. Early phylogenetic investigations implied a function as an oxygen buffer with PPO evolving as plants adapted to land (*12*), but these data have been updated and are presented in another chapter in this volume.

Aside from the intellectual exercise of understanding the biology of PPO, the enzyme is interesting and compelling in several respects. First, PPO has been reported from virtually every genus of plant examined (*1-5*). It is often an undesirable enzyme from the standpoint of the food industry in that it causes unwanted browning or flavor of tissues. Manipulation of the levels of PPO in various organs is a worthwhile pursuit to improve both flavor and quality. PPO is one of the most extensively investigated enzymes for which no *in vivo* function can be ascribed. Secondly, PPO is somewhat enigmatic being found sequestered in the chloroplast while its potential substrates are sequestered in the vacuole. It is desirable to ascertain its function and to identify the reasons for its sequestration in the chloroplast and whether this compartmentation is important to the metabolism of the chloroplast, perhaps playing a role in photosynthesis or in stabilizing the photosynthetic membrane.

Our interest in PPO began with investigations of the mode of action of tentoxin, a cyclic peptide toxin produced by the fungus, *Alternaria alternata*. Tentoxin causes extreme chlorosis in treated tissues of sensitive species with the plastids in affected tissues showing little or no internal membrane structure (*13-16*). Early experiments with tentoxin demonstrated that tentoxin induced the loss of plastidic PPO while it had no effect *per se* upon the activity of the native enzyme *in vitro* (*15*). Western blot analysis of proteins extracted from treated tissues and immunocytochemistry showed that an enzymatically inactive PPO protein was present in toxin treated tissues and that the inactive protein accumulated at the plastid envelope. Western blots indicated that the inactive protein had the same molecular mass as the active protein and led to the belief that PPO may have a unique pathway for import into the chloroplast (*13-16*). However, this apparent similarity of molecular mass has caused considerable confusion and will be discussed in detail later.

It was determined that tentoxin had no effect on proteins, other than PPO, that were synthesized in the cytoplasm and subsequently transported into the

nucleus. Because tentoxin inhibited chloroplast coupling factor 1 (CF1), it was believed that inhibition of this enzyme caused the chlorosis associated with tentoxin treatment (17). However, the lack of effect on the other proteins indicated that tentoxin induced inhibition of PPO import into the chloroplast resulted from factors other than inhibition of CF1 (10). Indeed a mutant of *Oenothera* having a non-functional CF1 showed chlorophyll accumulation and grana accumulation unless treated with tentoxin (10). The toxin treated plastids were chlorotic and ultrastructurally identical with those of other species indicating a mechanism of chlorosis for tentoxin different from its known effect on CF1. It is perhaps significant that tissues of plants which lack PPO, such as Bruce's Sport that lack PPO, are pale yellow (18,19) and lack internal chloroplast membranes implying an important although unidentified role for PPO in chloroplast metabolism or development.

Molecular Mass of PPO

PPO from a number of species has been characterized with molecular masses ranging from 20 kDa to 180 kDa (1, 4, 7, 20, 22). There are several reasons for such diverse estimated molecular masses, most of which involve the estimation of such molecular masses under non-denaturing or partially denaturing conditions to preserve enzyme activity. Tobacco shows a multiplicity of forms of PPO after electrophoresis of partially denatured chloroplast proteins (6), and there appear to be small gene families for PPO in all of the species in which the genes for the enzyme have been characterized (7-9, 23-26). Steffens, et al. (7) have extensively reviewed the reasons for the observed heterogeneity of forms upon electrophoresis some of which are explained by the heterogeneity of the gene families, but many of which are due to artifacts of protein isolation and electrophoresis. We have found that one of the major reasons for the discrepancy of molecular mass determinations lies in the fact that in many cases the proteins were not fully denatured in order to preserve enzyme activity for staining of enzyme in partially denaturing gels. Figure 1 shows the anomalous migration of *Vicia faba* PPO upon electrophoresis without complete denaturation. Proteins were solubilized from isolated chloroplasts using lithium dodecyl sulfate but to preserve enzyme activity samples were unheated nor did they contain thiol reagents. Identical samples were heated in the absence or presence of thiol reagents for comparison of the molecular masses. Proteins were subjected to electrophoresis and identified through western blotting using anti-PPO polyclonal antibody. Undenatured PPO migrates as an enzymatically active species (data not shown) having an estimated molecular mass of 45 kDa, while PPO denatured with heat and the addition of 2-mercaptoethanol migrates at a molecular mass of approximately 62 kDa.

Another factor leading to disparate estimations of molecular mass has been attributed to C-terminal proteolysis during isolation which yields an active peptide of 40-45 kDa and an inactive 18-20 kDa carboxy-terminal peptide (25). Proteolytic processing of the enzyme may the play a role in the heterogeneity of the observed forms as well as participate in activating the latent forms of the

protein which are observed in many species (*1-4*). Furthermore, crosslinking because of the enzymes activity has been reported and may contribute to some of the observed variation in mass determination.

Cloning and Characterization of PPO Genes

The molecular weight and the form of PPO have long been a matter of speculation because of the various forms of the enzyme upon electrophoresis and their interconversion as described above.   In addition to elucidating the molecular mass of PPO, the cloning of PPO genes would allow levels of the enzyme to be manipulated in order to investigate the physiological role of the enzyme *in vivo*. PPO genes have now been characterized from broad bean, tomato, potato, and grape (*7-9, 22-26*). Characterization of PPO genes in various plant species has shown that these genes are present in the plant genome as gene families. We have identified a small PPO gene family in *V. faba* consisting of a minimum of three genes based on DNA sequence analysis of two full-length and one partial cDNA isolated from mature leaf tissue (*24*). Seven genomic PPO genes have been identified in tomato (*8*). These clones have been differentiated into three classes based upon restriction mapping and DNA sequence data. Pulsed field electrophoresis has indicated that the tomato PPO genes are all clustered within an approximate 165 kb region of chromosome 8. Shahar, et al. (*26*) have also identified what appears to be a small PPO gene family in tomato based on analysis of both cDNA and genomic clones. In addition, Hunt, et al. (*22*) have isolated two PPO cDNAs from potato leaf.   All plant PPOs cloned to date have been found to encode mature peptides of 57-62 kDa with 8-11 kDa putative transit peptides (*7-9, 22-26*). While the overall homology at the amino acid level between *Vicia* and tomato is approximately 42% the copper A binding regions believed to be the catalytic centers of the enzyme have homologies in the range of 92%. Homology for the copper B region is 60% (Figure 2). Northern blot analysis of total leaf RNA from *V. faba* identified a PPO transcript of approximately 2.2 kb (*24*). This is consistent with the size expected for a precursor protein of about 68 kDa. Transcripts of about 2 kb have been observed from Northern hybridizations of total leaf RNA from tomato, potato and grape (*7-9, 22-26*).

PPO is found in virtually all organs of plants regardless of the maturity of that organ. In many cases the activity is present but reduced without some activation either by solubilization of the enzyme or by some mechanism of enzyme activation. In contrast with the long-lived presence of the enzyme in the majority of plant species in which PPO has been investigated, mRNA for PPO is found only transiently in young tissues of the plants. The only known exception to this is in the case of *Vicia faba* in which mRNA has been found in all ages of leaves investigated, including mature leaves (*7, 24*). These data indicate that PPO has a very long half-life at least in species in which no transcript can be found at developmental stages in which enzyme activity is present. We can offer no explanation for the transient nature of transcript at the present time, but further
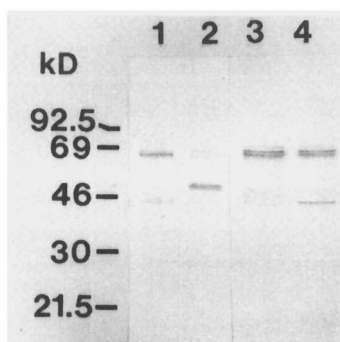
Figure 1. Western blot analysis of polyphenol oxidase from *Vicia faba* leaves. Lane 1, purified PPO, undenatured; lane 2, purified PPO, boiled only; lane 3, PPO treated with 2-mercaptoethanol and boiled; lane 4, puriifed PPO treated with 2-mercaptoethanol only. Note the multiple forms interconverted through denaturation. Molecular mass markers indicated are prestained markers (Amersham, Arlington Heights, IL): myosin, 200 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa, trypsin inhibitor, 21.5 kDa; and lysozyme, 14.3 kDa.

Cu A binding site

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ST-A    HFSWLFFPFHRWYLYFYERILGSLINDPTFALPYWNW
PPOE    HFSWLFFPFHRWYLYFYERILGSLINDPTFALPYWNW
A1      HGSWLFFPFHRWYLYFYERILGSLINDPTFALPFWNY
B5      HGSWLFFPFHRWYLYFYERILGSLINDPTFALPFWNY
Q1      HGSWLFFPFHRWYLYFYERILGSLINDPTFALPFWNY
P2/B    HFSWLFFPFHRWYLYFYERILGSLINDPTFALPYWNW
        * ****************************** **
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Cu B binding site

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ST-A    HTPVHIWTGDKPRQKNGEMNGNFYSAGLDPLFYCHHANVDRMWDEWKLIGGKRRD
PPOE    HTPVHIWTGDKPRQKNGEDMGNFYSAGLDPIFYCHHANVDRMWNEWKLIGGKRRD
A1      HAPVHTWTGDNT-QTNIEDMGIFYSAARDPIFYSHHSNVDRLWYIWKTLGGKKHD
B5      HGPVHTWTGDNT-QTNIEDMGIFYSAARDPIFFSHHSNVDRLWSIWKTLGGKKHD
Q1      HGPVHTWTGDNT-QPNIEDMGIFYSAARDPIFFSHHSNVDRLWSIWKTLGGKKHD
P2/B    HTPVHIWTGDKPRQKNGEGMGNFYSAGLDPIFYCHHANVDRMWNEWKLIGGKRRD
P2/A    HTPVHIWTGDSPRQGNGEDMGNFYSAGLDPLFYCHHANVDRMWNEWKLIGGKRRD
        * *** ****   * * * ** ****  ** *   ** **** *  **  *** *
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Figure 2. Comparison of the deduced copper A and copper B binding regions of several plant polyphenol oxidases. St-A is Steffens potato clone A, PPOE is clone E from tomato from Steffens, A1, B5 and Q1 are the three *Vicia faba* clones and P2/B is the tomato clone from Shahar et al. Asterisks indicate identity of the deduced amino acid. Conserved amino acid substitutions are common but are not indicated.

investigation is warranted to determine whether this phenomenon is a general trend or is confined primarily to the *Solanacae*.

This transient transcriptional regulation offers the possibility of expression of PPO in specific plant organs if appropriately designed coordinately controlled antisense constructs can be designed. This could be required for maintenance of enzyme if PPO is required for chloroplast metabolism or for resistance to pathogens or predation.

Chloroplast Import of PPO

The presence of a putative transit sequence identified in all PPO clones is consistent with the presence of transit peptides in other known proteins which are synthesized in the cytosol and imported into the chloroplast (*2, 7-9, 22-26*). This is in contrast to the earlier suggestion that PPO was uniquely synthesized as a protein having its mature molecular mass transported intact into the chloroplast (*2, 16*). This hypothesis was based upon the molecular mass discrepancy earlier described. PPO has now been shown to be targeted to the thylakoid lumen in a two step process (*9*).   At least several forms of the enzyme appear to have regions which could associate with the thylakoid membrane, while species in tomato also exist which contain no such binding domains (*7 ,9*). It remains to be shown whether all of these forms are in fact transcribed and imported into the chloroplast *in vivo* and what funtions each plays in the chloroplast metabolism. Our findings have shown that the *V. faba* plastid PPO genes encode a protein with a predicted mature molecular mass of 58 kDa and a putative transit peptide of 10 kDa (*24*). Similarly, the genes encoding PPO from tomato, potato and grape also contain a putative transit sequence homologous with that identified for *Vicia faba* (*7-9, 22-26*). The 92 amino acid *V. faba* transit peptide displayed the expected characteristics found in the transit peptides of other known plastid-targeted proteins. Namely, an amino-terminal domain rich in the hydroxy-amino acids serine and threonine characteristic of the stromal-targeting domain of imported plastid proteins is found for all of the PPO genes identified to date. There is also a central region that has interspersed basic amino acid residues but almost no acidic residues. In addition, the *V. faba* PPO transit peptide has a hydrophobic carboxy-terminal region typical of nuclear-coded proteins targeted to the lumen. Both potato and tomato putative transit peptides share a high degree of homology with that of *V. faba* suggesting a common mechanism of processing and targeting both into and within the chloroplast among these plant species (*7-9, 22-26*). Interestingly, *V. faba* PPO genes showed no homology to the copper-containing and thylakoid membrane-localized *V. faba* plastocyanin (data not shown). There was very little homology between the transit peptides of these two enzymes indicating a possibly dissimilar mechanism of subchloroplast routing. Immunolocalizations of *Vicia faba* PPO clearly show an association with the stacked regions of thylakoid membranes and appear to be concentrated near the margins of the grana (*10*). In tomato there appears to be possibly two forms of the enzyme, one form loosely attached to the thylakoid while the other form is a soluble lumenal form of the enzyme (*9*). Clearly further work is required to

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resolve the exact subcellular location of the various forms of PPO and to determine whether one or more functional roles is played by these forms.

In vitro translated PPO has been successfully imported into chloroplasts recently and has been shown to be processed in a manner similar to that of other known proteins synthesized in the cytoplasm but destined for the chloroplast (9). Interestingly this transport is inhibited by relatively low concentrations of copper ions (9). This may indicate that copper is inserted into the enzyme following transport into the chloroplast or that some component not present in the import assay is required for import competence. Alternatively copper binding may alter the tertiary structure of the protein thus affecting in vitro import. Significantly, tentoxin also inhibited the *in vitro* import of PPO into the chloroplast (9) further validating the findings of Vaughn and co-workers (2, 10, 16) concerning the molecular lesion responsible for the chlorosis induced by tentoxin. In all of the species studied to date tentoxin sensitive species (based upon chlorosis induced by the toxin) have demonstrated inhibition of transport of PPO into the chloroplast while synthesis of the PPO was unaffected based upon immunoblotting after solubilization and electrophoresis of the leaf proteins. Having the availability several clones and an *in vitro* import assay (9) will allow us to study more fully the molecular biology of chloroplast uptake of the enzyme.

Physiological Role of Polyphenol Oxidase

Polyphenol oxidase has been ascribed many potential functions in the plant and has long been of interest because of its many undesirable side effects especially in the food industry (*1-4*). Enzymologists have long been aware of the unpleasant effects of the enzyme upon purification of other plant enzymes. Having identified the gene(s) involve in its biosynthesis in a variety of plant species offers the potential to produce transgenic plants having regulated levels of PPO using antisense or overproduction constructs (*7-9, 22-26*). This should finally allow the determination of the function of PPO not only in the well characterized insect and host plant/pathogen resistance but also would allow the determination of any contribution to the metabolism of the chloroplast itself.

As indicated above tentoxin selectively inhibits the import of PPO into the chloroplast *in vivo* and *in vitro*. This inhibition is accompanied *in vivo* by the marked chlorosis of affected tissues. Plant tissues which lack PPO because of nuclear mutation are also chlorotic and are ultrastructurally indistinguishable from tissues treated with the toxin (2, 16). The role of PPO inhibition of this chlorosis is still unresolved, however understanding the timing and the regulation of PPO biosynthesis may allow us to more fully investigate this question. Clearly there is an effect of the toxin on PPO levels and the toxin has been shown to specifically inhibit the transport of PPO. This raises the intriguing possibility of the use of tentoxin to prevent the accumulation of unwanted PPO by judicious application of the compound.

Steffens and his co-workers have reported the construction of antisense mutations in several species in which no detectable PPO was present. These

tissues did not exhibit abnormal appearance nor were they reported to be chlorotic (7). These seemingly disparate findings must be further investigated to determine what if any role the import inhibition of PPO plays in the chlorosis induced by tentoxin, and whether all forms of PPO were inhibited using the described antisense constructs.

Conclusions

We now have at hand more tools for the further characterization of PPO in plants and are beginning to develop the ability to manipulate the levels of PPO in tissues for their desirable (anti-predation) and undesirable characteristics (discoloration and oxidation). Clearly this research will lead us to further understanding not only of the regulation of this enzyme, but also will abet our understanding of the physiological role which has so long eluded our understanding.

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Chapter 10

Reactions of Enzymically Generated Quinones in Relation to Browning in Grape Musts and Wines

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The role of enzymically generated caffeoyltartaric acid quinones in browning development was investigated in grape musts and model solutions. Caffeoyltartaric acid quinones are only slightly colored and undergo condensation rather slowly, yielding mostly colorless products. On the other hand, they are powerful oxidants and, in particular, oxidize flavan-3-ols to unstable secondary quinones which proceed readily to brown polymers. The structure of dimers resulting from caffeic acid oxidation was elucidated, and the involvement of nucleophilic attack by water in the process of their formation demonstrated. Seven dimers, including both colorless and brown compounds, were obtained by oxidation of catechin and characterized. The involvement of caffeoyltartaric acid quinone in anthocyanin oxidative degradation was also established. Trapping of primary quinones by glutathione competes with reactions leading to must discoloration but maintains high levels of oxidizable compounds, especially flavan-3-ols, which serve as browning precursors in wine.

Phenolic compounds are important grape constituents, responsible for major organoleptic properties of wines, in particular color (*1, 2*). The phenolic composition of wines depends on the grape initial level of phenolics and also of other compounds which may interfere with them as well as on the wine-making process used, which greatly influences the extraction of various components from grapes and their subsequent reactions. Phenolic compounds are easily degraded (e.g. by oxidation or hydrolysis). They may also form covalent products and non-covalent complexes with various types of molecules. Some of these reactions start when the grapes are crushed or pressed whereas others take place during storage and ageing.

In particular, phenolic compounds largely contribute to oxidative browning in white grape musts and wines as well as in other plant-derived food products (*3-5*).

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The fundamental step in enzymatic browning is the enzymically catalyzed oxidation of phenols into quinones (*3, 4*) which further react and form more or less colored products. The quinones produced are colored, with absorbance maxima around 400 nm in the visible region (*6, 7*). They are powerful oxidants, susceptible to oxidize other compounds and be reduced to the original phenol. They may also react with nucleophilic compounds including thiols, amines, and other phenols (*3*). Some of the secondary reaction products are colored or may be oxidized to colored quinoidal forms. Thus, the discoloration resulting from enzymatic oxidation, although it also depends on the nature of the primary quinone formed, is largely determined by its subsequent reactions.

In grape products, the major enzyme involved in phenolic oxidation is catecholoxidase (*o*-diphenol : oxygen oxidoreductase [EC 1.10.3.2]), which catalyzes hydroxylation of monophenols to *o*-diphenols and oxidation of the latter to *o*-quinones, in the presence of molecular oxygen (*8, 9*). Another polyphenoloxidase of fungal origin, laccase ([EC 1.10.3.1]), which oxidizes both *o*- and *p*-diphenols to the corresponding quinones, may also be present, in particular when grapes are infested by *Botrytis cinerea* (*9*).

The major substrates of polyphenoloxidases in grapes are caftaric (caffeoyltartaric) acid and, to a lesser extent, coutaric (*p*-coumaroyltartaric) acid (*10, 11*) which are both transformed to caftaric acid o-quinone. The *o*-quinone spontaneously reacts with the available glutathione to yield 2-S-glutathionyl caftaric acid (*12*), called Grape Reaction Product or GRP (*13, 14*). Formation of GRP, which is neither colored nor a substrate for grape catecholoxidase, was first believed to limit browning of musts and wines by trapping caftaric acid quinone and preventing it from proceeding further to brown polymers (*14*). However, GRP can be oxidized to the corresponding *o*-quinone by laccase (*15*) and by the *o*-quinones of caftaric acid (*16*). Besides, grapes and wines contain other phenolic substrates, including various flavan-3-ols (catechin, epicatechin and their derivatives) (*17-20*), flavonols (*21, 22*), and, in the case of red grapes, anthocyanins (*21, 23*). All these compounds serve as poor substrates for PPO but are rapidly degraded by enzymically generated *o*-quinones, due either to coupled oxidation or to condensation reaction between phenol and quinone (*24*). Among grape constituents, flavan-3-ols (condensed tannins) greatly increase must and wine browning susceptibility (*25-27*).

The purpose of the work presented herein was to determine the exact nature (substrates involved, products formed) and relative importance of the major reactions of quinones responsible for grape must browning, as well as their consequences on the resulting wines. These reactions include oxidation of various reductants, formation of glutathionyl adducts, and condensation with different phenolic compounds. Although laccase attacks a wider range of substrates than grape catecholoxidase (*9, 15*) and may induce further browning, our studies were restricted to the case of sane grapes (i.e. devoid of any mold or rot). Therefore, the general term polyphenoloxidase (PPO) refers to grape catecholoxidase in the following discussion.

Experimental

Chemicals.   Sodium benzenesulfinate, sodium metabisulfite, sodium periodate, caffeic acid, (+)-catechin, ascorbic acid, and Amberlite IRA 904 were purchased from Fluka (Buchs, Switzerland). *Trans*-caftaric acid was isolated from grape juice

and GRP prepared by oxidizing it enzymatically in the presence of glutathione. Both were purified by preparative HPLC (16). Procyanidins were extracted from grape seeds, purified by HPLC on the semi-preparative scale, and identified in our laboratory (20). The major grape anthocyanins were purified from a grape skin extract prepared following the procedure of Glories (28) by semi-preparative HPLC and identified as described by Wulf and Nagel (23). Standard malvidin-3-glucoside from Sarsynthèse (Mérignac, France) was also available.

Preparation of Crude Grape PPO Extract and Assay of PPO Activity. The crude grape polyphenoloxidase extract was prepared as described by Singleton and co-workers (14) and stored at -18 °C until used. PPO activity was measured by polarography using 4-methyl catechol or catechin as substrate.

Model Solution Studies. All the enzymatic oxidations were carried out in must-like solutions consisting of 0.013 M potassium hydrogen tartrate (pH 3.6), at 30 °C, with air agitation on a magnetic stirrer, unless otherwise specified. The enzyme was suspended in the potassium hydrogen tartrate solution and sonicated for 5 min before use to break the protein aggregates and homogenize the suspension. The reactions were started by addition of the enzyme suspension. All incubations were performed in triplicate. One-milliliter aliquots were withdrawn at regular intervals throughout the incubation period. Sodium benzenesulfinate was immediately added to trap the free quinones eventually present (29) and the enzyme inhibited by addition of a sodium metabisulfite solution (0.1% w/v). Sodium fluoride (25 mM), another strong PPO inhibitor (30), was used to stabilize the samples in replacement of sodium metabisulfite in order to study the effect of sulfiting on both the enzyme and the quinones under wine-making conditions. All samples were filtered through 0.45 μm membranes and analyzed by HPLC.

Organic solution of caffeic acid o-quinone was prepared by chemical oxidation, using Amberlite IRA 904-supported periodate as the oxidant (31). After filtering off the resin, the o-quinone solution was diluted with appropriate solvents and its reactions monitored by spectrophotometry or HPLC.

Iron-catalyzed autoxidation of (+)-catechin (4mM) was studied in aqueous 0.02 M potassium hydrogen tartrate (pH 3.7) containing 20% ethanol to stimulate wine conditions and prevent microbial growth. Iron was added as ferric sulphate at 0, 2, 5, 8, 10, 15 and 20 mg/L. The solutions were maintained at room temperature and samples were taken at regular intervals througout the incubation period (one month) for HPLC analysis and browning measurements (at 430 nm).

Controlled Oxidation of Musts. The grapes were carefully sorted to avoid any mold contamination or skin damage. Berry samples (230 to 250g) were randomly prepared and crushed under vacuum using the experimental device described by Rigaud et al. (32). The musts were oxidized by successive injections of oxygen into the reactor by means of a gas syringe. Oxygen consumption was monitored using an Oxi-191 oxymeter (WTW, Weilheim, Germany) equipped with a Clark electrode. Samples were taken when the added oxygen was totally consumed, stabilized as described above, filtered through 0.45 μm membranes and analyzed by HPLC. The oxidation procedure, from injection of oxygen to sampling of the oxidized must, was

repeated until oxygen consumption levelled off. Each oxidation experiment was performed in triplicate.

HPLC Analyses. Phenolic acids and flavan-3-ols were analyzed by reverse-phase HPLC as described earlier (*16*), using a Waters-Millipore system, equipped with a M990 diode array detector.

Another HPLC method was developed to analyze solutions containing anthocyanins. The HPLC apparatus was a Kontron Instruments (Milano, Italy) system including a 465 autosampler, a 325 pump system, a 440 diode array detector and a 450-MT2 data system. The column was reversed phase Lichrospher 100-RP18 (5-μm packing) (250 x 4 mm i.d.) protected with a guard column of the same material (Merck, Darmsdadt, FRG). The elution was carried out as follows: 1 mL/min flow rate; oven temperature, 30 °C; solvent A, water / formic acid (98:2, v); solvent B, acetonitrile / water / formic acid (80:18:2, v); elution with linear gradients from 3 to 20% B in 25 min, from 20 to 30% B in 10 min, and from 30 to 50% B in 10 min, followed by washing and reconditioning of the column.

For quantitation purposes, the detector was set at 280 nm. Calibrations were performed for each compound by injections of known dilutions. The response factor of malvidin-3-glucoside was used for all anthocyanins.

HPLC on the semi-preparative scale was performed under the same conditions with 7.8 mm i.d. columns and 2 mL/min flow rate.

Ascorbic acid was assayed by direct injection of the must samples onto the HPLC system using isocratic elution with water / acetic acid (97.5:2.5, v) containing 10mg/L EDTA, with the detector set at 254 nm.

Identification of Oxidation Products. The isolated products were identified by ^{1}H and ^{13}C NMR (400 and 500 MHz). Caffeic acid derivatives were analyzed by electrospray mass spectrometry in the negative mode (*31*) and catechin derivatives by FAB MS in the positive mode in a glycerol matrix.

Color Measurements. Color was estimated as absorbance at 430 nm or as tristimulus color values. The latter, expressed in the L*a*b* scale (CIE 1976) (*33*) were measured by means of a Minolta CR 200 tristimulus color analyser using 1-cm width cuvettes and against a white background.

Results and Discussion

Several series of oxidation experiments were performed both in model solutions and in musts in order to establish the major reagents and chemical reactions responsible for browning. Musts were prepared from various *Vitis vinifera* varieties differing by their composition and eventually enriched in some grape components which were believed to play a major role in the discoloration process. Experimental wines were also made in order to evaluate the influence of pre-fermentation reactions on the final wine quality.

Influence of Glutathione on White Must Browning. The first series of studies was carried out to specify the respective roles played by caftaric acid, glutathione and

GRP in grape must oxidation and browning. The latter was estimated by measuring the tristimulus colour values of the oxidized musts. Controlled oxidation of white musts obtained from 24 *Vitis vinifera* varieties by pressing under vaccuum allowed us to distinguish three classes of varieties, on the basis of their enzymatic browning capacity (*34*). These classes can be defined as follows: A: very little browning, low oxygen consumption, low amounts of hydroxycinnamic acids; B: moderate browning, moderate oxygen consumption, moderate level of hydroxycinnamic acids; C: large browning, high oxygen consumption capacity and hydroxycinnamic acids level. The kinetics of caftaric (and coutaric) acid oxidation, GRP formation and GRP degradation were monitored in each oxidizing must and compared with those observed in model solutions containing various proportions of caftaric acid and glutathione oxidized in the presence of PPO (*35*).

The three must classes were characterized by three different patterns of phenolic compounds oxidation resembling those of model solutions containing respectively, equimolar amounts of caftaric acid and glutathione (A), caftaric acid to glutathione between 1 and 3 (B) and larger excess of caftaric acid (C). Thus, the color of oxidized musts appeared largely related to their initial hydroxycinnamic acids to glutathione molar ratio. As well, a Grenache blanc must (class C) added with glutathione in excess consumed less oxygen than the control and showed almost no discoloration (*36*), confirming the role of glutathione in the prevention of browning. However, the actual hydroxycinnamic acids to glutathione molar ratio was more variable within each must class (comprised, respectively, between 0.9 and 2.2, between 1.1 and 3.5, and between 3.8 and 5.9, in classes A, B, and C). Besides, the concentrations of glutathione adducts and of *o*-quinones were usually lower in musts than in model solutions containing equivalent initial levels of hydroxycinnamic acids and glutathione, suggesting that some competition between trapping by glutathione and other reactions of quinones occurred in musts. The compounds involved in such reactions include nucleophilic compounds, which form adducts with the quinones in the same way as glutathione, and substances of lower reduction potential, which are easily oxidized by the quinones. Some phenolic compounds act both as reductants (especially those possessing *o*-diphenol moieties) and as nucleophiles.

Condensation Reactions of Quinones with Phenols.

Products of Caffeic Acid Oxidation. Oxidation products of caffeic acid were obtained in model solutions either by PPO-catalyzed oxidation (*37*) or by periodate oxidation (*31*). The rate of quinone disappearance was almost constant in the pH range 3 to 5 but increased dramatically at higher pHs. The nature and relative amounts of the major products formed from the quinone depended on the pH. In particular, some of them were observed only at pH under 4.6 (*31*).

The major products formed at pH 3.6 were isolated and characterized by NMR and mass spectrometry (*31*). Two of them (6 and 9) are isomers of 2,5-(3',4'-dihydroxyphenyl) tetrahydrofuran 3,4-dicarboxylic acid, formed by addition of a water molecule onto the methylene quinone resulting from dimerization. The dimerization may proceed either by nucleophilic addition of caffeic acid on the electrophilic quinone or by radical coupling of two caffeic acid semiquinones.

A third compound, found at all pH values but more abundant in less acidic media, is identical to caffeicin F formed by autoxidation of caffeic acid in alkaline medium (*38*). The products formed enzymically at pH 3.6 were isolated and shown to be the same as products 6, 7, and 9 by NMR spectrometry. Note that the major products formed by caffeic acid oxidation were colorless, with the exception of the quinone. However, compounds 6 and 9 were spontaneously degraded to a series of new products and the solution gradually turned pink when kept under acidic conditions. Besides, compounds 7 and 9 were oxidized by grape PPO (*37*).

Products of Caftaric Acid Oxidation. In enzymically oxidized solutions, caftaric acid quinone was much more stable than caffeic acid quinone. The major secondary product formed showed a UV-visible spectrum resembling that of caffeicin F, suggesting a similar structure. No product equivalent to products 6 and 9 was detected, probably meaning that the steric hindrance of tartaric acid moiety prevents reactions on the hydroxycinnamic double bond.

Products of Catechin Oxidation. PPO-catalyzed oxidation of catechin was studied in the pH range 3 to 7, using the same level of PPO activity at all pHs. The nature and amounts of products formed was highly pH dependent (Figure 1). The solutions oxidized at pH below 4 contained mostly colorless products (λ max 280 nm) whereas yellow compounds (λ max 385-415 nm), eluting later than the colorless ones, predominated at higher pHs. Seven of them were purified by semi-preparative HPLC. MS, ^{1}H and ^{13}C NMR data indicated that colorless products were dimers and trimers presenting C-C and C-O-C linkages between the B-ring of one catechin unit and the A ring of another. The yellow products are dimers with conjugated ketone moieties and additional oxygenated heterocycles. Similar structures have been previously obtained by chemical (*39*) or peroxidase-catalyzed (*40*) oxidation of catechin. Spontaneous oxidation of the C-C linked colorless dimers at pH above 4 yielded the yellow compounds whereas dimers with ether linkages were more stable.

Autoxidation of (+)-catechin in wine-like solutions containing 10% EtOH, 2.5 g/L potassium hydrogen tartrate (pH 3.6) and 0 to 20 mg/L of ferrous ions, provided as iron sulfate, was followed by HPLC with diode array detection and spectrophotometry. Catechin oxidation rate (Table I) increased with increasing amounts of Fe^{2+}.

The HPLC profiles of the autoxidized solutions resembled those of the enzymically oxidized solutions, showing colorless products along with less polar yellow compounds. Colorless autoxidation products, like those formed in the presence of PPO, were resistant to acid hydrolysis and thus different from biosynthetic catechin oligomers (*i.e.* procyanidins). However, the products obtained in the presence of iron coeluted neither with the products of enzymatic oxidation nor with those formed in the control solution (no iron, no PPO). Besides, the yellow products resulting from iron-catalyzed and PPO-catalyzed oxidation exhibited different UV-visible spectra (Figure 2), with absorbance maxima comprised between 420 and 440 nm and in the range 385-412 nm, respectively. Thus, the structure of products seemed related to oxidation conditions (PPO- or iron-catalyzed), suggesting that catechin is degraded by different mechanisms in grape must and in wine.
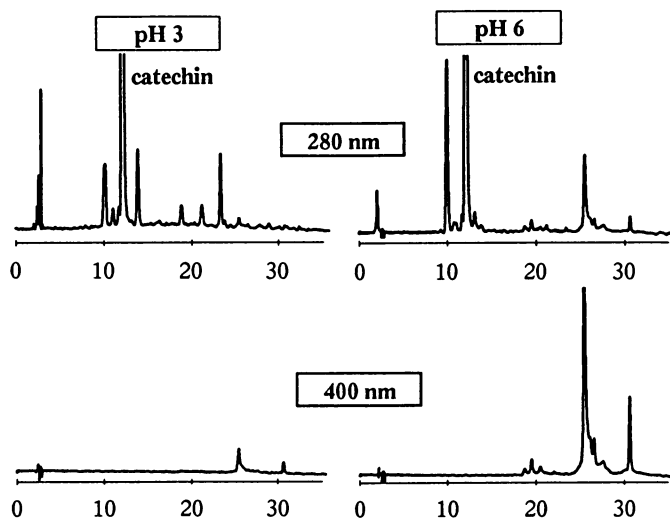
Figure 1. HPLC traces obtained by oxidizing catechin (4mM) for one hour in the presence of PPO (17 nkat/ml) at pH 3 and 6.

Table I. First order apparent rate constants of catechin oxidation in the presence of iron

Iron (Fe^{2+}) (mg/L)	rate constant (10^{-5}h^{-1})*	correlation coefficient (n=8)
0	16	.995
1	23	.979
2	49	.995
5	82	.980
8	106	.966
10	128	.958
15	149	.957
20	180	.954

* calculated as the Ln ([Cat]$_t$ / [Cat]$_o$) / t, with t = incubation time

Reactions of Caftaric Acid Quinone with Anthocyanins.   PPO-catalyzed degradation of anthocyanins in the presence of caftaric acid was studied in model solutions. Although it cannot be oxidized to the corresponding *o*-quinone, malvidin-3-glucoside was degraded in the presence of enzymically generated caftaric acid quinones. HPLC analysis of the oxidized malvidin-3-glucoside / caftaric acid solution showed the presence of several products including red pigments (Figure 3). All of them exhibited two absorbance maxima in the UV region at 280 and 320 nm, suggesting that they contained a caftaric acid moiety. Therefore, the pigments (λ max 530-532 nm) are likely to be condensation products formed by nucleophilic addition of the anthocyanin on caftaric acid quinone. All colorless compounds were different from caftaric acid oxidation products. Thus, they may also be copolymers, arising from the degradation of the colored products or formed by other reactions competing with the formation of pigments.

Oxidation of cyanidin-3-glucoside in the presence of caftaric acid and PPO first produced a series of red pigments, with absorbance maxima at 286, 317 and 518 nm, similarly suggesting that they were cyanidin-3-glucoside / caftaric acid condensation products. After one hour of incubation, the HPLC profiles showed only colorless compounds which probably result from the degradation of the chromophore of the primary products formed. As well, the major products formed apparently did not include pure cyanidin derivatives.

Oxidative Reactions of Quinones

Role of Sulfur Dioxide.   Addition of bisulfite ions to an oxidizing must delayed the conversion of caftaric acid to GRP and increased the oxygen consumption by approximatively one oxygen atom per bisulfite ion (*35*), suggesting that they were oxidized by caftaric acid *o*-quinones. Part of the quinones formed during pressing under regular white wine-making conditions were reduced by sulfiting at 50 mg/L SO_2 (41, *42*). For example, sulfiting after hyperoxidation (*41*) regenerated up to half the initial amount of caftaric acid. On the other hand, must samples taken after sulfiting in a commercial winery still contained caftaric acid quinones but no GRP quinones (*42*). Model solutions containing approximately 0.15 mM of both *o*-quinones were prepared by incubating caftaric acid (0.45 mM), glutathione (0.15 mM) and grape PPO (0.16 g/L crude extract, as found in must) for 6 min and immediately sulfited with 50 mg/L sulfur dioxide. No quinone remained after sulfiting in the control solution, in which PPO was inhibited by addition of sodium fluoride, whereas caftaric acid *o*-quinones were found in the solution stabilized by sulfiting only. Since caftaric acid quinones are more powerful oxidants than GRP quinones, this indicates that the level of bisulfite ions used was sufficient to reduce all the quinones initially present but not to inhibit PPO. The initial loss of quinones was due to simultaneous reduction of quinones and PPO inactivation by bisulfite ions. The latter reaction is irreversible (*43*) but the residual PPO activity produced more caftaric acid quinones which oxidized bisulfite ions to sulfates, thus limiting enzyme inhibition. Although sulfur dioxide may also serve as a quinone trapping agent (*44*), no caftaric acid-sulfur dioxide adduct was detected under the acidic pH conditions prevailing in musts.
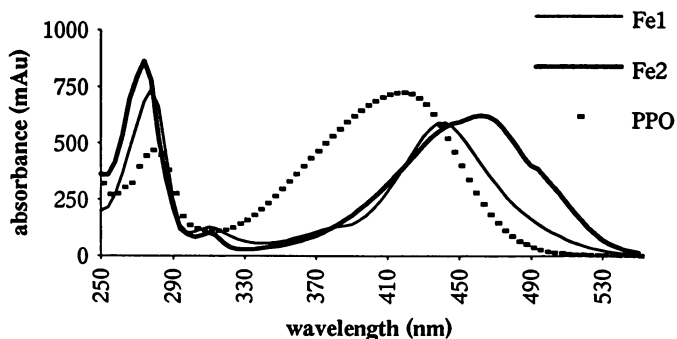
Figure 2. UV-visible spectra of the major colored products obtained by iron-catalyzed autoxidation (Fe1,Fe2) and enzymatic oxidation (PPO) of (+)-catechin.
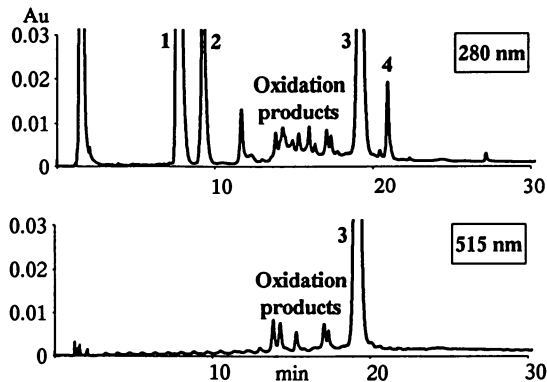
Figure 3. HPLC traces at 280 nm and 515 nm of a solution of malvidin-3-glucoside and caftaric acid (0.5 mM each) oxidized for 10 min with PPO (2.5 nkat/ml). 1: benzene sulfinic acid; 2: caftaric acid; 3: malvidin-3-glucoside; 4: caftaric acid benzene sulfone.

Oxidation of Ascorbic Acid. In most musts, an initial lag phase was observed before degradation of caftaric acid and formation of GRP started although oxygen was consummed rapidly (*35*). Addition of ascorbic acid further delayed caftaric acid oxidation and GRP formation (*35, 36*). It also increased oxygen consumption by one atom per ascorbic acid molecule (*36*). Measurement of ascorbic acid in the oxidizing must confirmed that oxidation of ascorbic acid took place during the lag phase. Besides, the *o*-quinones formed in model solutions are instantly reduced by ascorbic acid (*7*). Thus, the observed lag phase is likely to be due to oxidation of ascorbic acid by the o-quinone, as shown by other researchers (*45, 46*), although it may also result from competition between caftaric acid and ascorbic acid towards oxygen.

Coupled Oxidation of *o*-diphenols by Caftaric Acid Quinone. Since GRP is not directly oxidizable by PPO (*14-16*), the formation of its *o*-quinone, detected as the corresponding benzenesulfone, in the presence of caftaric acid (*34, 35*) demonstrated unequivocally the occurrence of coupled oxidation.

In model solutions containing caftaric acid and (+)-catechin in different proportions (*47*), the rate of caftaric acid degradation and the concentration of its quinone decreased with increasing concentrations of catechin whereas larger amounts of catechin *o*-quinones were formed in the presence of caftaric acid, meaning that coupled oxidation of catechin by caftaric acid quinones took place. However, catechin quinones proceeded rapidly to brown polymers. Besides, incorporation of caftaric acid into condensation products was faster in solutions containing catechin, suggesting that catechin-caftaric acid products formed more readily than pure caftaric acid oligomers. Such copolymers have actually been observed in oxidized catechin-chlorogenic acid mixtures (*48*). Procyanidins were not susceptible to enzymatic oxidation but they were oxidized to very unstable quinones in solutions containing PPO and caftaric acid (49)

Degradation of the *o*-diphenolic anthocyanins in the presence of caftaric acid was faster than that of non *o*-diphenolic anthocyanins (Cheynier, V. et al., *J. Sci. Food Agric.*, in press). The disappearance of caftaric acid was slower and the level of caftaric acid *o*-quinones lower in model solutions containing cyanidin-3-glucoside or delphinidin-3-glucoside. The influence of cyanidin-3-glucoside on caftaric acid oxidation is shown in Figure 4. Although no anthocyanin quinone was detected, the sparing of caftaric acid is believed to result from reduction of its quinone in coupled oxidation of the anthocyanin.

Oxidative Browning in Musts and Wines. The color of the oxidized solutions was highly variable. Oxidation of caftaric acid induced very little discoloration whereas oxidized catechin solutions became very brown. Solutions containing anthocyanin became gradually less red and more brown throughout oxidation; white musts added with anthocyanins and the corresponding controls reached similar final a* values after oxidation. Degradation of flavan-3-ols in oxidizing musts induced browning both of the must (*36*) and of the resulting wine (*27, 50*). Trapping of the enzymically generated caftaric acid *o*-quinones by glutathione protected other phenols from oxidation and limited browning. However, the maintenance of large amounts of potential browning precursors in musts containing high glutathione levels led to increased browning susceptibility of the corresponding wines.

In fact, browning reactions in wines are known to be largely related to the flavanol content (25, 26). Data concerning flavanol composition and color of white wines prepared by different wine-making procedures are presented in Table II. Wines prepared with pomace contact contained larger amounts of flavanols than control wines. Must oxidation induced large losses of flavan-3-ols and increased absorbance at 420 nm (used as browning estimate) in all types of wines. However, the wine browning capacity as evaluated by the procedure of Simpson (26) was correlated to the level of remaining flavanols (27), confirming that they may serve as browning precursors during long term storage. Iron-catalyzed autoxidation is one of the possible discoloration mechanisms as browning of catechin autoxidized solutions increased with the amounts of Fe^{2+} (Figure 5). In particular, autoxidation of catechin and related compounds in wines containing 5 to 10 mg/L iron should induce intense browning. This may be partly prevented if flavan-3-ols are depleted due to enzymatic or coupled oxidation, in the course of prefermentation treatments.

Conclusions

The examples presented in this paper illustrate the complexity of the mechanisms responsible for browning in grape musts and wines. The role of various o-quinones generated enzymically or as secondary products of enzymatic oxidation in the development of browning in musts and must-like solutions has been demonstrated. O-quinones are highly reactive both as oxidants and as electrophiles. Competition between these two types of reactions depends on the nature both of the quinone itself and of the other species (reductants or nucleophiles) present and on their respective concentrations. Caftaric acid o-quinone is one of the most powerfull oxidants among must constituents. It oxidizes readily well-known reductants such as ascorbic acid and sulfur dioxide but also phenols like GRP, flavanols and o-diphenolic anthocyanins. As electrophile, it also reacts with various nucleophiles including glutathione, malvidin-3-glucoside and caftaric acid itself. In comparison, flavanols have a lower redox potential and are stronger nucleophiles than caftaric acid. Therefore, they are rather involved in condensation reactions, forming both flavanol-flavanol and flavanol-caftaric acid products. Detection and characterization of various reaction products led to a better understanding of the mechanisms taking place in oxidizing must-like solutions. However, most of these products have not been detected in musts, probably owing to the numerous interfering reactions (e.g. competition with other grape components for reaction with the quinone, enzymatic degradation of the products). The availability of pure quinones obtained by chemical synthesis should be of great help to determine their reactivity in increasingly complex media. Browning reactions in wines appeared governed by totally different mechanisms and essentially related to the flavanol content. Autoxidation of flavanols (e.g. catalyzed by Fe^{2+}) is one of the reactions possibly involved in wine discoloration but spontaneous acid-catalysed hydrolysis and polymerization reactions of flavan-3-ol oligomers (proanthocyanidins) may also lead to larger, less soluble, and colored compounds.
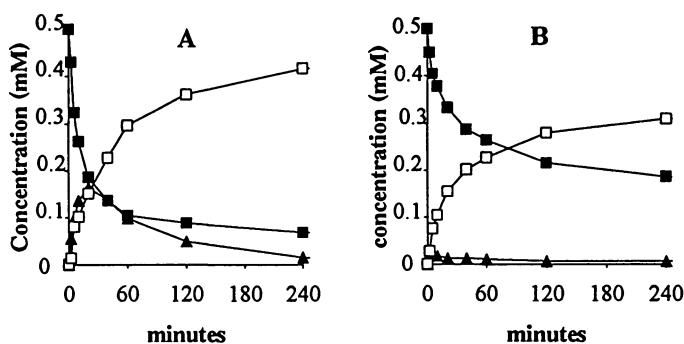
Figure 4. Kinetics of caftaric acid oxidation in solutions containing initially A: caftaric acid (0.5 mM); B: caftaric acid and cyanidin-3-glucoside (0.5 mM each), oxidized with PPO (2.5 nkat/ml). ■: caftaric acid; ▲: caftaric acid *o*-quinone; □: caftaric acid involved in condensation products.

Table II. Wine flavanol content and color in relation with wine-making process

Wine code[a]	Concentration (mg/L)			A_{430}[b]	
	Catechin	Epicatechin	B1	at zero time	after 28 days
GO	1.3	0.5	0.1	0.045	0.39
GP	6.0	3.6	2.2	0.049	0.53
GPO	2.3	0.3	0.3	0.051	0.37
C	3.9	1.2	1.4	0.080	0.56
CO	1.7	0.3	0.4	0.085	0.39
CP	6.0	3.5	3.2	0.076	0.80
CPO	2.6	1.5	0.5	0.078	0.51

[a] G: Grenache blanc; C: Chardonnay; O: addition of oxygen (16 mg/L); P: pomace contact (for 4 h at 15 °C).
[b] wines were stored in bottles at 15 °C until zero time, then at 50 °C in half-filled screwcap tubes with an air head space.
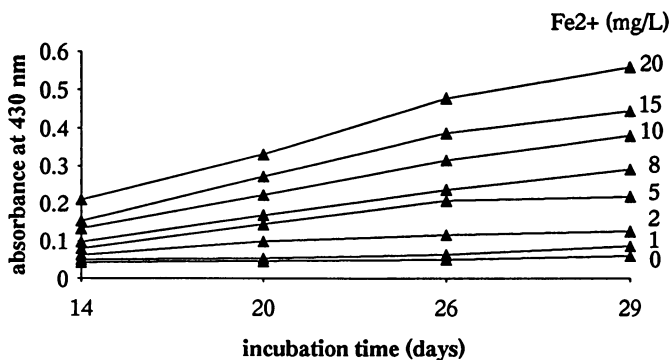
Figure 5. Influence of iron (Fe^{2+}) on the autoxidative browning of catechin.

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Chapter 11

Evolution of Chlorogenic Acid *o*-Quinones in Model Solutions

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The oxidation of phenolic compounds into their corresponding *o*-quinones catalyzed by polyphenoloxidase (PPO) is now well documented. However, in spite of their considerable significance with regard to browning, few studies of non-enzymatic secondary reactions involving *o*-quinones are available. The present study describes the evolution of chlorogenic acid *o*-quinones. Following the oxidation of chlorogenic acid by purified apple PPO, chlorogenic acid *o*-quinone and some secondary products have been characterized. Two evolution pathways of *o*-quinones have been suggested depending on the pH conditions. After addition of (-)-epicatechin to oxidized solutions of chlorogenic acid, three condensation products have been purified and identified. These products are themselves enzymatically and non-enzymatically (by cooxidation with chlorogenic acid *o*-quinone) oxidizable. In addition, they exhibit competitive inhibition properties towards polyphenoloxidase.

Discoloration phenomena occurring in apple juice, purée and slices are mainly related to enzymatic browning. However, browning can also originate from non-enzymatic reactions such as the Maillard reaction which occurs in heat processed apple products. Basically, enzymatic browning can be defined as an initial enzymic oxidation of phenolic compounds into slightly colored quinones, catalyzed by polyphenoloxidases. The most prevalent substrates in apples for PPO are chlorogenic acid and (-)-epicatechin, chlorogenic acid being more readily oxidized than (-)-epicatechin *(1)*. Moreover, according to Amiot et al. *(2)*, apple browning susceptibility is mainly dependent on the relative proportions of flavan-3-ols and hydroxycinnamic acids derivatives. Apples

contain hydroxycinnamic esters, flavanols, anthocyanins, flavonols and dihydrochalcones *(2-4)* the contribution of which to browning has been reported by numerous authors *(5-7)*.

The *o*-quinones formed are very reactive entities and are subjected to further secondary reactions, both enzymic and non-enzymic, leading to pigments. Depending on the phenolic compound from which they originate and on environmental factors of the oxidation reaction, the *o*-quinones show great differences in stability and reactivity and the colors of the corresponding pigments differ widely in hue and intensity *(8-10)*. Different evolution pathways of *o*-quinones involving phenolic or nonphenolic compounds have been described in the literature:

i. nucleophilic additions involving sulfites *(14,15)*, thiols or amino groups of aminoacids or peptides have been identified *(16,17)*. Recently, the addition of water to DOPA or 4-methylcatechol *o*-quinone has been described yielding the triphenols *(8,18)*. The so-formed triphenols are readily oxidized by polyphenoloxidase or by an excess of quinones, leading to the formation of *p*-quinones.

ii. *o*-quinone can react with another molecule of the parent phenol, leading to the formation of dimers. These dimers have been suggested to undergo further oxidation, yielding larger oligomers with different color intensities *(9,19)*. Oszmianski and Lee *(20)* have pointed out that catechin produced mainly dimers and polymers with a low polarity while chlorogenic acid produced mainly polymers.

iii. *o*-quinones are also reported to react with a different phenol molecule, either leading to a copolymer or regenerating the original phenol and giving a different *o*-quinone by coupled oxidation *(11,14)*. Following this last pathway, chlorogenic acid *o*-quinones have been postulated to oxidize tyrosine *(21)*, some anthocyanins *(22)*, flavonol and dihydrochalcone glycosides *(5,6)* and procyanidins *(5,6)*. However, due to the difficulties of discriminating between enzymatic and nonenzymatic mechanisms, these coupled reactions have not been clearly demonstrated by previous authors. *o*-Quinones are also known to form copolymers with other phenolic compounds. Following the oxidation of (+)-catechin-chlorogenic acid mixture, six copolymers have been observed and spectrophotometrically characterized by Oszmianski and Lee *(20)*. The structures and the susceptibility to oxidation of these compounds remain unknown.

The purpose of the present work was to investigate the secondary reactions involving chlorogenic acid *o*-quinone both in model solutions containing chlorogenic acid alone and in mixtures of chlorogenic acid with a second phenolic compound. Specific attention will be given to the condensation products formed from the oxidation of a (-)-epicatechin-chlorogenic acid mixture.

Experimental

Materials. PPO was extracted from apple flesh (*cv* Red Delicious, picked at commercial maturity) and 120-fold purified in two steps: fractional precipitations by ammonium sulphate and hydrophobic chromatography with Phenyl-Sepharose CL4B (Pharmacia), according to the method of Janovitz-Klapp et al. *(23)*. Chlorogenic acid, (-)-epicatechin and (+)-catechin, kaempferol, quercetin and its glycosides (quercetin-3-galactoside, -3-glucoside, -3-rhamnoside and -3-rutinoside), phloretin and phlorizin, cyanidin and its glycosides (cyanidin-3-glucoside and -3-galactoside) were from Extrasynthèse (Genay, France); 4-methylcatechol and all other chemicals were of reagent grade from Sigma (St. Louis, MO).

Assay of PPO activity. PPO activity was polarographically assayed according to the method of Janovitz-Klapp et al. *(23)* using 20 mM 4-methylcatechol as substrate. Activity was expressed as nmol. of O_2 consumed per second (nkat) under the assay conditions. To study the susceptibility to oxidation of the three copolymers, analyses were carried out by polarography with a reaction mixture containing 30 μl of purified copolymer and 10 nkat PPO in a total volume of 3 ml of a Mc Ilvaine's buffer at pH 4.5. For inhibition studies with copolymers, 4-methylcatechol varied between 1 and 20 mM in the control and two concentrations of copolymers. All assays were performed in duplicate and apparent Vm and Km values were determined using a nonlinear regression data analysis program developed for IBM PC by Leatherbarrow *(24)*.

Preparation and HPLC analysis of model solutions. All of the enzymatic reactions were carried out with purified apple PPO (5 or 20 nkat.ml^{-1}) in a reaction vessel at pH 4.5 (5 for copolymers analysis) and 30°C, in the presence of vanillic acid (internal standard for HPLC analysis) using air agitation unless otherwise specified. The concentrations of chlorogenic acid and (-)-epicatechin varied between 1 and 10 mM. For incubation period tested, 0.5 ml was withdrawn from the reaction vessel and immediately mixed with an equal amount of stopping solution containing 2 mM NaF *(15)*. The residual phenols and oxidation products were separated by HPLC (9010 pump and 9050 UV detector driven by a 9020 workstation from Varian) on 10 μl samples using the isocratic and the gradient conditions described by Richard et al. *(15)*. The spectra were obtained using a diode array detector (Waters 990).

Preparation of the (-)-epicatechin- chlorogenic acid copolymers. The (-) epicatechin-chlorogenic acid copolymers were enzymically prepared in a Mc Ilvaine buffer at pH 5 and 30°C. Purified apple PPO (250 nkat) was added to 50 ml of buffer solution containing 10 mM chlorogenic acid and 4 mM (-) epicatechin. After 20 minutes oxidation, the reaction was stopped by adding 10 ml of a stopping solution (60% acetonitrile-40 % H_2O at pH 2.6) containing 10 mM NaF. Copolymers were isolated by semi-preparative HPLC using a Lichrosorb column (25 cm x 1 cm, 10 μm, Interchrom) at 30°C. Detection

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was monitored at 280 nm. The elution solvent was 10% acetonitrile - 90% H_2O (adjusted to pH 2.6 with H_3PO_4) delivered at a flow rate of 2.5 ml.min^{-1} by a Varian 9010 pump. Copolymers were concentrated by adsorption on C_{18} Sep Pack cartridges (Millipore UK Ltd) following by elution with 2 ml methanol.

HPTLC Characterization. Copolymers isolated (10 μl of methanolic solution) were applied to 10 x 10 cm silica gel TLC plates (DC-Fertigplatten, Merck ref n˚5577). The TLC eluent used was 15% HOAc. After developing, the plates were immediately dried under cold air and observed under 366 nm excitation before and after fuming with ammonia and /or spraying the following reagents: vanillin (1% in 11 M HCL) to detect flavan-3-ols, which turn red, and, 2,4 aminoethyldiphenylborate (1% in methanol), revealing caffeoyl derivatives as green fluorescent spots under 366 nm after fuming with ammonia.

Fast Atom Bombardment Mass spectrometry (FAB-MS). Positive mode spectra were recorded with a MR-JEOL-DX 300-3 KeV spectrometer using NBA as a matrix.

Results and Discussion

Oxidation products of chlorogenic acid. The disappearance of chlorogenic acid and the formation of oxidation products were monitored by HPLC at 280 nm. A typical chromatographic profile (corresponding to 15 min oxidation) is shown in Figure 1. The initial reaction products were mainly four peaks more polar than the parent phenol, labelled CG_1, CG_2, CG_3 and QCG. The use of NaF stopping solutions supplemented with ascorbic acid (2 mM) allowed us to identify QCG as the chlorogenic acid o-quinone and also to characterize CG3 as a quinonic structure with CG2 as its corresponding parent phenol. Chlorogenic acid oxidation also produced compounds which were more apolar, the number and the amount of these increased with reaction time so that at least 20 were present after 30 min oxidation. These products have been supposed to be chlorogenic acid oligomers: using different experimental conditions, Oszmianski and Lee *(6)* reported the presence of 6 condensed products. When the detection was carried out at 400 nm, QCG, CG_3 and the less polar products were still observed. Figure 2 shows the kinetics of the oxidation of chlorogenic acid catalyzed by apple PPO and of the formation of the quinone of chlorogenic acid (QCG) and of CG_2 and CG_3. Only QCG reached a maximum after 3-5 min and then steadily decreased to almost zero after 30 min. CG_2 increased in the reaction mixture. CG3 increased also, but more slowly. CG3 seemed to be a compound produced from QCG with an important lag time or a secondary product from CG2. When the enzymic oxidation was stopped with NaF after 3 min, a slight decrease in chlorogenic acid concentration was apparent whereas the quinone content decreased rapidly and the areas of CG_2 and CG_3 increased. The same experiment was carried out with reaction
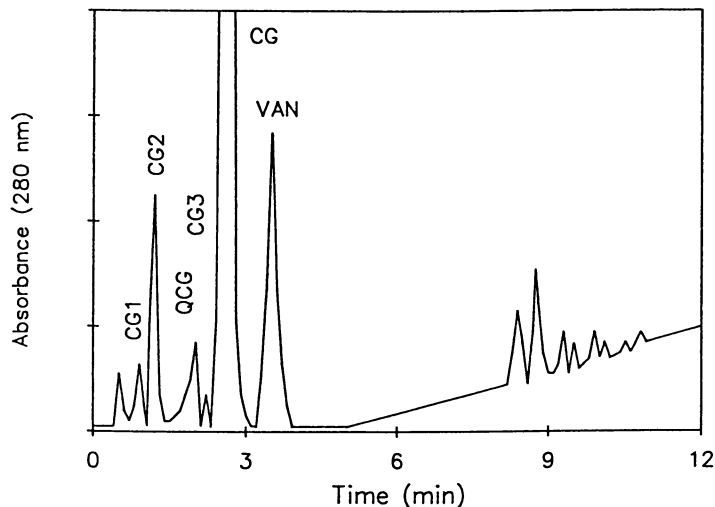
Figure 1. HPLC elution profile of the products formed by the PPO-catalyzed oxidation of chlorogenic acid. Conditions : 1 mM chlorogenic acid was oxidized by 10 nkat.ml⁻¹ PPO in a Mc Ilvaine Buffer at pH 4.5. The reaction mixture was analyzed after 15 minutes of oxidation following the addition of the stopping solution. CG: chlorogenic acid, CG1: unknown compound, CG2: triphenol of chlorogenic acid, CG3: *p* -quinone of CG2, QCG: *o*-quinone of chlorogenic acid. The putative structures of CG2 and CG3 and the structure of QCG are given in Figure 4.
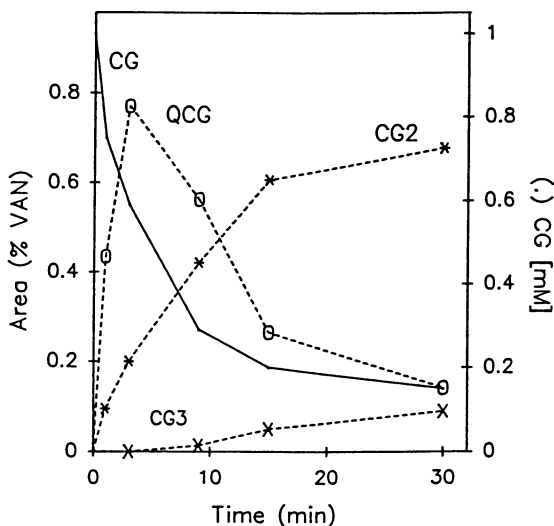
Figure 2. Chlorogenic acid disappearance and the formation of QCG, CG2 and CG3 during oxidation of 1 mM chlorogenic acid by 10 nkat.ml⁻¹ PPO.

mixtures stopped and simultaneously acidified to pH 4 and pH 3. Firstly, it was apparent that as the pH became lower, the QCG degradation became more rapid (the pseudo-first-order rate constant of $0.035.min^{-1}$ determined at pH 4 and $0°C$ increased to $0.049.min^{-1}$ at pH 3). Secondly, a chlorogenic acid regeneration was observed at pH values lower than 4 whereas the increase in CG_2 and CG_3 areas seemed not to be influenced by pH.

The spectra obtained for QCG, CG_2 and CG_3 using a diode array detector are given in Figure 3. The QCG spectrum exhibited three maxima close to 255, 325 and 400 nm, consistent with the presence of an *o*-quinone structure. Three maxima (255, 300 and 400 nm) were also observed in the CG3 spectrum. The CG2 spectrum with two maxima at 300 and 340 nm displayed a bathochromic shift compared to that of chlorogenic acid, which showed a maximum at 325 nm characteristic of a cinnamic structure.

The same experiments as previously described were carried out with 4-methylcatechol *(25)*. The results obtained with the two phenolic compounds exhibited many similarities. Therefore, two pathways of QCG degradation could be proposed (Figure 4). The first one corresponds to the polymerization pathway as described by Singleton *(19)* and Nicolas et al. *(11)* and leads to the less polar products observed in our chromatograms, for which only hypothetical formulae have been suggested by Cheynier et al. *(26)*. The second pathway (favoured by acid pH) may correspond to the hydroxylation of QCG by water *(17,25)*, yielding a triphenolic compound (CG_2). CG2 may react with another QCG to give its corresponding *p*-quinone (CG_3) and regenerate chlorogenic acid. CG2 may also be substrate of apple PPO. However, in comparison with 4-methylcatechol, two slight differences have to be explained:
- the triphenol (CG_2) resulting from the hydroxylation of QCG was present in appreciable amounts in our reaction mixtures, meaning either that it was a very poor substrate of apple PPO or that its cooxidation involving QCG was slower.
- the *o*-quinone of 4-methylcatechol was more stable than QCG and the polymerization pathway seemed to be dominant (considering the number of condensed products), certainly to to a less steric hindrance of 4-methylcatechol than chlorogenic acid. The same observation has been made with (-)-epicatechin (data not shown).

Coupled oxidations involving chlorogenic acid *o*-quinone. The enzymatic oxidation of model solutions with chlorogenic acid supplemented with another phenolic compound has been monitored by HPLC at 280 nm. Among the flavonoids tested, only the monomers of flavanol, the aglycones of flavonols (quercetin and kaempferol) and the dihydrochalcones (phloretin and phloridzin) were substrates of apple PPO *(11,24)*. The enzyme was unable to oxidize either the anthocyanins (cyanidin, cyanidin-3-galactoside and 3-glucoside) or the glycosides of quercetin (quercetin-3-galactoside, 3-glucoside, 3-rhamnoside and 3-rutinoside). Furthermore, the previous quercetin glycosides have been characterized as competitive inhibitors with inhibition constants close to 1 mM
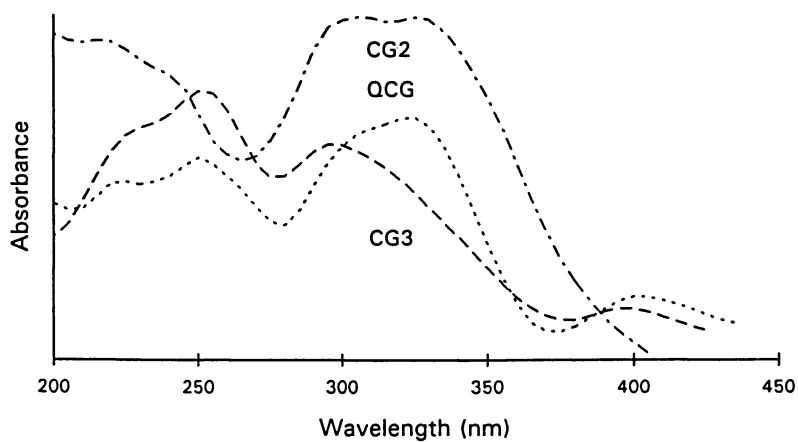
Figure 3. Spectra of the oxidation products (QCG, CG2 and CG3) of chlorogenic acid
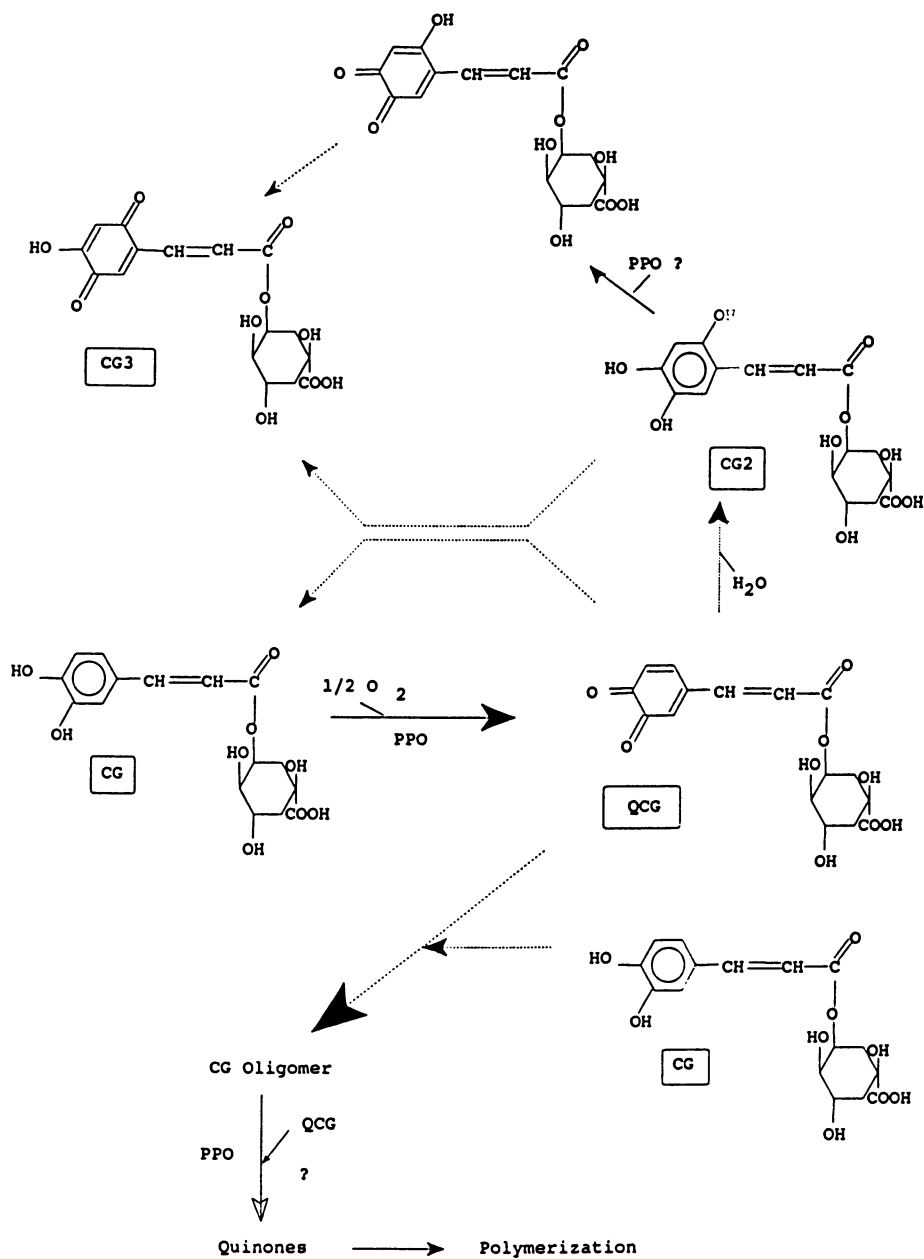
Figure 4. Proposed pathways for the formation of oxidation products from chlorogenic acid.

(data not shown). In all the former mixtures, compared to the oxidation rate of the phenol alone, the flavonoid degradation was significant or enhanced while that of chlorogenic acid was slower, as it was found by Oszmianski and Lee *(20)*, Cheynier et al. *(26)* and Cheynier and Ricardo da Silva *(27)* for caffeoyltartaric acid/flavanol mixtures. The mechanism proposed involved enzymatic oxidation of the two phenols followed by the chemical oxidation of the flavonoid compound by caffeoyl quinone, so that caffeoyl quinone was reduced to its phenol form. Such a pathway has not been clearly demonstrated. The following experiments were carried out to demonstrate its occurrence. An aliquot of chlorogenic acid (1 mM) was oxidized by purified apple PPO (20 nkat. ml^{-1}) with air agitation. After 3 minutes (corresponding to the maximum of the o-quinone formation), the reaction was stopped by two stopping solutions, one with NaF, and, one with NaF containing different amounts of flavonoids ranging from 0.25 to 4 mM. The content of the two final mixtures were analyzed by HPLC. The results obtained with (-)-epicatechin are reported in Table I.

Table I: Effects of the addition of different amounts of (-)-epicatechin (EP) on the oxidation of chlorogenic acid (CG) by apple PPO after stopping the enzymic reaction with NaF

Residual amounts before addition of NaF		Amounts of added EP	Residual amounts after addition of NaF + EP		
CG (mM)	QCG (mM)	EP (mM)	CG (mM)	QCG (mM)	EP (mM)
0.49	0.25	4	0.72	-	3.80
0.49	0.25	3	0.73	-	2.82
0.55	0.22	2	0.74	-	1.85
0.53	0.23	1	0.73	-	0.86
0.57	0.21	0.5	0.73	-	0.38

QCG: *o*-quinone of chorogenic acid

Before the addition of (-)-epicatechin, the residual amounts of chlorogenic acid ranged from 0.49 to 0.57 mM and the *o*-quinone of chlorogenic acid varied between 0.21 and 0.25 mM. After the addition of the flavanol, the *o*-quinone QCG disappeared, while the chlorogenic acid content increased and the (-)-epicatechin was partially degraded. Whatever the amounts of flavanol added, the stoicheiometry of the chloro-genic acid regenerated was, for the (-)-

epicatechin degraded, close to 1.3, which suggested the occurrence of other mechanisms of phenol regeneration from *o*-quinone. This could be either the protonation pathway of two semi-quinone free radicals formed from *o*-quinone *(19)* or the hydroxylation pathway of QCG previously described. In addition, three condensation products were detected, the structures of which will be discussed in the following section.

Similar results were obtained with stopping solutions supplemented with flavonol, dihydrochalcone or anthocyanin. However, no condensation products were detected, except with the addition of quercetin.

Thus, the previous experiments demonstrated that QCG and flavonoids were able to react nonenzymatically, leading to the regeneration of CG and the formation of pigments. Moreover, it appeared that QCG played a determinant role in secondary reactions with other phenolic compounds, including those phenolics which are not substrates of PPO.

Oxidation products of chlorogenic acid/(-)-epicatechin mixtures. During enzymic oxidation of an equimolar (1 mM) chlorogenic acid/(-)-epicatechin mixture by PPO (10 nkat.ml^{-1} of PPO), three new peaks with a higher polarity than the two initial phenolic compounds (Figure 5), labelled COD1, COD2 and COD3, were detected by HPLC. The amounts of COD1, COD2 and COD3 increased during the first 15-20 minutes remaining steady as the reaction proceeded further. Whatever the reaction time, ascorbic acid had no effect on the three compounds, suggesting that COD1, COD2 and COD3 were neither quinonic forms nor parent phenols of quinonic forms, present in the reaction mixtures. After the reaction was inhibited by the NaF addition, a slight degradation of the three copolymers was apparent, suggesting the occurrence of non- enzymic oxidative reactions. Different factors, including pH and temperature, enzyme and phenolic substrates concentrations, were analyzed in order to establish the best conditions to accumulate the three copolymers. Thus, the highest amounts were obtained after 20 minutes oxidation of a chlorogenic acid (10 mM)/(-)-epicatechin (4 mM) mixture at pH 5 and 30°C with 5 nkat.ml^{-1} PPO.

UV-visible spectra (200-500 nm) were recorded for the three compounds by means of a photodiode array detector (Figure 6). COD1 exhibited a characteristic spectrum with two absorption maxima at 295 nm and 300 nm and a shoulder at 325 nm. This spectrum showed great similarities to that given by Oszmianski and Lee*(6)* for a (+)-catechin/chlorogenic acid copolymer. Two maxima (at 280 and 315 nm) were also observed in the COD2 spectrum. The COD3 spectrum with a maximum at 325 nm and a shoulder at 285 nm was reminiscent of chlorogenic acid.

The mass spectral data of the three copolymers exhibited great similarities. In the COD1 and COD2 fragmentation patterns, the highest mass fragment was observed at m/z 644 and considered as the molecular ion (M+H)$^+$. This suggested that COD1 and COD2 were two codimers resulting from the
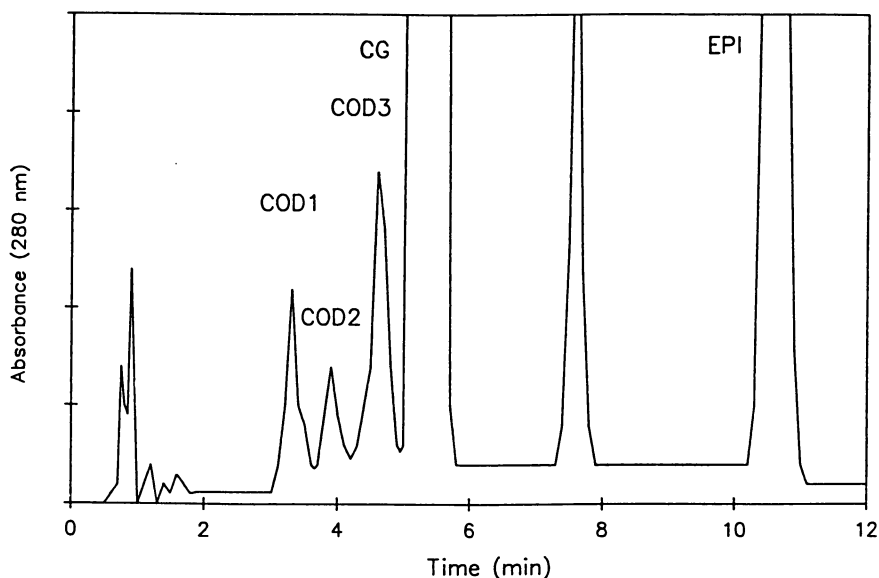
Figure 5. HPLC elution profile of the products formed by the PPO-catalyzed oxidation of a chlorogenic acid / (-)-epicatechin mixture. Conditions: a 1 mM chlorogenic acid / 1 mM (-)-epicatechin mixture was oxidized by 10 nkat.ml^{-1} of apple PPO. The reaction mixture was analyzed after 20 minutes of oxidation following addition of the stopping solution. CG: chlorogenic acid, EPI: (-)-epicatechin, COD1, COD2, COD3: codimers resulting from the complexation of CG and EPI.
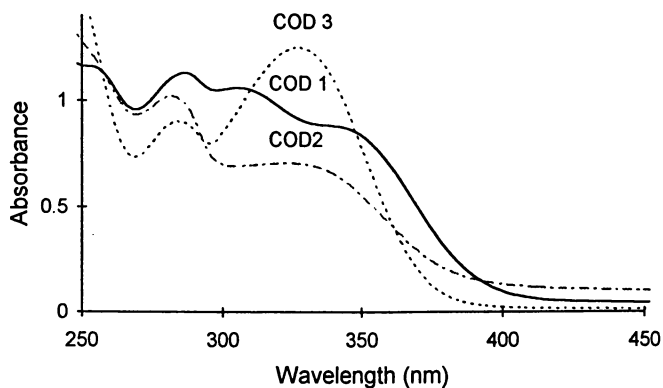
Figure 6. Spectra of the oxidation products (COD1, COD2, COD3) of a chlorogenic acid/(-)-epicatechin mixture.

complexation of 1 mole of epicatechin and 1 mole of chlorogenic acid. Among the daughter ions, peaks at m/z 291 (epicatechin + H)$^+$ and 355 (chlorogenic acid + H)$^+$ were observed. The COD3 fragmentation pattern exhibited also a peak at m/z 644 and numerous peaks previously noticed for COD1 and COD2; however, higher mass fragments and especially an intense peak at m/z 664 were apparent and remain unidentified. Moreover, COD2 was also an oxidation product of a (+)-catechin/chlorogenic acid mixture (data not shown) and could be the result of the condensation of 1 mole of chlorogenic acid and 1 of (+)-catechin which is always contaminant of commercially available (-)-epicatechin. In accordance with the hypo-thesis of three codimers, we have estimated molecular extinction coefficients for COD1, COD2 and COD3 to be 2720, 5080 and 8570 M^{-1}.cm^{-1}, respectively. The 8570 value ascribed to COD3 was certainly high due to the incomplete purification of this compound shown by HPTLC.

On HPTLC plates, when sprayed with vanillin reagent, the three copolymers turned red, indicating that the flavanol A ring was still present in their structure. When sprayed with 2,4- aminoethyldiphenylborate and fumed with ammonia vapour, only COD1 gave a green fluorescence under 366nm, revealing the presence of a caffeic ester in its structure. Thus, the HPTLC characterization results suggested that, in the COD1 struc-ture, the (-)-epicatechin/chlorogenic acid complexation involved the B ring of the flavanol and the benzene ring of the hydroxycinnamic acid. No conclusions concerning the COD2 and COD3 structures could be advanced. The isolation of larger amounts of COD1, COD2 and COD3 is needed to achieve their structural identification by NMR.

Additional experiments were performed to study the susceptibility to oxidation of the three compounds and their possible inhibitory effects on apple PPO. These analyses indicated that only COD1 was a substrate of polyphenoloxidase. No quinone or oxidation products were apparent in our chromatograms. When COD1, COD2 or COD3 were added to chlorogenic acid o-quinone, the three compounds were oxidized but no new condensation products were observed with our HPLC conditions. Nevertheless, after the addition of COD3, the yellow colour of the oxidized chlorogenic acid solution became bright orange. The effects of COD1, COD2 and COD3 on apple PPO were studied by polarography using 4MC as substrate and two concentrations of copolymers. The three compounds exhibited a competitive inhibition pattern. The inhibition constants evaluated: 0.8 mM for COD1, 0.4 mM for COD2 and for COD3, indicated that the three compounds had high inhibitory effect, close to that of benzoic and p-hydroxybenzoic acids (28). Moreover, the three codimers exhibited a greater affinity for apple PPO than chlorogenic acid (Km = 4.2 mM) and (-)-epicatechin (Km = 5.9 mM) (28).

Thus, the previous experiments illustrated the reactivity of the three copolymers and showed how secondary reaction products were able to react further with the products of primary enzymatic reaction and hence influence
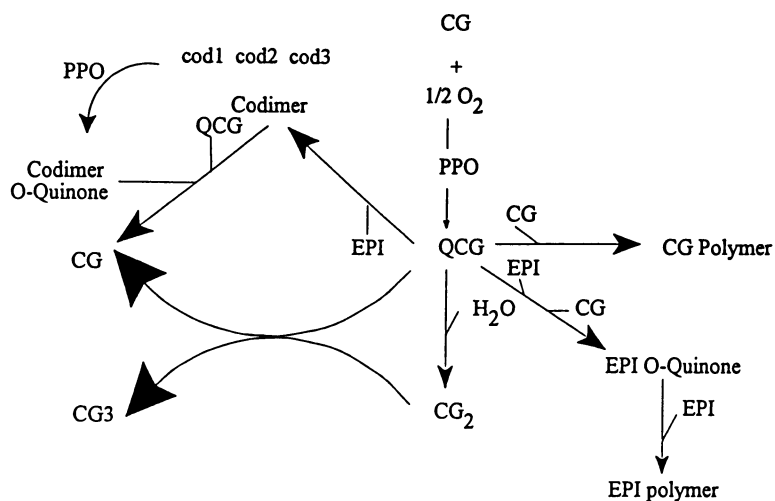
Figure 7. Proposed pathways for chlorogenic acid *o*-quinone evolution in a reaction mixture supplemented with (-)-epicatechin.

the final coloration, in agreement with the results of Cheynier and Moutounet *(29)* concerning caffeic acid oligomers.

Conclusion

Figure 7 summarizes the different pathways that we propose for the evolution of chlorogenic acid *o*-quinones in reaction mixtures supplemented with (-)-epicatechin. To achieve a better understanding of *in vivo* enzymatic browning, further work will need to concentrate on more complex model solutions. Flavonols, dihydrochalcones and anthocyanins will be introduced into our model mixtures and the structures of the resulting oxidation products will be elucidated, evaluating their contribution to the final coloration. Enzymatic oxidation will be performed at pH values ranging between 3 and 4, close to that observed in apple. Moreover, the influence of some enzymatic browning effectors (endogenous PPO inhibitors, ascorbic acid, thiol compounds, ...) will be taken into account.

Acknowledgements

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Chapter 12

Exploiting Tyrosinase Activity in Aqueous and Nonaqueous Media

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Methods of utilising tyrosinase (polyphenol oxidase), E.C. 1.14.18.1, to prepare *o*-diphenols and *o*-quinones from phenols are described and discussed. Characteristics of enzyme activity under low-water conditions in organic solvents are reviewed including the use of salt hydrate pairs as water buffers. Recent progress in the use of the enzyme in synthetic chemistry, polymer modification, bioremediation and biosensors is highlighted.

The last decade has seen a rapid increase in our knowledge of the molecular biology of tyrosinase. The tyrosinase gene has been sequenced from both plant and animal sources allowing comparisons of size and sequence homology. It has also provided extra evidence for the location of the enzyme in leaves - namely that it is in the lumen of the chloroplast. The next logical development of over-expression of the gene has been accomplished (*1*) heralding the prospect of ready availability of the enzyme and also the opportunity to undertake site-directed mutagenesis. It is therefore an appropriate time to consider how we might use the enzyme in biotechnology.

Role in Nature

In nature, the enzyme plays an essential part in melanogenesis in animals and sclerotization in insects (*2, 3*). Although it occurs widely in plants its role in intact tissue is not always obvious (*4*); it is, however, involved in adventitious browning caused when cell structure is lost allowing substrate and enzyme to mix. Figure 1 provides a simplified view of these processes, emphasizing that the enzyme is involved at an early stage of a complicated sequence. It utilises oxygen to hydroxylate phenols and to oxidise *o*-diphenols, in each case the product being the corresponding *o*-quinones. These undergo addition reactions with nucleophiles such as the amino and sulfhydryl groups of amino acids (XH in Figure 1). In melanogenesis leading to the polymer eumelanin, tyrosine provides both the phenolic

and amino groups; when cysteine is also present pheomelanin is formed. In sclerotization the nucleophiles are present as protein, instead of free amino acid, leading to the formation of a tanned protein. A somewhat similar process occurs in egg shell formation in helminths (*5*) and in the formation of the adhesive protein in mussels (*6*). In the latter case tyrosinase may be involved in the hydroxylation of tyrosine residues in the polyphenolic protein since the protein contains a significant amount of dopa. Subsequent oxidation of this *o*-diphenol side chain is expected to lead to nucleophilic attack by lysine side chains leading to the formation of crosslinks, although none have yet been identified.

Applications

A major theme of this symposium is the prevention of adventitious browning in plants by inhibiting tyrosinase, or by terminating the sequence at the *o*-diphenol level by addition of a reducing agent such as ascorbate (AH_2 in Figure 1), or sulfite. By contrast I wish to take the opposite approach and discuss how the enzyme may be usefully employed; it is helpful to group applications loosely under three sections (a) production of diphenols (b) polymeric products formed from *o*-quinones (c) use in biosensors.

Tyrosinase as an Alternative to Fremy's Salt. The Teuber reaction which utilises potassium nitrosodisulfonate (Fremy's salt) in the conversion of para-substituted phenols to *o*-quinones (*7*) is superficially similar to the reaction catalysed by tyrosinase. However, there are differences as Table I illustrates.

Fremy's salt participates as a reactant supplying the oxygen atom which is inserted into the phenol ring whereas the enzyme utilises molecular oxygen and is thus required in much smaller amounts. The enzyme is also a completely reliable ortho-hydroxylating agent whereas Fremy's salt yields *p*-quinones with phenol and 4-chlorophenol. For synthetic use in production of *o*-diphenols the quinone must be reduced, usually by ascorbate. In aqueous media the *o*-quinone must be reduced as it is formed to prevent it polymerising or reacting with water. However, there is a problem with this system in obtaining the diphenol in good yield. During the progress of the reaction the *o*-diphenol concentration rises and the monophenol concentration decreases proportionately. Thus the enzyme has a choice of substrates - it can hydroxylate the monophenol or it can oxidise the diphenol. The latter reaction, which has a higher Vmax, is non-productive since it results in a futile cycle which removes reducing agent and causes inactivation of the catalyst.

Diphenol + $\frac{1}{2}O_2$	$\rightarrow$	*o*-Quinone + H_2O
o-Quinone + Ascorbate	$\rightarrow$	Dehydroascorbate + Diphenol
sum Ascorbate + $\frac{1}{2}O_2$	$\rightarrow$	Dehydroascorbate + H_2O
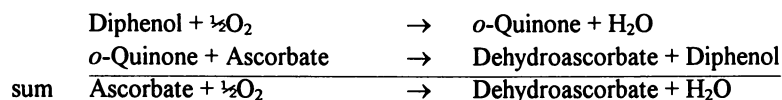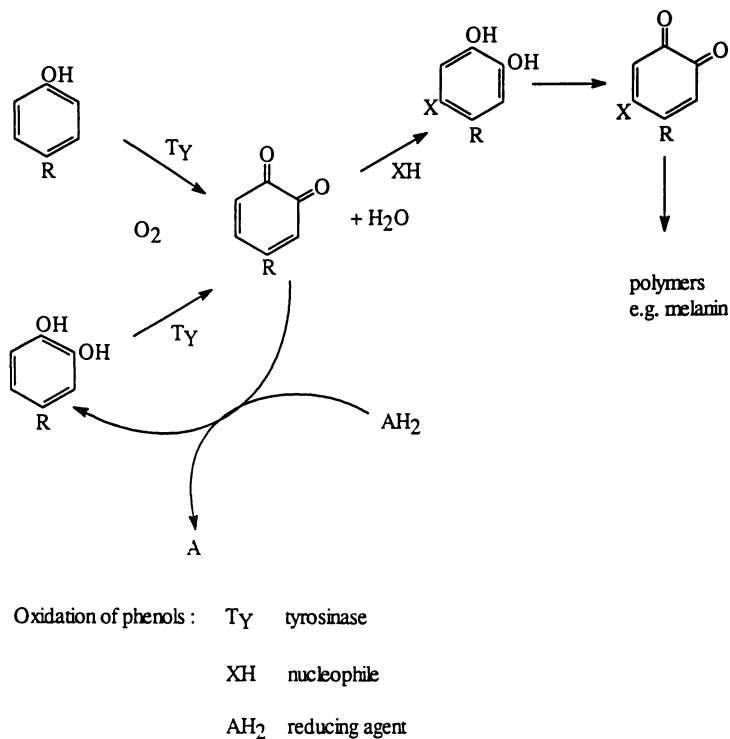
Figure 1: A schematic representation of the role of tyrosinase in nature.

Table I. Comparison of Hydroxylation of Phenols by Tyrosinase and Fremy's Salt (potassium nitrosodisulfonate)

	Biocatalyst	Fremy's salt
Preparation	easy	easy
Function	catalyst	reagent
Regioselectivity	ortho	ortho or para
Conditions	aqueous or low water	aqueous or biphasic
Stability	good at 5°C	Unstable unless purified

In spite of this obstacle good yields have been reported (*8*). Doddema (*9*) showed that yields were improved by high concentrations of borate; presumably borate forms a complex with the *o*-diphenol rendering it unavailable to the enzyme and thereby inhibiting the futile cycle. Tyrosinase hydroxylates a wide variety of *para* and *meta* substituted phenols but *ortho* substituted phenols are unreactive (*2,3,10*). Substitution with groups such as, alkyl, halogen, $-OCH_3$, $-CH_2OH$, $-CHNH_2COOH$, $-CH_2CHNH_2COOH$ is compatible with enzyme activity and reactivity with the *para* isomer is at least twofold greater than with the *meta* isomer. It is convenient to monitor oxidations by HPLC and colorimetrically (*9, 11*). Since dopa is an important drug, various groups (most recently, *12*) have investigated its formation from tyrosine but commercial exploitation is unlikely. There seems to be more interest in an enzymatic synthesis based on tyrosine phenol lyase for this compound (*13*).

An alternative approach to *o*-diphenol synthesis, which avoids the futile cycle, is to employ tyrosinase in nearly non-aqueous conditions. Here the *o*-quinones can have enhanced stability and the reaction is simply

$$\text{Phenol} + O_2 \quad\quad \rightarrow \quad\quad \textit{o}\text{-Quinone} + H_2O$$

The enzyme is employed as an insoluble powder, usually supported on glass or celite. Chlorinated (e.g. chloroform) solvents are particularly suitable as the reaction medium containing the phenol and dissolved oxygen. Water is required but only in small amounts (0.4% v/v for chloroform) and this may be added directly or more conveniently as a salt hydrate pair (*14*) e.g. $Na_2SO_4 10H_2O$: Na_2SO_4 anhyd. The advantage of the salt hydrate pair is that it acts as a water buffer, the higher hydrate ($Na_2SO_4 10H_2O$) donates water to start the reaction and the lower hydrate (Na_2SO_4) absorbs the water produced as the reaction proceeds, thereby maintaining the water activity at a constant level. The properties of mushroom tyrosinase in solvents have been established in several studies conducted by ourselves and others (*15-18*). The picture which emerges is that the enzyme functions best when almost fully hydrated i.e. a water activity > 0.7; other enzymes including certain lipases require virtually no water to function under these conditions (*19*). The solvation of the substrate in the solvent relative to water seems to be an important parameter and thus the K_m determined in aqueous and low water conditions is quite different (*20*). Some representative values for tyrosinase are given in Table II.

Table II. Km and Relative Vmax Values for the Oxidation of *o*-Diphenols by Tyrosinase

Substrate	4-methylcatechol		4 butylcatechol	
	Km (mM)	Vmax	Km (mM)	Vmax
aqueous conditions	0.29	1.0	0.66	0.5
in chloroform	0.52	0.1	35.2	0.12
corrected for solvation	0.78	--	5.0	--

It shows that there is a large discrepancy between the K_m values determined in buffer and solvent for the hydrophobic substrate, 4-butylcatechol. When a correction is made for solvation using the substrate partition coefficient between chloroform and water, the K_m is still significantly larger than in aqueous media, suggesting that other factors, such as chloroform dissolved in the aqueous layer, have an effect on catalysis. Table II also shows that Vmax is lower than in free solution, partly due to restricted access of substrate to the enzyme. Selection of solvent is based on the criteria of substrate and product solubility and compatibility with enzyme activity. Some satisfactory combinations are given in Table III.

Table III. Preferred Solvents for Tyrosinase

Substrate	Solvent	Ref
alkylphenols and	chlorinated solvents and	15-18
alkylcatechols	alkylbenzenes	20
N-acetyltyrosine ethyl ester	chloroform	9
catechin	heptanol	
catechol	butanol	21

Spectra typical of *o*-quinones can be obtained for various *m*- and *p*-substituted phenols and whilst stability is improved it is not absolute. The quinone may be reduced or can undergo Michael addition with cysteine for example, by shaking the solvent with the appropriate aqueous solutions.

Although the role of tyrosinase in the biosynthesis of *o*-diphenols remains unclear, the techniques described above could lead to its adoption *in vitro*. For example, the early stages in the biomimetic synthesis of the tyrosine-related alkaloid, codeine, are:

    (a)   tyrosine   →   dopa
    (b)   dopa   →   dopamine
    (c)   dopamine   →   3, 4-dihydroxyphenylacetaldehyde
                      →   norlaudanosoline

The conversions can be achieved with mushroom tyrosinase, a tyrosine decarboxylase from *Streptococcus fecaelis* and an amine oxidase from *Aspergillus niger* (Legge, R.L.; University of Waterloo, personal communication 1994, modified from [*22*]).

Polymeric products formed from *o*-quinones.   Since tyrosinase readily catalyses the formation of homopolymers from tyrosine or simple phenols (*3*) it can be used to produce phenolic resins. However, polymerisation of simple phenols by peroxidase has received more attention (*23*). The *o*-quinone products of enzyme action readily undergo addition reactions, a recent biomimetic example being the synthesis of coumestans from 4-hydroxycoumarins and catechol (*24*). In another interesting application tyrosinase is employed in the manufacture of biocompatible adhesives which cure under moist conditions and which may be suitable for *in vivo* surgical application (*25*). The mussel gene producing the adhesive protein has been expressed in yeast and then tyrosinase has been used to convert the tyrosine residues in the protein to dopa residues *in vitro*. Adhesion is achieved when dopa residues are

subsequently oxidised to the quinone state, allowing crosslinks to form. In a third variation the *o*-quinones are allowed to react with a carbohydrate polymer. Polyglucosamine (chitosan) is a potential waste product of the shellfish industry and its use in water purification has been advocated. Chitosan and tyrosinase are added to phenol-contaminated water (*26*) or chemical feedstock (*27*). Phenolic substrates present are removed from solution by oxidation to *o*-quinones and covalent attachment to the polymer. It is possible that this technique may also be of use when the enzyme is being extracted from tissue prior to its purification.

Tyrosinase based biosensors. In this area also tyrosinase has been used in aqueous and non-aqueous media. Since the enzyme uses oxygen, an obvious method of employment is in conjunction with an oxygen electrode (*28*). Thus if the enzyme is immobilised within a Clark electrode, the electrode responds when dipped in media containing phenol or *o*-diphenol substrates. Addition of ascorbate amplifies the oxygen consumed (*29*).

Tyrosinase was employed in the first biosensor to be adapted for use with organic solvents (*30*). In this application the *o*-quinone is reduced at a graphite electrode producing a current which is related to the substrate concentration. Since the enzyme is insoluble in the organic phase, immobilisation is easy and even tissue slices containing tyrosinase (e.g. banana) have been shown to be suitable (*31*). A low amount of water is necessary for function and the solvent is made conducting by addition of a supporting electrolyte. There is currently much interest in developing such devices to monitor effluents from chromatographic columns for phenols and catecholamines (*29, 32*).

Acknowledgement

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Chapter 13

Enzymatic Browning of Muscadine Grape Products

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Muscadine grape polyphenoloxidase (PPO) was purified by filtration through a 0.2μ membrane filter and by HPLC. Reactivity of the active fraction from the "Welder" muscadine grape per unit protein increased 33-fold over the values recorded for the crude extract. Electrophoretic analysis and activity stain showed that the enzyme in this cultivar displayed a single band with a molecular size lower than the non-PPO proteins. Optimum pH for activity was 4.5, and the enzyme was stable at temperatures up to 30 °C. Browning of muscadine wine does not appear to be significantly enzyme catalyzed. Total phenol content of the wine was unaffected by the browning process which was effectively inhibited by potassium metabisulfite. Tiron and EDTA had no significant effect, and diethyldithiocarbamate slightly retarded the rate of browning. The reaction appears to proceed by way of a heterolytic reaction pathway that does not involve phenolic compounds, possibly a Maillard-type reaction. Enzymatic catalyzed browning of muscadine grape products thus appear to be limited to juices and non-wine products of the grape.

The influence of phenolic compounds on the sensory qualities of grapes and grape products is well known (1-4). Oxidation of phenolics results in browning reactions that in most cases result in color and flavor deterioration in these products. In most cases, browning of grape products results from catalysis of phenolic oxidation by polyphenoloxidase (PPO), although there is evidence of some non-enzymic browning reaction pathways (5). In immature grapes, PPO is primarily located in the non-soluble particulate portion of the grape juice (6). PPO becomes more soluble with fruit ripening. Enzymic oxidation of grape products is influenced by factors such as available oxygen, phenolic substrate content, temperature, pH and

metal ions *(5, 7)*.   Quinones formed in the initial stages of the reaction rapidly polymerize or may combine with amino and sulfhydryl groups or proteins *(5, 7, 8)*.

Phenolic compounds present in grapes can be broadly classified as phenolic acids, anthocyanins, flavonols, flavan-3-ols and tannins, and flavanonols *(7)*. The nature and amounts present in grape products vary according to cultivar, maturity and processing methods *(7, 9, 10)*.   Optimum conditions for PPO activity also vary with cultivar as well as the nature and amount of available substrates *(11-15)*. Browning occurs mainly during pre-fermentation in crushing and pressing, and in juices. In most wines they are weak or non-existent *(7, 16)*. Previous attempts to isolate and characterize grape PPO using methods such as hydrophobic chromatography *(17)*, DEAE-cellulose and phenyl-sepharose CL-4B *(18)*, CM phenyl cellulose and hydroxylapatite *(19)* column chromatography have resulted in different degrees of success. These reports indicate that PPO isolation can be difficult, time consuming and generally requires more than one chromatographic separation.

Muscadinia rotundifolia (muscadine) grapes are widely grown in the Southeastern United States. Many muscadine cultivars are known to be highly susceptible to browning during processing and storage. While some reports on the non-enzyme catalyzed browning of their pigments and products *(20-22)* are available, very little is known about their enzyme-catalyzed browning potential. The enzymatic browning of muscadines and the factors that affect muscadine grape PPO activity were recently reported *(21, 23, 24)*. Enzymatic oxidative activities are weak or nonexistent in wines *(7)*.   Muscadine wines, however, brown excessively. In the study described here, a rapid method for the isolation and partial purification of muscadine grape PPO by high pressure liquid chromatography is presented.   Some characteristics of PPO isolated from the "Welder" muscadine grape cultivar and the possible involvement of enzyme catalysis in the browning of muscadine wine are also discussed.

Experimental

Preparation of Acetone Powder.   Fresh grape samples (200 g) were homogenized with cold acetone (300 mL;-10 °C and polyethylene glycol (1%; average mol. wt. 3350) in a Waring blender for 10 sec. After the seeds were discarded, the homogenate was further blended and then filtered through double layered nylon cloth (100 mesh) to separate the residue. The residue was further washed with cold acetone (900 mL) and filtered. Dry acetone powders were then ground in a mortar and sieved (65 mesh). The fine powder was used as the source of PPO extraction.

Extraction of PPO from Acetone Powder.   Dry acetone powder (15 g) was suspended in 0.05 M potassium phosphate buffer (pH 7.0; 200 mL) containing potassium chloride (1.0 M) and stirred for 2 h at 10 °C to extract PPO.   The suspension was centrifuged in a Sorval RC2-B refrigerated centrifuge at 12,000 X g and 0 °C for 20 min. The supernatant was then filtered through a Whatman No.

4 filter paper. The crude PPO extracts were kept at -20 °C until they were analyzed.

PPO Assay Procedure. Crude PPO extract was thawed at 10 °C. Assays of the PPO activity on the extracts (0.2 mL) were carried out in sodium phosphate buffer (0.01M, pH 7.0) using freshly prepared catechol (0.5 M, 0.4 mL) dissolved in the same buffer. Enzyme activity was determined on a Perkin-Elmer Lambda 3B spectrophotometer. The reaction mixture was transferred into a cuvette and increase in absorbance at 420 nm over a period of 60 sec was recorded using the Perkin Elmer TDRV software. First order kinetics of the data were analyzed using the Perkin Elmer KINI software. Enzyme activities were determined from the slope of a plot of log_e absorbance vs time. Measurements of enzyme reactivity were therefore based on the initial velocities of reaction, and were recorded as the rate of conversion of catechol to o-benzoquinone. Molar extinction coefficient (ϵ) for benzoquinone (Sigma Chemical Co.) was determined in the laboratory to be 184 $M^{-1}cm^{-1}$. PPO yields are also expressed as fold purification in terms of relative specific activity, with the specific activity of each crude extract = 1.0.

Determination of Protein. Protein content of crude extracts before and after filtration through a 0.2μ membrane filter was determined using bovine plasma albumin standards and the Bio-Rad protein assay dye reagent. Five mL reagent (20%) diluted in deionized water was added to 1 ml of standard solutions (0-1.5 mg/mL) and mixed thoroughly. Absorbance at 595 nm of the solutions was read after 15 min. Protein content of enzymes was determined from the absorbance values obtained for the standards, after the extracts were reacted with the dye reagent using a similar procedure.

HPLC Purification. Separation of PPO was carried out by injecting the extract (1 mL), (after filtration through 4.5μ teflon filter) on a LiChrospher Si 100 Diol 250 mm (L) x 4.00 mm (I.D.) column with a particle size of 10 μm. Elution was carried out using three solvent systems A (1 mM potassium) phosphate buffer; pH 7), B (ethylene glycol) and C (methanol), a Hitachi 655A-11 HPLC system and an L3000 multichannel photodetector. Eluents were monitored at 280 nm. With the initial concentration of A at 100% and flow rate at 0.5 ml/min, the concentration of B was increased to 50% after 5 min and held at this level for 20 min. After 25 min, the eluting solvent consisted of a mixture of 10% B and 90% C. Elution of the fractions were carried out over a period of 50 min. Peaks were integrated on a Hitachi D-2000 Chromato-Integrator. Eluted fractions were collected at 5 min intervals.

Polyacrylamide Gel Electrophoresis. An Ephortec vertical gel electrophoresis apparatus and 16 x 75 mm gel tubes were used. Electrophoresis was performed in 7.5% polyacrylamide gel in 0.05 M Tris - 0.4 M glycine buffer (pH 8.4) with a current of 2.0 mA per tube at 10 °C. Bromophenol blue was used as the tracking dye. Gels for protein were stained with Coomassie brilliant blue G-250 and destained with a solvent (methanol:acetic acid:H_2O = 3:1:6, volume ratio). PPO

activity on the gel was detected by immersing the gel in sodium acetate buffer (0.1 M; pH 6.0) containing 20 mM catechol and 0.05% p-phenylenediamine for 10 min at 25 °C *(19)*. After the reaction, the gel was placed in a solution of 10 mM L - ascorbic acid for 5 min and then washed with distilled water. The electrophoresis procedure separates on the basis of charge density and molecular size. Previous studies *(25)*, however, indicate minor variation in charge density of twenty proteins of the Welder muscadine cultivar. Molecular weights were thus estimated from a plot of $\log_{10}$ values of molecular weights of standards against their relative mobilities after they were analyzed using a similar procedure.

Effect of pH. PPO activity as a function of pH was determined by measuring activity in McIlvaine buffers ranging from pH 2.5 - 8.0. Rates of reaction at different pH values were monitored as described above.

Effect of Temperature and Time on PPO Stability. The effects of temperature and incubation time on PPO activity were determined. Enzyme extracts (0.2 mL) were subjected to 10 - 60 °C using a water bath, for times ranging from 0 to 60 min. They were then transferred into buffer solutions containing catechol (0.5 M) that were prewarmed to the corresponding temperatures. Initial reaction rates of these enzymes were assayed as described using the same procedure in 1 cm cuvettes around which water circulated at the respective temperatures of reaction.

Wine Analysis. The wine-making procedure was as previously described *(21)*. Color changes in wine samples were determined on a Perkin-Elmer Lambda 3B spectrophotometer equipped with an integrating sphere and a data station. Transmittance values from scans between 780 and 380 nm at 1-nm intervals were used to obtain chromaticity coordinates; luminance "L" and tristimulus "a" and "b" values were determined using the CIE standard source with the aid of the Perkin-Elmer COLOR software.

a-Amino nitrogen contents of wines were estimated as follows: 200-μL samples were transferred into small screw-capped tubes. To each was added 100 μL of 0.25% ascorbic acid and 1 mL of ninhydrin (3%), and each was boiled for 10 min. After cooling to room temperature, the samples were diluted with ethanol (3 mL) and absorbances at 570 nm were recorded. a-Amino nitrogen contents of samples were determined from standards (0 - 186 mg/L) that were prepared with leucine (1 mg/mL) in 80% ethanol.

Results and Discussion

Membrane filtration decreased protein content considerably, but increased PPO specific activity of the extract. Further purification by HPLC increased PPO specific activity by 33-fold over that of the crude extract (Table I). The PPO active fraction was eluted between 5 and 10 min (Figure 1). The recovery level after filtration and HPLC purification very likely indicates the removal of some inhibitory substances and/or the activation of these enzymes during the purification procedure.

Electrophoretic bands of the filtered extracts and HPLC fractions, detected with p-phenylenediamine, indicate that the molecular weight of PPO in muscadine grapes is relatively low when compared to proteins in the corresponding cultivars *(21, 25)*. PPO extract from Welder cultivar had a molecular weight of 15,000 Daltons (Rm = 0.8). This indicates that muscadine grape PPO is smaller in size than PPO of other grape species such as *V. vinifera* grapes *(18, 19, 26)*. The relative concentration of muscadine PPO proteins was also very low when compared to those of non-PPO bands (Figure 2). Unlike the non-PPO proteins, the

Table I. Protein Contents and Activity Recovery of Extracts

Sample	Protein Content (mg/100 mL)	Protein yield[a] (%)	Activity Recovery[b] (%)
Crude Extract	29.0 ± 1.4	100	100
Filtered Extract	4.0 ± 1.0	13.8	920
HPLC Fraction	3.0 ± 0.1	10.3	3300

[a]Protein yield has been adjusted for dilution by the eluting solution.
[b]Activity recovery per unit protein
Reproduced with permission from ref. 23.
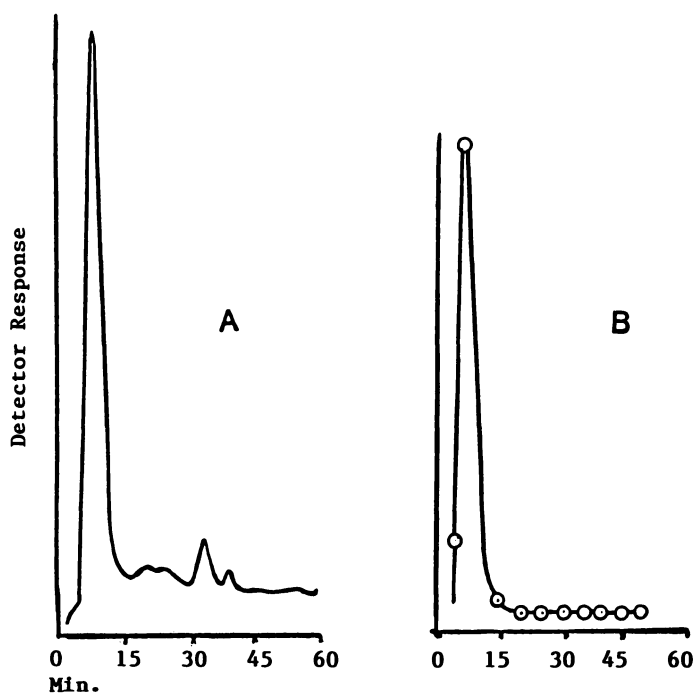
Figure 1. HPLC elution profile of PPO extract (A) and reactivity of fractions with catechol (B). Reproduced with permission from ref. 23.
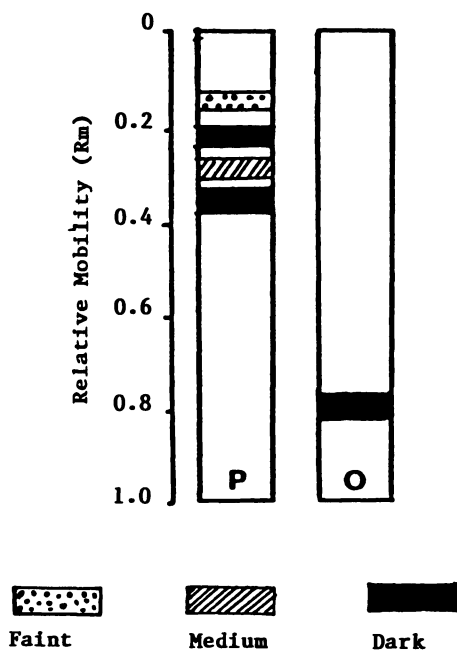
Figure 2. Protein (P) and PPO (O) bands of Welder muscadine cultivar on separated by acrylamide electrophoresis. Reproduced with permission from ref. 23.

PPO band was too weak for detection with Coomassie blue. Another possible reason for the inability of the PPO band to be detectable with Coomassie Blue stain is that the PPO might be bound to some other phenolic compounds (27). A low concentration of the muscadine grape PPO was recently suggested by Sims et al. (24). In their study to determine the effect of grape PPO on the color and quality of muscadine wines, the very weak PPO activity observed was attributed to a lack of indigenous PPO in the grapes and/or lack of readily oxidizable phenolics. That study was carried out on unisolated PPO in grape juices. The addition of mushroom PPO produced darker colored muscadine juices and wines thereby suggesting that the lack of PPO activity might be due to the absence of indigenous PPO rather than a deficiency of readily oxidizable phenolic compounds. Browning tendency is, however, more commonly related to the available phenolic substrates than PPO content of the grape (15). Muscadine grapes lack oxidizable phenolic acids such as caffeoytartaric acid (28), and differ considerably in anthocyanin composition from non muscadine grape species (29, 30).

The pH for maximum activity for PPO was 4.5. (Figure 3). At pH 3.0, activity was about 20% of the observed maximum activity. Therefore, harvesting of grapes for vinification at pH 3.0 or lower should depress the activities of these enzymes in muscadine grapes considerably. In grape juice, where the use of sulfite may be undesirable and the usual pH is between 3 and 4, PPO activity could be relatively high. At pH 3.5 and pH 4.0, for example, PPO activities were about 65% and 95% of the maximum activities, respectively.

The study to determine browning capability of muscadine grape PPO after the enzyme had been stored at various temperatures prior to contact with the substrate showed that the reaction rate was highest when the enzyme was kept at 45 °C for less than 50 min (Figure 4). Reactivity of the enzyme at this temperature, however, decreased with time of incubation of the PPO extract. When the enzyme was kept at 60 °C for 30 min. a loss of about 70% of the optimum activity occurred. Activity of the enzyme was stable when stored at temperatures below 30 °C. Based on the reactivity trend obtained with time of storage at these temperatures, for long term storage, the enzyme will be most active at 30 °C. This temperature is consistent with optimum temperatures reported for PPO activities in Concord (11) and Japanese Koshu grapes (19), but lower than that of de Chaunac grapes (18).

Muscadine wines appear to be more susceptible to browning than wines from other grape species. A set of experiments were thus carried out to determine the possible involvement of PPO in browning reactions of muscadine wines and the pathway of reaction. Browning of the Welder muscadine wine was accelerated by subjecting the wine to 49 °C over a period of time. This method has traditionally been used to simulate the effect of ageing and appears to correlate well with browning reactions at ambient temperatures (28, 31-33). The absorbance at 420 nm and tristimulus values were recorded over a period of time. A progressive increase in absorbance and tristimulus b values was observed (Figure 5). It was possible to inhibit the reaction by the addition of potassium metabisulfite (3 mM). The addition of 4,5-dehydroxy-1,3 benzene-disulfonic acid (tiron, 0.5 mM), a free radical scavenger, and ethylenediaminetetraacetic acid (EDTA, 10 mM) a metal ion complexing agent, had no effect on the rate of color development in the wine. The addition of diethyldithiocarbamate (0.1 mM), resulted in a slight decrease (15%) in the rate of browning. The wine pH decreased from 2.98 to 2.93 after 3 days. Protein level in the wine, determined as described by Amerine and Ough, (26) decreased from 3.03 mg/100mL to 1.45 mg/100mL over the same period of time.

Autooxidation of phenols occur in some non-enzymic browning reactions *(5)*. Kon *(34)* reported that enzymic browning of some fruits and vegetables was not inhibited by the addition of oxygen scavengers such as superoxide dismutase (SPO) and suggested that PPO does not fix molecular oxygen in the form of hydroxy derivatives by single electron transfers resulting in free radical or active oxygen formation, but rather by transferring a pair of electrons which avoids free radical intermediates in the formation of quinone. Post quinone oxidation, however involves free radicals *(5)*. The reaction steps of the non-enzyme catalyzed autooxidation is similar to the post-quinone enzymic oxidation and they ultimately add to enzymic degradation. Heterolytic mechanisms involving nucleophile
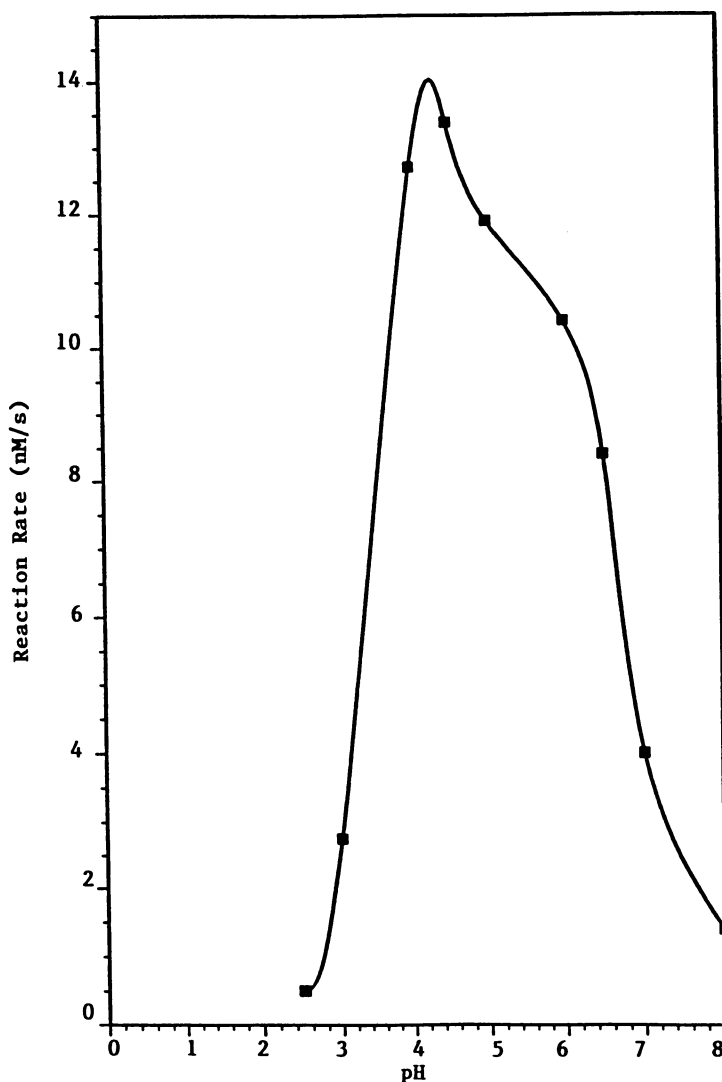
Figure 3. Effect of pH on muscadine grape PPO activity.

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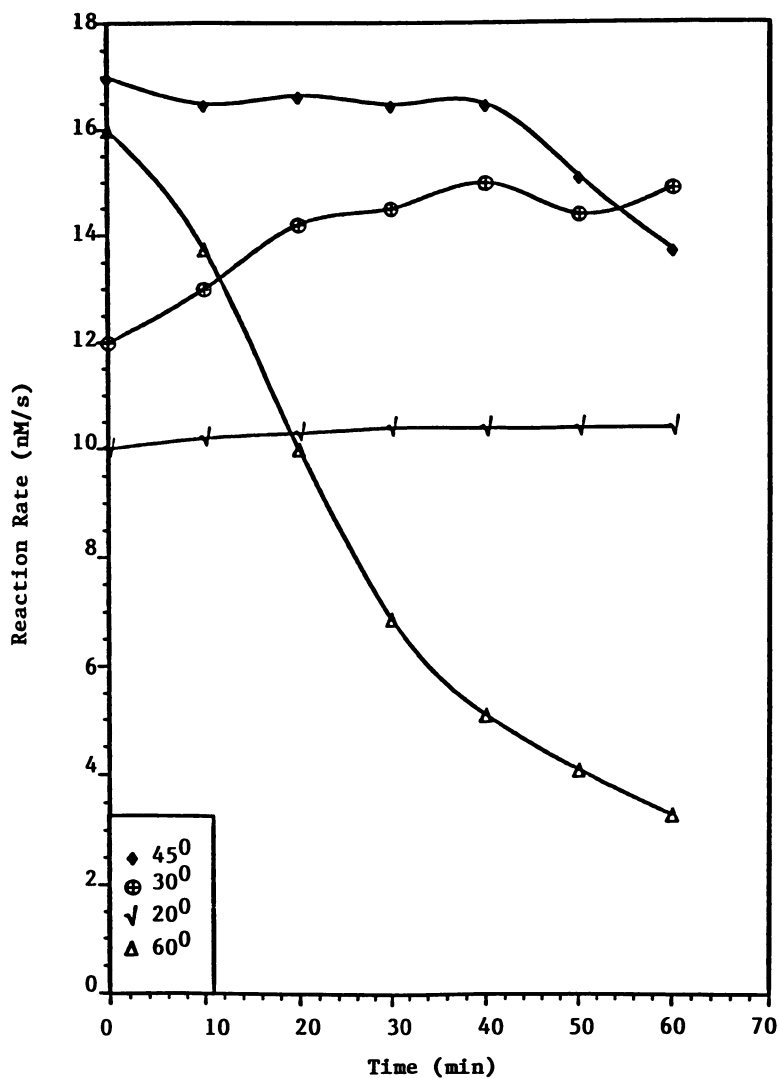
Figure 4. Effect of temperature and time on PPO stability.
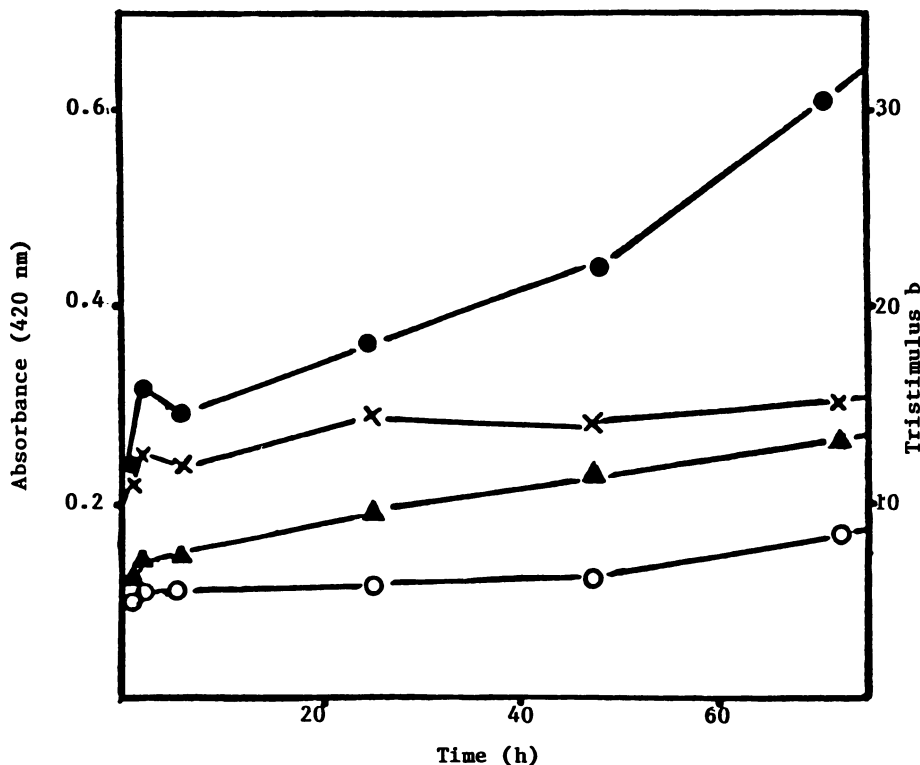
Figure 5. Browning of Welder muscadine grape at 49 °C: Increase
in absorbance at 420 nm in the presence (x) and absence (●) of sulfite
and tristimulus b values in the presence (o) and absence (▲) of
sulfite. Reproduced with permission from ref. 21.

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additions between amine and oxogroups (Maillard reaction) such as those between
reducing sugars and α-amino groups often result in non-enzyme catalyzed
browning. The extent to which enzymic and non-enzymic browning occur in
grapes appears to be directly related to the flavonoid concentrations in wines *(35)*.
    The ability of bisulfite to inhibit these reactions in food systems is well
known *(36-38)*. The inhibitive effect of potassium metabisulfite on browning of
the wine was therefore expected. The lack of sensitivity of the reaction to tiron,
a radical scavenger and complexing agent, suggests the absence of free radical
intermediates in the browning of muscadine wines. The catalytic effect of metal
ions in solution on PPO catalyzed reactions and other homolytic reactions is well
known *(39, 40)*. In the Welder wine, concentrations of Ca^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+},
Mg^{2+}, and Zn^{2+} are 37.0, 0.5, 0.4, 0.9, 13.0, and 3.9 mg/L respectively *(21)*. Cash
et al. *(11)* demonstrated that EDTA is just as effective as bisulfite in inhibiting
PPO activity in Concord grapes. The lack of sensitivity of the reaction to EDTA
indicates that PPO catalyzed browning is unlikely to be significant. This fact is

also supported by the inability of diethyldithiocarbamate that would be expected to inactivate PPO by removing Cu^{2+} from the native enzyme *(12)* to effectively retard browning. The absence of PPO and phenolic compound involvement in the browning reaction was also confirmed by the fact that total phenol contents of the fresh and brown wine, determined as described by Amerine and Ough *(26)*, were the same. It was not possible to conclusively determine the extent to which Maillard type reactions contributed to the observed browning by following available α-amino nitrogen content of the wine with time. Initial α-amino nitrogen contents was 65 mg/L and it increased to 77mg/L over four days instead of the expected decrease if they are involved in the browning process. The increase is apparently due to protein hydrolysis and modification of peptide bonds over the period of time *(21)*. The possible involvement of a Maillard type reaction is also supported by the decrease in wine pH during the browning process.

Conclusions

Isolation and partial purification of muscadine grape crude PPO can be achieved by membrane filtration of the acetone powder extract, followed by HPLC separation using a Si Diol column. Concentration of the muscadine grape PPO and its molecular size are relatively lower than those of the non-PPO proteins. The pH for optimum activity was lower than those of the non-muscadine cultivars but the influence of temperature on activity was similar. If uncontrolled, the activity of the PPO may be high under harvesting and processing conditions. During juice production, pasteurization should be effective in inactivating muscadine grape PPO activity. The potential deterioration of muscadine grape products through enzyme catalyzed browning appears to be limited to its non-wine products. In wines, while enzymatic oxidation could occur during the crushing and pressing stage of the winemaking process, browning of muscadine wines proceed by way of a non-enzymic heterolytic mechanism, possibly a Maillard-type reaction.

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Browning Mechanism of Water Convolvulus (*Ipomoea aquativa* Forsk.) Stored at Low Temperature

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The young leaves and stems of water convolvulus, a leafy vegetable that originates in tropical regions, showed browning as a symptom of chilling injury at temperatures below 9 °C. The main compound associated with browning in this vegetable was found to be chlorogenic acid, synthesized by caffeic acid:CoA ligase (CL) and caffeoyl CoA:quinate hydroxycinnamoyl transferase (CQT). The activity of CQT was higher at 1 °C than at 20 °C. Three phenolic fractions, free, esterified and bound, were extracted from water convolvulus. At 1 °C, a part of the bound form was transformed into a free reactive form. Electron-dense deposits produced by action of polyphenoloxidase on DOPA were observed with electron microscopy. These deposits were only seen in grana- and stoma-thylakoids. Chloroplast structure in the mesophyll cells was destroyed before the appearance of chilling injury. Three fractions: F-I, chloroplast rich precipitate (300~3,000Xg); F-II, a precipitate (3,000~100,000Xg) and F-III, supernatant (100,000Xg) were obtained by centrifugation. The activities of CQT and PPO in F-I increased, reaching a maximum just before the occurrence of chilling injury.

A great deal of attention has been focused on browning associated with chilling injury of fruits and vegetables during low temperature storage. In water convolvulus, an important leafy vegetable located in southeast Asia and southern Japan, browning was observed in the young leaves and stems during low temperature storage. In higher plants, phenolic acids consist of free and bound compounds and the free form is more reactive in browning than the bound one (*1, 2*). Shomer et al. (*3*) reported that polyphenoloxidase (PPO) is localized in the chloroplast thylakoid membrane in olive fruit. The objectives of our studies were to identify the levels and precursors of browning, changes in organization of the cells, the cellular localization of CQT and PPO in water convolvulus stored at low temperature, and to examine the mechanism of browning associated with chilling injury.

Changes in Appearance of Water Convolvulus at Various Temperatures

Growth and Storage Conditions. Water convolvulus (*Ipomoea aquatica* FORSK.) was grown in the field from June to October, harvested, packed in polyethylene bags (30 μm) and stored at 1 to 30 °C. Seedlings of water convolvulus were also stored over the same range of temperature and the sensitivity of chilling injury to temperature was investigated.

Changes in Appearance. Chilling injury was observed in the young leaves and stems during storage below 9 °C. Brown spots first appeared on the tips and young leaves; then the stems became dark brown and the leaves changed to dark green. The period leading to chilling injury was dependent on temperature and differed with harvest time. During July to August, injury occurred within 1 to 3 days, compared with 4 to 7 days during the September to October harvest. During storage at 1 °C, chilling injury appeared between 1 to 2 days but at 6 to 9 °C storage, injury appeared after 3 to 7 days. When stored at 12 to 15 °C, there was no indication of injury for more than a week, but both mature and green leaves turned yellow. The mature leaves turned yellow after 3 to 5 days at 20 °C and after 1 to 2 days at 30 °C. In seedlings of water convolvulus stored at different temperatures, the cotyledon was first damaged. Cotyledons, young leaves and stems stored at 1 to 9 °C showed browning injury. Seedlings were relatively insensitive to low temperature compared to harvested leaves. The seedlings at 15, 20 and 30 °C generally showed epinasty.

Changes in Content of Free and Bound Phenolics

Extraction and Fractionation of Phenolic Acids. Young folding leaves were frozen and powdered in liquid N_2 and extracted 4 times with MeOH:acetic acid:water (7:7:6), the extract was then centrifuged at 20,000Xg for 20 min. The method of Krygier (*4*) was used to extract the free phenolic acid fraction and esterified phenolic fraction from the supernatant; the insoluble bound phenolic fraction was extracted with the same solvent.

Phenolic Compound Determination and Gas Chromatographic Separation. A spectrophotometer was used to measure total phenolic and orthodiphenolic compounds. Phenolic acids were separated and determined by a gas chromatograph equipped with FID according to the method of Casteele (*5*).

Changes in Phenolics. When water convolvulus was stored at a low temperature, there was an increase in the amounts of caffeic and chlorogenic acids. We observed that K^+ leakage was higher at 1 °C than at 15 °C before the chilling injury occurred (*7*). It was suggested that changes in the membrane permeability may induce an unbalance in metabolism (*6*).

We examined the quantitative changes of the phenolic forms in water convolvulus stored at low temperature (Figure 1). Phenolic compounds were extracted as free, esterified and insoluble bound forms. The percent of total phenolics in the esterified form was about 62%, while the free form was about 14% and the insoluble bound form, about 24%. Total phenolic compounds in the free form increased from day 1 to 4 when stored at 20 °C. At 1 °C, the total free phenolic levels were even higher that at 20 °C for all storage days but the level decreased on the day 2 and then increased to the highest level by day 4. There was a decrease in the esterified form at both 1 °C and 20 °C, but the decrease was greater at 1 °C than 20 °C. During storage at 1 °C, the insoluble bound phenolic

compounds increased slightly and remained low. The changes in the three forms of orthodiphenolic compounds were almost the same as the changes in the total phenolics. The total amount in total and orthodiphenolics of these three fractions was slightly greater at 1 °C than 20 °C. These results suggest that phenolic compounds in water convolvulus were transformed from the esterified to free form which is more reactive and presumably the form responsible for the browning during low temperature storage. On the other hand, levels of insoluble bound phenolics were low and had little effect on browning. The main free phenolics identified at harvest were *trans*-cinnamic, *para*-coumaric, ferulic, caffeic and chlorogenic acids. The esterified fraction contained the same phenolics. Only ferulic acid was detected in the insoluble bound form.

Phenolic compounds exist in free forms and in bound forms with amino acids, proteins, sugars and other substances. Following metabolic turnover, the bound forms are converted to free forms and visa versa. It was reported that phenolic compounds in beans and peas (*8*) played a role in unspecified inhibition of enzyme activity by binding with the protein. The esterified phenolics mainly exist in the vacuole (*9*). The free form of phenolics browned rapidly because the aglycone is reactively reactive (*2*). Barley seeds contain syringic, protocatechuic and ferulic acids as free phenolics and form vanillic and *para*-coumaric acids in their esterified and glycoside forms (*1*). Some interconversion between the two occur during ripening and/or storage.

Enzyme Activity Related to Chlorogenic Acid Biosynthesis

Enzyme Assays. Activities of hydroxycinnamoyl CoA ligase (CL) and caffeoyl CoA:quinate hydroxycinnamoyl transferase (CQT) were determined according to Rhodes (*11*). The protein content was determined by the Lowry method (*12*).

Enzyme Activities. In water convolvulus, the main phenolic compounds were caffeic and chlorogenic acids synthesized through phenylpropanoid metabolism. Of the enzymes related to biosynthesis of chlorogenic acid, the main enzyme was CQT, which conjugates quinic acid and caffeoylCoA. CL and CQT activities increased during both 1 °C and 15 °C storage, but the increase at 1 °C was faster for CL and reached a higher activity for CQT before the occurrence of chilling injury (Figure 2).

Observation of Ultrastructure in Young Leaves and Electron-dense Deposits with DOPA

Electron Microscopy of Cells and Electron-dense Deposits with DOPA. The mesophyll tissues at harvest and after 5 days storage at 1 °C and 20 °C were fixed, dehydrated and embedded by the method of Spur (*13*). The mesophyll tissues were incubated with and without 10 mM DOPA in 0.1 M phosphate buffer for 2 h (*3, 14*); after that they were treated by the above method.

Observation of Ultrastructure. The ultrastructure of chloroplast, mitochondria and tonoplast of young leaves of water convolvulus at harvest (Figure 3(a)) was observed clearly. The ultrastructure at 20 °C stored for 5 days (Figure 3(b)) showed little change compared with that at harvest. When stored at 1 °C for 5 days, the chloroplasts were swollen and the number of grana stacks and chloroplast ribosomes were decreased (Figure 4). Mitochondria cristae disappeared (Figure 5) and tonoplast were invaginated into the vacuole and disappeared (Figure 5) after storage for 5 days at 1 °C. These subcellular changes were presumed to be associated with chilling injury. When water convolvulus was
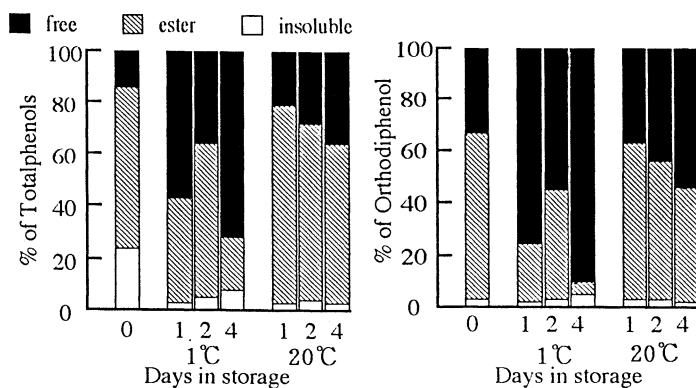
Figure 1. Contents of total and orthodiphenolic compounds extracted from the free, esterified and insoluble bound phenolic compounds in water convolvulus stored at 1 and 20 °C. Adapted from ref. 10.
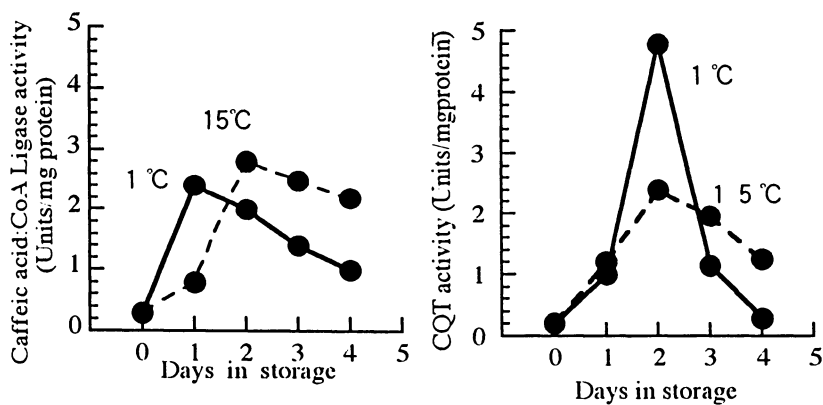
Figure 2. Changes in the activities of caffeic acid:CoA ligase (CL) and caffeoyl CoA:quinate hydroxycinnamoyl transferase (CQT) extracted from the young leaves of water convolvulus stored at 1 and 15 °C, (1 unit=0.01ΔOD/min). Adapted from ref. 7.
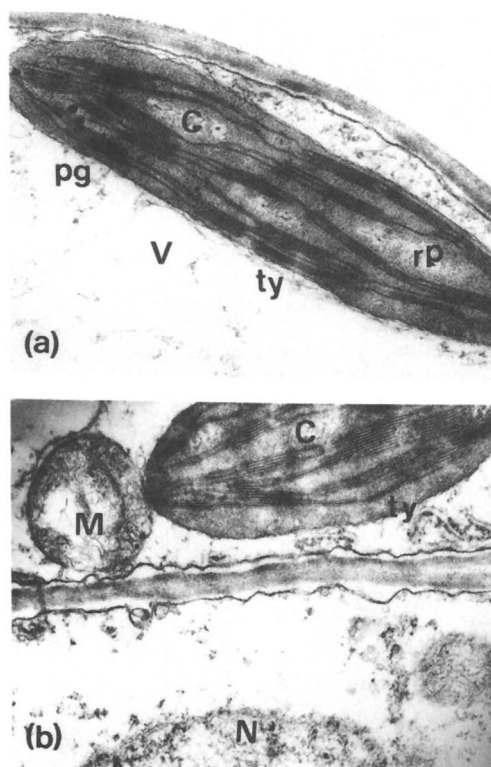
Figure 3. Electron micrograph of young leaf of water convolvulus at harvest (a) and stored at 20 °C for 5 days (b), showing vacuole (V), chloroplast (C), plastoglobule (pg), stoma thylakoid (ty), chloroplast ribosome (rp), nucleous (N) and mitochondria (M). Adapted from ref. 15.
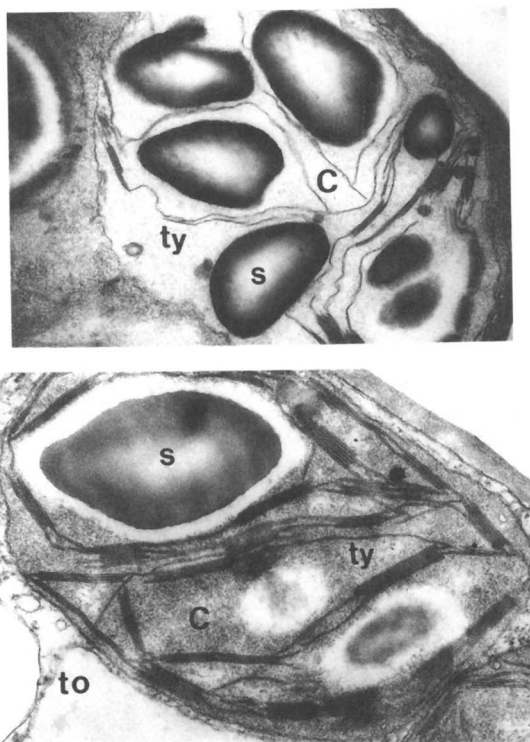
Figure 4. Electron micrograph of young leaves of water convolvulus at 1 °C for 5 days, showing chloroplast (C), thylakoid (ty), tonoplast (to), starch grain (S). Structural changes are compared with Figure 3. Adapted from ref. 15.
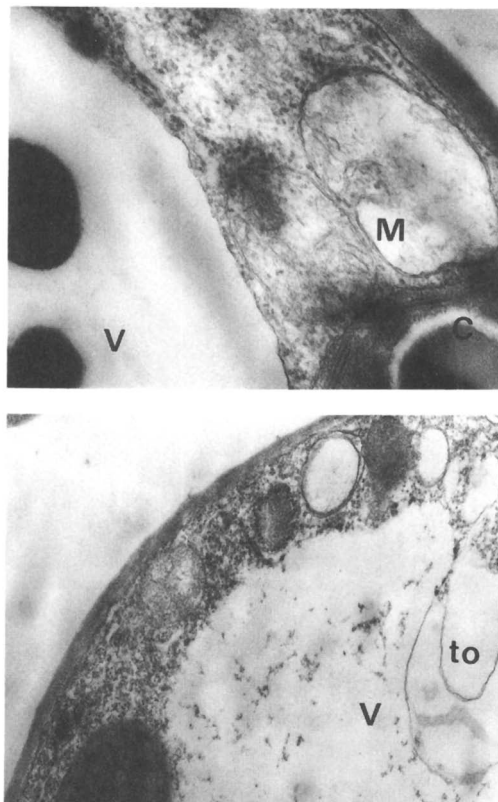
Figure 5. Electron micrograph of young leaves of water convolvulus at 1 °C for 5 days, showing mitochondria (M), vacuole (V), and tonoplast (to). Structural changes are compared with Figure 3. Adapted from ref. 15.

incubated with DOPA, a substrate for PPO, electron-dense deposits were found in the thylakoids of chloroplast (Figure 6). This observation suggests that a part of PPO was bound to thylakoid membranes and not to mitochondria or other organelles. Under chilling and excess light for photosynthesis (16), chloroplast in cucumber leaves showed thylakoidal and stomal changes. Lax and Vaughn (17) reported that the functions of PPO are unclear, but various roles were postulated such as host defense and photosynthetic electron transport. PPO in broad bean leaves was localized in the thylakoid membrane and the distribution pattern was consistent with photosynthesis II proteins (80% grana and 20% stoma).

Cellular Localization of CQT and PPO Enzymes

Separation of Cellular Organization by Differential Ultracentrifugation.
Mesophyll tissues were homogenized in Tris-HCl buffer (pH 8.0) containing 0.4 M sucrose and 2 mM EDTA, and the homogenate was squeezed through Millarcloth. The 300 to 3,000Xg pellets were chloroplast rich and the 3,000 to
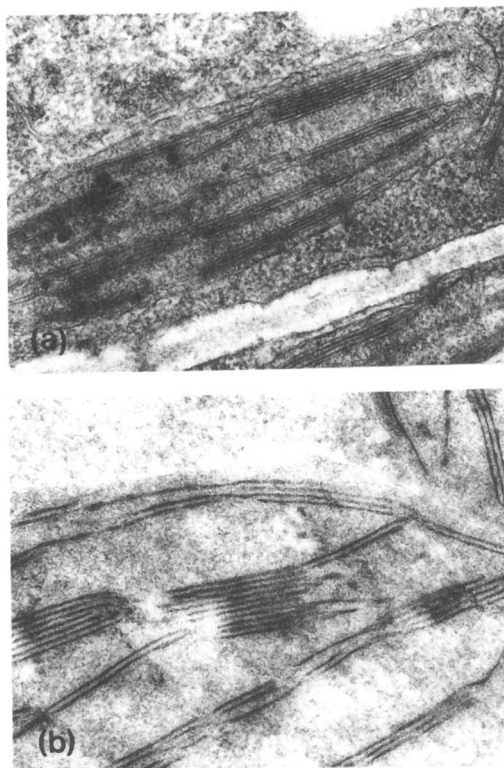
Figure 6. Section of chloroplast of young leaves of water convolvulus incubated without (a) and with (b) DOPA. Electron dense-deposits are visible within the thylakoid incubated in DOPA.
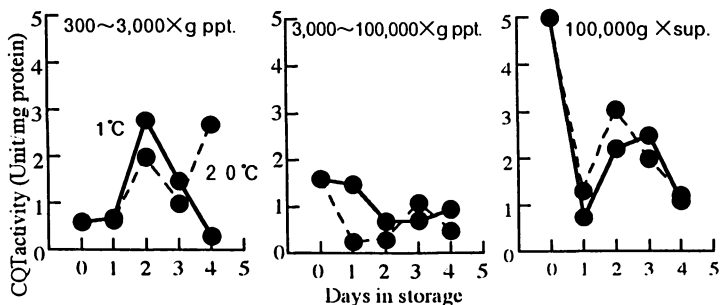
Figure 7. Changes in caffeoyl COA:quinate hydroxycinnamoyl transferase (CQT) activities isolated from the leaves of water convolvulus stored at 1 °C (●━━●) and 20 °C (●━ ━●), separated by three differential centrifugations.
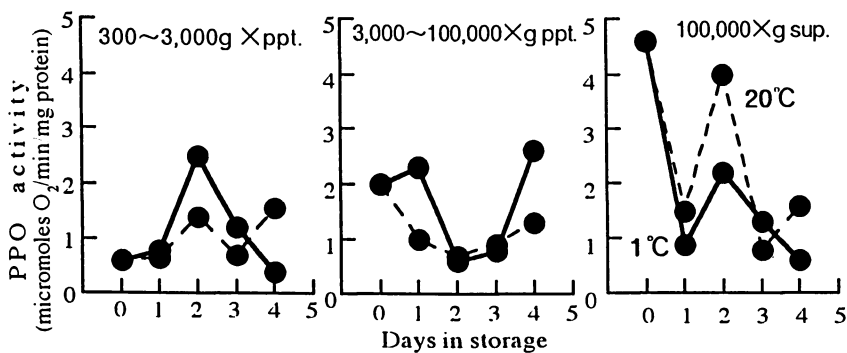
Figure 8. Changes in polyphenoloxidase (PPO) activities isolated from the leaves of water convolvulus stored at 1 °C (●━━●) and 20 °C (●━ ━●), separated by three differential centrifugations.

100,000Xg pellets were various membranes, except chloroplast, and the 100,000Xg supernatant was the soluble fraction. PPO was assayed using an oxygen electrode with chlorogenic acid as the substrate. The localization and activity of CQT and PPO were investigated in each fraction.

Changes in Enzyme Activities. In the three fractions, both enzymes showed the same changes (Figures 7 and 8) during 4 days of storage. The activities of CQT and PPO in F-I increased, reaching a maximum before the occurrence of chilling injury. In F-II and III, they decreased during the storage.

Conclusion

The mechanism of browning induced by a low temperature storage in water convolvulus was considered to be as follows. Free phenolic acids, which are the precursors of browning, increased due to hydrolysis of esterified phenolics and by biosynthesis. The main phenolic acids in water convolvulus are caffeic and chlorogenic acids, synthesized by CL and CQT through the phenylpropanoid metabolic pathway. The destruction of chloroplast, where PPO is located, and tonoplast may be related to browning due to oxidation of the phenolic compounds by the above processes.

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Chapter 15

Difference Spectra Spectrophotometry for Polyphenol Oxidase Assay

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We utilized difference spectra in spectrophotometry in order to estimate polyphenol oxidase (PPO) activity and amounts of polyphenols that are substrates for PPO in plant foods. Difference spectra produced peaks in the ultraviolet-wavelength range during enzymatic polyphenol oxidation. Peak height (ΔA) was found to be directly proportional to the concentration of polyphenol. A close relationship was seen between the ΔA value and oxygen uptake measured by the manometric method, and between the rate of polyphenol oxidation (ΔA/min) and PPO concentration. These results indicate that difference spectra spectrophotometry is a useful way of rapidly and accurately determining PPO activity and polyphenols, which are closely related to enzymatic browning in plant foods.

Chlorogenic acid and catechin analogues are major phenolic compounds (1) and natural substrates in enzymatic browning (2) in foods. We isolated PPO (EC 1.10.3.1) which showed strong oxidative activity on chlorogenic acid and catechin from eggplant (3), head lettuce (4) and Japanese pear (5). We also purified PPO, which particularly catalyzed the oxidation of triphenols such as phloroglucinol and pyrogallol, from Satsuma mandarin orange (6), turnip (7) and edible burdock (8).

PPO assay has usually been performed by manometric (9) and polarographic (10) methods based upon the fact that the rate of oxygen consumption is proportional to the amount of enzyme present during polyphenol oxidation. Several investigators (11-13) have employed a spectrophotometric method in which PPO activity was estimated by the increment of absorbance at a visible wavelength (around 420 nm) during polyphenol oxidation. The spectrophotometric method is an indirect assay of PPO activity by measuring the oxidative conversion of the substrate. In enzyme inhibition studies, therefore, the polarographic method is more desirable when compared with the spectrophotometric method. However, by measuring change of

absorbance at the initial stage in enzymatic oxidation, direct inhibition of PPO from inhibitory reactions affecting product intermediates can be avoided. Recently, Neujahr *(14)* reported the determination of phenol and catechol concentrations with oxygen probes coated with immobilized enzymes or immobilized cells. Such biosensor assay coupled to polarograph, however, involves numerous complicated procedures and is unsuitable for dealing with many samples at the same time. Therefore, we employed crude or partially purified enzyme solutions in difference spectrophotometric method for PPO assay.

Using Satsuma mandarin orange PPO, we found a considerable difference between the relative rate of phloroglucinol oxidation measured by a manometric method and that measured with an absorbance increment of 420 nm *(15)*. The same discrepancy was found in enzymatic chlorogenic acid oxidation by Japanese pear PPO *(5)*. Although measuring absorbance is simpler than measuring oxygen uptake, the actual change of absorbance at that wavelength following enzymatic oxidation of polyphenols, such as chlorogenic acid and phloroglucinol, is very small. Therefore, we utilized peak height of difference spectra in the ultraviolet-wavelength range.

We originally developed the enzymatic method using difference spectra as a rapid and sensitive determination of *L*-ascorbic acid in foods *(16)* and biological samples *(17)*. The present paper describes difference spectrophotometric procedures in estimating PPO activity and polyphenol content in plant foods *(18-21)*.

Experimental

Preparation of PPO. Crude PPO solutions were usually prepared by extracting acetone powder of plant foods with a buffer solution. Enzyme solutions which strongly oxidized chlorogenic acid and catechins were purified by fractionation with ammonium sulfate from extracts of Japanese pear (*Pyrus serotina* Rehd.), apple (*Malus Pumila* Mill.), sweet pepper (*Capsicum annuum* L.), eggplant (*Solanum melongena* L.) and head lettuce (*Lactuca sativa* L.). Phloroglucinol oxidase (1, 3, 5-trihydroxy-benzene : oxygen oxidoreductase) was obtained from Satsuma mandarin orange (*Citrus unshiu* Marc.), turnip (*Brassica rapa* L.) and cabbage (*Brassica oleracea* L.) by a similar procedure.

Measurement of Absorbance and Difference Spectra. When a conventional spectrophotometer (1 cm light path) is used, the ΔA_{325}, ΔA_{380} and ΔA_{272} values are calculated by measuring the absorbance at 325, 380 and 272 nm, respectively, of control and test reaction mixtures after incubation. The absorption spectra of control and test reaction mixtures and the difference spectra of test reaction mixture against control are measured with a recording spectrophotometer (Hitachi Model 557) using a 1 cm light path within 15 sec. Peak height (ΔA) of the difference spectra is read. The activities of chlorogenate oxidase, epicatechin oxidase and phloroglucinol oxidase are determined from the oxidized amount per unit time of these polyphenols which are obtained using the calibration curves of these polyphenols.

Enzyme solutions were prepared by extraction with 0.1 M citrate / 0.2 M sodium phosphate (McIlvaine) buffer from the acetone powder of sweet pepper seeds or other plant tissue. Sample solutions were made from plant tissue by ethanol extraction.

Measurement of Oxygen Consumption. Oxygen consumption was measured

by a manometric method using a Warburg manometer. The contents were composed of 1.3 mL of 0.1 M phosphate buffer (pH 7) and 0.2 mL of the enzyme solution in the main chamber, 0.5 mL of 20 mM aqueous solution of phloroglucinol in the side chamber and 0.1 mL of 20% potassium hydroxide in the center well. After preincubation for 15 min at 30 °C, phloroglucinol solution was added to the enzyme solution in the main chamber and the time course of oxygen consumption was measured.

Results and Discussion

PPO Assay. Peak height ($\Delta A325$) in the difference spectra of chlorogenic acid oxidation increased in proportion to the reaction time for about 10 min at 30 °C. The time course of enzymatic oxidation is given in Figure 1 (A). $\Delta A325$ increase appears to be directly proportional to the concentration of chlorogenic acid utilized, as shown in Figure 2. A linear relationship was observed between the rate of chlorogenic acid oxidation (expressed as $\Delta A325/5$ min) and the concentration of the enzyme, as illustrated in Figure 3. From these results, within a certain range of enzyme concentration, we can quickly and precisely assay chlorogenate oxidase by measuring $\Delta A325/5$ min. In the difference spectra of phloroglucinol oxidation, peak height ($\Delta A272$) was increased in proportion to the reaction time for about 20 min at 30 °C as shown in Figure 1 (B). The $\Delta A272$ was found to be proportional to the concentration of phloroglucinol. $\Delta A272/5$ min was also proportional to the concentration of phloroglucinol oxidase.

These results suggest that the $\Delta A325/5$ min and $\Delta A272/5$ min values respectively represent the rate of enzymatic oxidation of chlorogenic acid and phloroglucinol. Nawa et al. *(22)* determined phloroglucinol oxidase activity by measuring the increment of absorbance at 328 nm utilizing manganese ion as the enzyme activator. However, the $\Delta A328$ value was lower than the $\Delta A272$ value. It is reasonable that utilization of $\Delta A272$ is superior to that of $\Delta A328$ for determination of enzyme activity. In addition, as shown in Figure 4, $\Delta A272/5$ min in phloroglucinol oxidation was found to be highly correlated with the rate of oxygen uptake. The spectrophotometric method is easier to use as a PPO assay than the manometric method, because the ΔA value directly indicates the change of polyphenol concentration during enzymatic oxidation.

In the case of catechin [(-)-epicatechin and (+)-catechin)], the absorbance in a 300 to 500 nm wavelength range markedly increased during enzymatic oxidation. Peak height ($\Delta A380$) in the difference spectra increased in proportion to the reaction time for about 20 min at 30 °C. The $\Delta A380$ was also proportional to the increase in concentration of (-)-epicatechin. Therefore, the $\Delta A380$ value can be adopted for the catechin oxidative activity of PPO. In this case, however, the $\Delta A420$ value may also be usable because it is only slightly different from the $\Delta A380$ value.

The procedure for PPO assay was set up as follows:
[Test solution]: a mixture of 0.5 mL of 0.5 mM aqueous solution of polyphenol (chlorogenic acid or phloroglucinol), 1.3 mL of McIlvaine buffer (pH 4 for pear polyphenol oxidase and pH 7 for cabbage phloroglucinol oxidase) and 0.2 mL of PPO solution is incubated for 5 min at 30 °C. After incubation, the oxidation reaction is stopped by adding 3 mL of 4 % metaphosphoric acid.
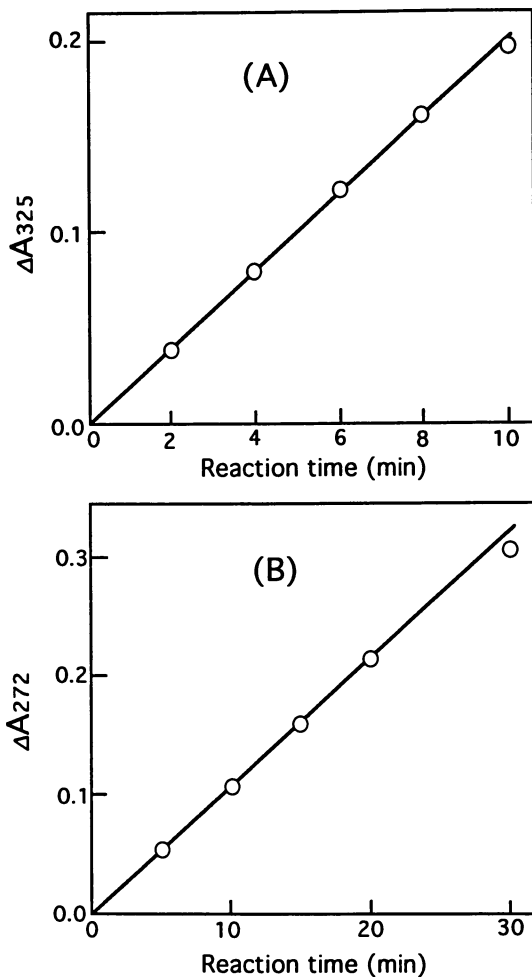
Figure 1. Time course of enzymatic polyphenol oxidation. (A) chlorogenic acid oxidation by Japanese pear enzyme. (B) phloroglucinol oxidation by cabbage enzyme.
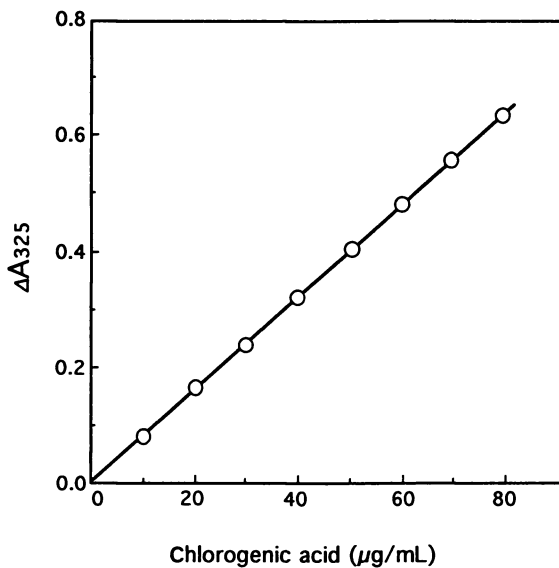
Figure 2. Relationship between concentration of chlorogenic acid and peak height (ΔA_{325}) in difference spectra during chlorogenic acid oxidation by sweet pepper enzyme.
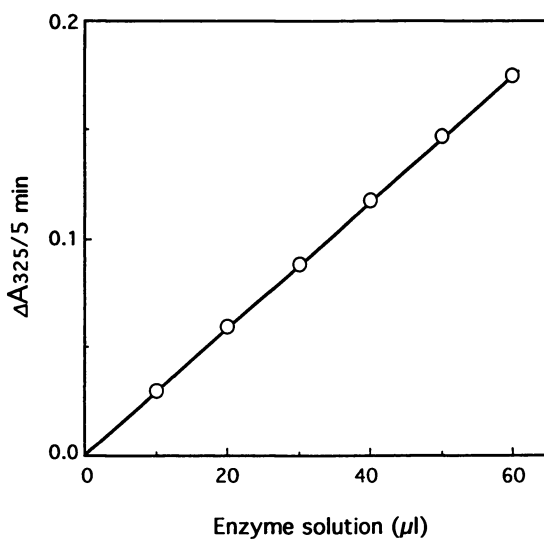
Figure 3. Dependence of rate of chlorogenic acid oxidation (ΔA_{325} /5 min) on increased enzyme concentration. Enzyme concentration is expressed as volume of stock enzyme solution, which was prepared by ammonium sulfate fractionation from crude extract of sweet pepper seeds.
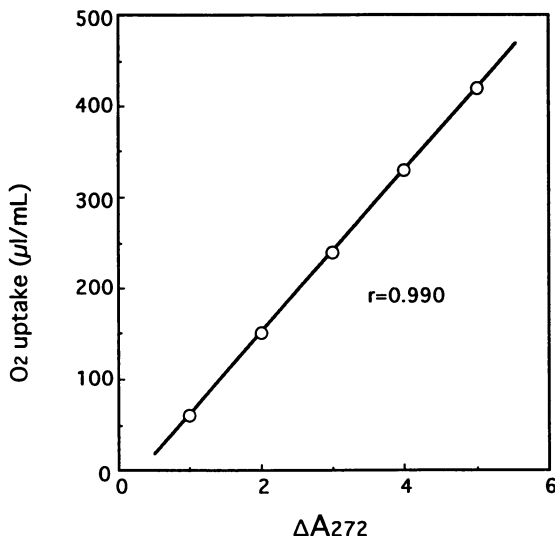
Figure 4. Relationship between oxygen uptake and peak height (ΔA_{272}) in difference spectra during phloroglucinol oxidation by cabbage enzyme.

[Control solution]: 0.2 mL of the enzyme solution is added to a mixture of 3 mL of 4% metaphosphoric acid, 0.5 mL of 0.5 mM polyphenol solution and 1.3 mL of the same buffer. In this reaction mixture, the enzyme is inactive due to the presence of excess metaphosphoric acid. PPO activity is suppressed below pH 2.5. $\Delta A_{325}/5$ min for chlorogenic acid, $\Delta A_{380}/5$ min for epicatechins and $\Delta A_{272}/5$ min for phloroglucinol are estimated.

The absorption spectra of control and test solution and the difference spectra of test solution against control are measured with a recording spectrophotometer as shown in Experimental section. In the phloroglucinol oxidase assay, distilled water is used instead of metaphosphoric acid because the peak shifts to a higher wavelength in difference spectra.

Table I shows chlorogenic acid and (-)-epicatechin oxidative activity of the enzymes from four plants, sweet pepper (seed), Japanese pear (immature fruits), apple (flesh) and eggplant (flesh). All the enzymes had optimum activity at about pH 4 for chlorogenic acid oxidation, and at 4 to 7 for (-)-epicatechin oxidation. The sweet pepper PPO showed highest activity among the four plant PPOs used as crude enzyme. The partially purified enzyme solution obtained from the immature fruit (1 to 3 cm in dia.) of Japanese pear had a specific activity of about 60 units/mg of protein. One unit of activity was defined as that which increased ΔA by 0.01 per min at pH 4.2 and 30 °C.

**Table I . Chlorogenic acid and (-)-Epicatechin Oxidizing
Activities in Various Plant PPOs**

Sample	Chlorogenate oxidase*		Epicatechin oxidase*	
	Opt. pH	Activity at pH 4	Opt. pH	Activity at pH 7
Sweet pepper (seeds)	4	24.3	7	43.5
Eggplant (flesh)	4	3.5	6	3.5
Japanese pear (immature fruits)	4	6.8	4	5.4
Apple (flesh)	4	4.9	5	4.4

*Activity was shown as units/ml of crude enzyme solution (1 unit = 0.01
of ΔA_{325} or ΔA_{380} per 5 min).

Analysis of Polyphenol (PPO substrate). As mentioned above, sweet pepper PPO showed high activity in the oxidation of chlorogenic acid and (-)-epicatechin at pH 4 and 7, respectively. Therefore, the sweet pepper PPO was used for determining polyphenols by the difference spectrophotometric method in this study. After incubation for about 10 and 20 min, ΔA_{325} and ΔA_{380} reached respectively plateau levels, which lasted up to 60 min. These results indicate that both chlorogenic acid and (-)-epicatechin are oxidized to o-quinone within about 10 to 20 min and that this o-quinone is spectrophotometrically stable for at least 60 min. Table II shows the effect of polyphenols and L-ascorbic acid on ΔA_{325} value of chlorogenic acid. ΔA_{325} values decreased with the increasing concentration of added (-)-epicatechin, which is widely distributed in plant foods with chlorogenic acid. For example, ΔA_{325} was decreased by 13 to 17% at a 1:2 ratio of epicatechin/chlorogenic acid (Table II). Recently, we found that a purified eggplant PPO oxidized chlorogenic acid more quickly than (-)-epicatechin at pH 4 (3). The oxidation rate of (-)-epicatechin by the enzyme was about one tenth of that of chlorogenic acid. Therefore, the effect of epicatechin will be considerably reduced by the use of this purified eggplant enzyme. The ΔA_{380} was also proportional to increase in concentration of (-)-epicatechin (20). Thus, the amount of chlorogenic acid and catechins in food can be estimated by measuring ΔA_{325} and ΔA_{380} with difference spectra spectrophotometry.

The procedure for analysis of polyphenols that are substrates for PPO was set up as follows:

[Test solution]: a mixture of 1 mL of sample solution and 1 mL of the PPO solution is incubated for 10 to 20 min at 30°C. After incubation, the oxidation reaction is stopped by adding 3 mL of 4% metaphosphoric acid.

[Control solution]: 1 mL of PPO solution is added to a mixture of 1 mL of sample solution and 3 mL of 4% metaphosphoric acid.

The difference in absorbance (ΔA) between the control solution and the test solution is measured by a spectrophotometer. The amount of polyphenol (PPO substrate) is estimated using a calibration curve which shows a linear relationship between ΔA and the amount of polyphenol oxidized by PPO (Figure 2).

**Table I . Effect of Polyphenols and Ascorbic Acid on ΔA_{325}
Value of Chlorogenic Acid**

Compound (M)	Concentration		Relative ΔA_{325} value (%)			
		Chlorogenic acid (M)	Polyphenoloxidase			
			Japanese pear	Apple	Sweet pepper	Eggplant
Chlorogenic acid	2.5×10^{-5}	—	100	100	100	100
Caffeic acid	2.5×10^{-5}	—	87	91	89	86
Dopamine	2.5×10^{-6}	2.5×10^{-5}	98	98	99	99
	5.0×10^{-6}	"	96	98	97	96
	12.5×10^{-6}	"	90	90	94	86
Pyrocatechol	2.5×10^{-6}	"	99	98	99	98
	5.0×10^{-6}	"	98	98	98	96
	12.5×10^{-6}	"	93	96	95	86
(-)-epicatechin	2.5×10^{-6}	"	97	97	98	98
	5.0×10^{-6}	"	93	93	95	95
	12.5×10^{-6}	"	84	83	87	84
Pyrogallol	2.5×10^{-6}	"	97	98	98	98
	5.0×10^{-6}	"	97	98	98	98
	12.5×10^{-6}	"	83	80	87	80
L-ascorbic acid	12.5×10^{-5}	"	100	100	100	100

.ch015

Several samples were analyzed by the difference spectrophotometric method and colorimetric methods [Zucker and Ahrens method (23) based on Hoepfner reaction was used for chlorogenic acid analysis and vanillin-sulfuric acid method for catechin analysis]. As shown in Tables III and IV, the difference between values obtained by the colorimetric method and the difference spectrophotometric method ranged from 1.4 to 16.5. The precision is higher by the difference spectrophotometric method (0.8 to 14.3%) than by the colorimetric method (5 to 40%). Recovery of polyphenols was good (19). Other polyphenols such as pyrocatechol, dopamine, pyrogallol and gallic acid have also been analyzed by our methd (Tono, T., Nishikyushu University, unpublished data).

Table III. Comparison of Chlorogenic Acid Content by Difference Spectra Spectrophotometry and Colorimetric Method

Sample \ Method	Chlorogenic acid (mg/100g)	
	Difference spectra Spectrophotometry (ΔA_{325})	Colorimetric Method* (A_{520})
Eggplant (flesh)	79.9 ± 0.7**	94.0 ± 7.7**
Sweet pepper (seeds)	131.3 ± 1.5	145.2 ± 12.4
Japanese butterbur (petiole)	46.1 ± 1.2	54.6 ± 10.3
Japanese pear(immature fruits)	172.3 ± 1.3	155.8 ± 8.4

* Zucker and Ahrens method (23), ** Mean (n=6) ± standard deviation.

Table IV. Comparison of Catechin Content by Difference Spectra Spectrophotometry and Colorimetric Method

Sample \ Method	Catechin (mg/100g)	
	Difference spectra Spectrophotometry (ΔA_{380})	Colorimetric Method* (A_{500})
Eggplant (flesh)	13.5 ± 0.3**	18.1 ± 7.3**
Potato (tuber)	1.4 ± 0.2	2.8 ± 1.1
Apple (peel)	25.4 ± 0.7	40.2 ± 8.5
Japanese pear (immature fruits)	29.7 ± 0.4	31.2 ± 4.6

* Vanillin-sulfuric acid method. ** Mean (n=6) ± standard deviation.

Assessment of Enzymatic Browning. As enzymatic browning proceeds in food, cut surfaces become dark, and the absorbance of the juice changes in a 220 to 500 nm wavelength range. The difference spectra show a characteristic pattern in each food. Figures 5 and 6, respectively, show changes in absorption spectrum and in difference spectra of reaction mixtures during enzymatic oxidation of chlorogenic acid and phloroglucinol. The difference spectra of phloroglucinol oxidation shows a single positive peak at 272 nm. The difference spectra of chlorogenic acid oxidation shows two positive peaks at 255 and 410 nm and a negative peak at 325 nm. The difference
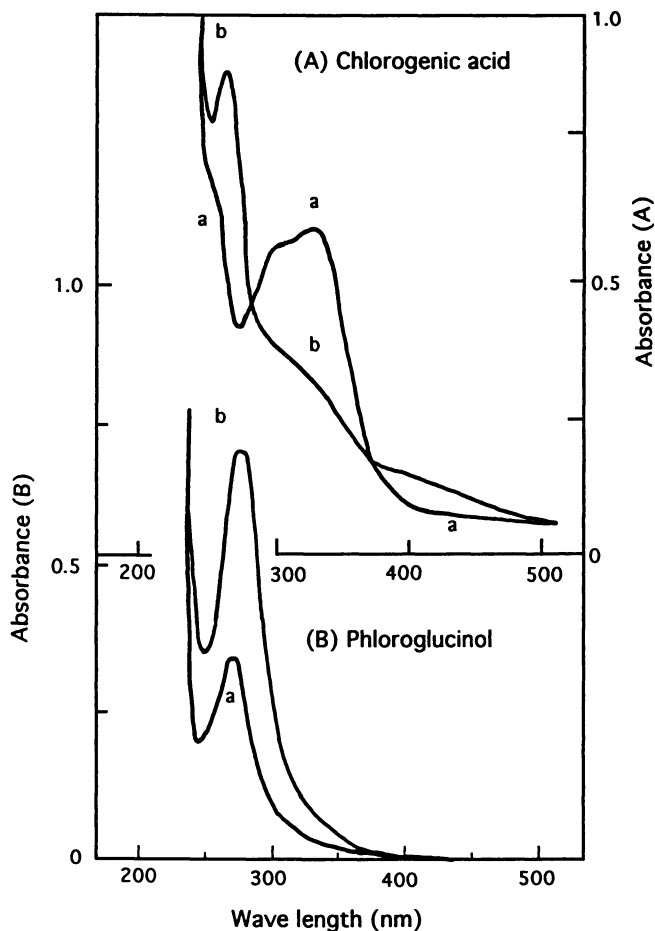
Figure 5. Changes in absorption spectrum during enzymatic oxidation of chlorogenic acid (A) and phloroglucinol (B). a and b indicate the absorption spectrum of control and test solutions (after 10 min at 30°C). Sweet pepper enzyme was used for chlorogenic acid oxidation at pH 4 and cabbage enzyme for phloroglucinol oxidation at pH 7.

spectra of (-)-epicatechin oxidation has two positive peaks at 255 nm and 380 nm with no negative peak *(20)*. The positive peak at 255 nm found in both chlorogenic acid and (-)-epicatechin oxidation may have been derived from an *o*-quinone formed in the initial stage of enzymatic polyphenol oxidation *(24)*. Thus, major natural substrates of enzymatic browning in plant foods can be easily assessed from the pattern of the difference spectra.

Conclusions

By the difference spectra spectrophotometry, PPO activity and concentration of polyphenol (PPO substrate) can be quickly and precisely determined without isolating these compounds using a chromatographic technique, because of the high substrate specificity of enzymes. By measuring difference spectra, the kind of the principal natural substrate of enzymatic browning in plant foods can be easily assessed. It is concluded that the difference spectra spectrophotometry is a useful method of rapidly and accurately determining the role of PPO and polyphenols in the enzymatic browning of food.
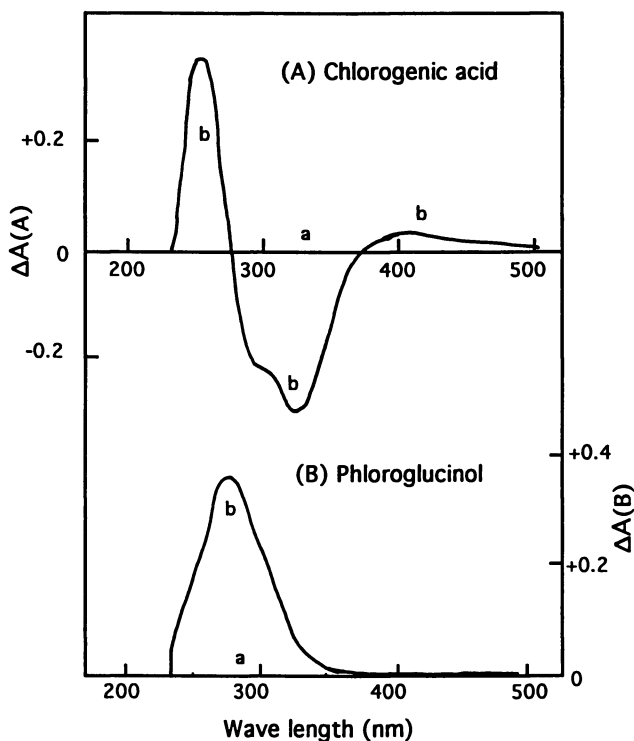
Figure 6. Changes in difference spectra during enzymatic oxidation of chlorogenic acid (A) and phloroglucinol (B). The difference spectra of test solution (b) against control solution (a) were measured. Reactions were carried out at the same conditions as shown in Figure 5.

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Chapter 16

Antioxidant Characteristics of Melanin-Related Products from Enzymatic Browning Reaction of Catechin in a Model System

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Antioxidant activity of melanin related products obtained from enzymatic oxidation of catechin was studied in a model system. Melanin related products were obtained from the catechin-polyphenol oxidase reaction at pH 6.5 and 25 °C at various time intervals. All catechin-enzyme reaction products (CERPs) were brown in varied intensities with increased absorption at 210-220, 380-390 nm, and 420-430 nm. CERPs obtained at the early stage of the reaction showed a higher antioxidant activity than those from the later stage. Antioxidant activity of CERPs may be explained by their abilities of hydrogen atom donation, free radical scavenging, and lipoxygenase inhibition. However, the exact mechanism of the antioxidant activity of CERPs cannot be explained until their chemical structures are elucidated.

Enzymatic browning of fruit and vegetable products is mainly caused by the oxidative reactions of various polyphenol compounds catalyzed by polyphenol oxidase (PPO) in the tissues. The enzyme catalyzes the oxidation of diphenols to *o*-quinones and further reaction leads to melanin, brown pigments. Some of these reaction products have shown specific chemical characteristics depending on the particular polyphenol compounds (*1-3*). The antioxidant activity from non-enzymatic browning reaction products in processed foods has been reported (*4-7*). There have been reports on the antioxidant activity of the enzymatic browning reaction products in apples (*8-10*). However, there has been no report on the antioxidant activity of the enzymatic browning reaction products from specific polyphenol compounds.

Catechin, one of the major phenolic compounds found in many fruits, has shown various antioxidant activities such as tocopherol as a free radical scavenger in the lipid oxidation systems (*11-13*). On the other hand, catechin itself is the major cause of enzymatic browning acting as a good substrate for polyphenol oxidase in many food products, such as tea and fruits (*14, 15*). The objective of this study was to obtain information on the antioxidant activity of the polyphenol oxidase reaction products in a model system using catechin.

Materials and Methods

Reaction Products and Their Absorption Spectra. Melanin related reaction products were prepared by the following method. A sample of 19.5 mL of catechin solution (2 mM) in Mc'Ilvaine's citric acid-phosphate buffer (0.02 M, pH 6.5) was mixed with 0.5 mL polyphenol oxidase (EC 1.14.18.1, tyrosinase; 1000 unit/mL) solution in the buffer and kept at 25 °C in a water bath while stirring. Samples of the catechin-enzyme reaction products (CERPs) were taken at 0, 1, 3, 6, 15, 30, 60, and 120 min intervals. Some samples were measured directly for antioxidant activity while other samples were freeze-dried for later use. The absorption spectra (190-700 nm) of CERPs were measured using a spectrophotometer (Shimadzu UV 2100, Japan). The catechin content during enzymatic browning was monitored by HPLC (*16*) and the rate of browning was measured spectrophotometrically at 420 nm.

Inhibitory Effect of CERPs on Peroxide Formation. Antioxidant activity of CERPs was determined by measuring peroxide produced from the linoleic acid oxidation system. A 2.5 mL of CERPs was mixed with linoleic acid solution (15 mg in 2.5 mL ethanol) and kept at a constant temperature of 37 °C for 2-4 days for autooxidation. The formed peroxides were then measured using a ferric thiocyanate method (*17, 18*).

Measurement of Free Radicals. Antioxidant activity of CERPs in terms of free

radical scavenging property was measured using α,α'-diphenyl-β-picrylhydrazyl (DPPH) (*19, 20*). DPPH (16 mg) was dissolved in 100 mL ethanol and diluted to 200 mL with H2O and then filtered using Whatman filter paper No. 2. A 5 mL DPPH solution was mixed with 1 mL of CERPs and the absorbance decrease at 528 nm was recorded.

Measurement of Conjugated Dienoic Acid. Antioxidant activity of CERPs was also measured by their inhibitory effects on the formation of conjugated dienoic acid from linoleic acid in the presence of lipoxygenase. A 0.1 mL of CERPs was mixed with 0.9 mL of the enzyme solution, kept at 25 °C for 10 min, and to which 2 mL of the linoleic acid solution was added. The conjugated dienoic acid formed in the linoleic acid-lipoxygenase system was measured by the increase in absorbance at 238 nm using a spectrophotometer (Cecil CE 599, England). The enzyme solution was prepared by dissolving lipoxygenase (EC 1. 13. 11.12, 52,000 unit/mg) in 0.17 M boric acid buffer at a weight ratio of 1:30 to linoleic acid. Linoleic acid was dissolved in ethanol at ratio of 1.7 mL to 1 mL (*21, 22*).

Enzymes used in this experiment were from Sigma Chemical Co. (St. Louis, MO). Other chemicals were acquired from Fluka Chemical Corp. (Ronkonkoma, NY).

Results and Discussion

Changes in Catechin and the Browning Rate During Catechin Oxidation Catalyzed by Tyrosinase. Catechin was oxidized rapidly during the first 15 min. More than 50% of the catechin was converted to CERPs within the first 12 min of the reaction time (Figure 1). The reaction rate slowed at 30 min when more than 80% of catechin was lost and remained at the low level thereafter. At the same time, the rate of browning (absorbance at 420 nm) was fast at the beginning, up to the 30 min reaction time, and then increased slowly at the steady rate reaching a maximum absorption (7.9) at 120 min reaction time. Practically 90% of the original catechin was converted to CERPs at the end of the experiment (at 120 min) with only a small amount of residual catechin remaining.

Absorption Spectra of CERPs. Figure 2 represents the absorption spectra of catechin and CERPs taken at 15, 30, and 120 min. Catechin (control) showed a maximum absorption at 200-219 nm with an increased absorption at 290 nm. CERPs obtained at the early stages of the reaction showed a similar spectral pattern but the reaction products obtained later (after 15 min) showed increased absorbance at 380-420 nm. A rapid visual color change was observed at 15 min which coincided with the increased absorption at 210 nm, 390 and 420-440 nm. This increase in absorbance progressed with the reaction time. It has been reported that the characteristics of brown color in the enzymatic browning reactions depend on the nature of the reactants, the rate of reaction, and the photo-absorption characteristics of the reactants (23). The characteristic absorbance of CERPs at 280, 380 and 420 nm indicates the presence of dimers, procyanidins and melanin as reported previously (24-27).

CERPs As an Oxidation Inhibitor of Linoleic Acid. Antioxidant activity of CERPs, measured by the inhibitory effect on the peroxides formation in the linoleic acid autooxidation system, is shown in Figure 3. The control (linoleic acid alone) produced peroxides very rapidly, reaching a maximum absorbance at 48 hr, and then decreasing thereafter, while the samples of CERPs added were lower in absorbance due to their inhibitory activity. There were significant differences in the antioxidant activity among CERPs obtained at different reaction times. The CERPs obtained during the early stages of the reaction (before 15 min) showed a higher level of activity as compared to that of the catechin alone (2 mM, original concentration), but those CERPs obtained after 15 min of reaction time exhibited lower activity that decreased with increased reaction time. Residual catechin content in CERPs at the 15 min reaction time was about one-third of the original catechin; therefore, antioxidant activity derived from the residual catechin in CERPs at 15 min should be minimal.

   The antioxidant activity of CERPs was shown to be concentration-dependant (Figure 4). Antioxidant activity of CERPs (at 120 min reaction time) at the level of 0.05% was higher than that at 0.01%. Antioxidant activity of various plant phenolic compounds has been well documented (28, 29). Recently, the chemical characteristics of phenolic compounds as a hydrogen donor or free radical scavenger and their structure-activity relationships have been reviewed extensively (29). However, information on the antioxidant activity of the phenolic compounds-polyphenol oxidase reaction products is limited (8-10, 30).

Antioxidant Activity of CERPs as a Free Radical Scavenger. Free radical scavenging activity of CERPs using a DPPH method is shown in Figure 5. DPPH is reduced by cysteine, glutathione, tocopherol, polyhydroxy aromatic compounds, and others, converting the purple color to colorless. Therefore, DPPH has been used often to test for antioxidant, hydrogen donor, or free radical scavenging activities (8, 19, 20, 30). We observed a varied range of scavenging activity among CERPs obtained at the different reaction times. CERPs collected from the early stages of the oxidation reaction had a higher free radical scavenging activity than those from the later stages of oxidation (data not shown) and the pattern of the antioxidant activity was similar to those observed in linoleic acid oxidation (Figure 3). Comparison of the antioxidant activity of CERPs (120 min) and other commercial antioxidants, such as butylatedhydroxyanisole (BHA) and a-tocopherol, showed that CERPs at the 0.05% level exhibited comparable antioxidant activity as 0.005% BHA, and that 0.01% CERPs had the same level of antioxidant activity as 0.01% α-tocopherol (Figure 5).
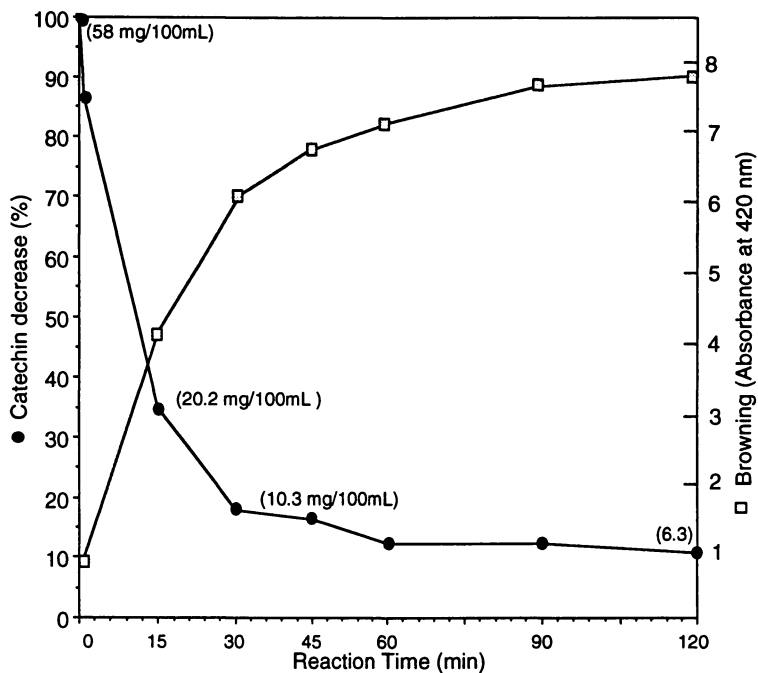
Figure 1. Changes in catechin content and the browning rate during the catechin-tyrosinase oxidation reaction at 25 °C, pH 6.5.
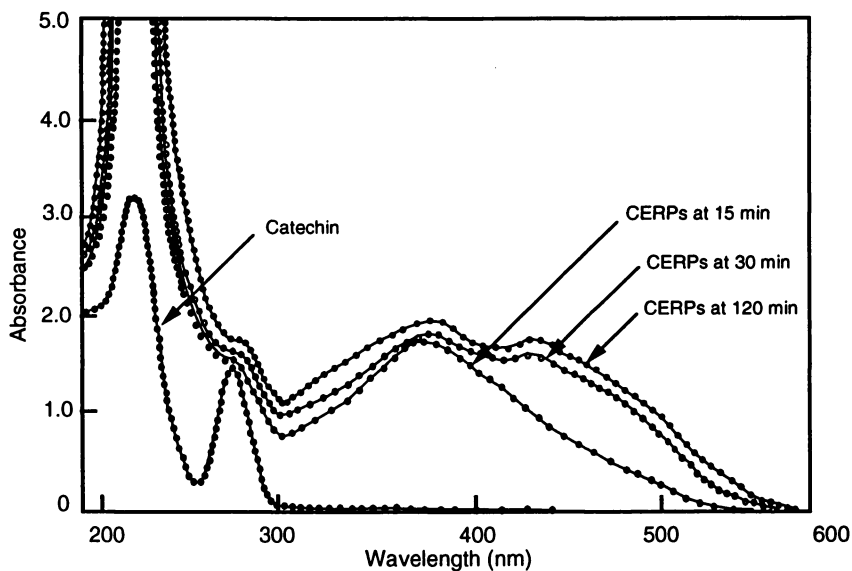
Figure 2. Absorption spectra of catechin and CERPs.
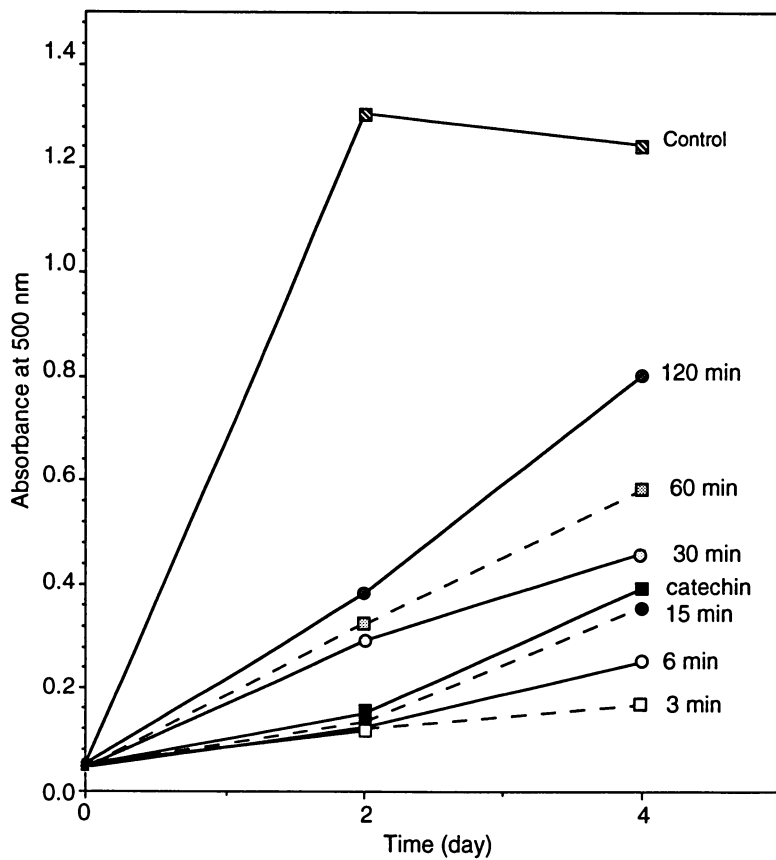
Figure 3. Antioxidant activity of CERPs at different reaction times on linoleic acid oxidation at 37 °C. Measurement was made on the formed peroxides.
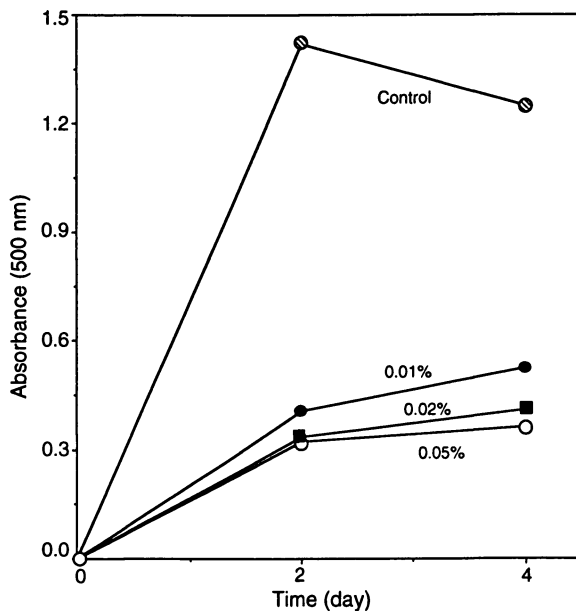
Figure 4. Antioxidant activity of different concentrations of CERPs (at 120 min) on linoleic acid oxidation at 37 °C. Measurement was made on the formed peroxides.
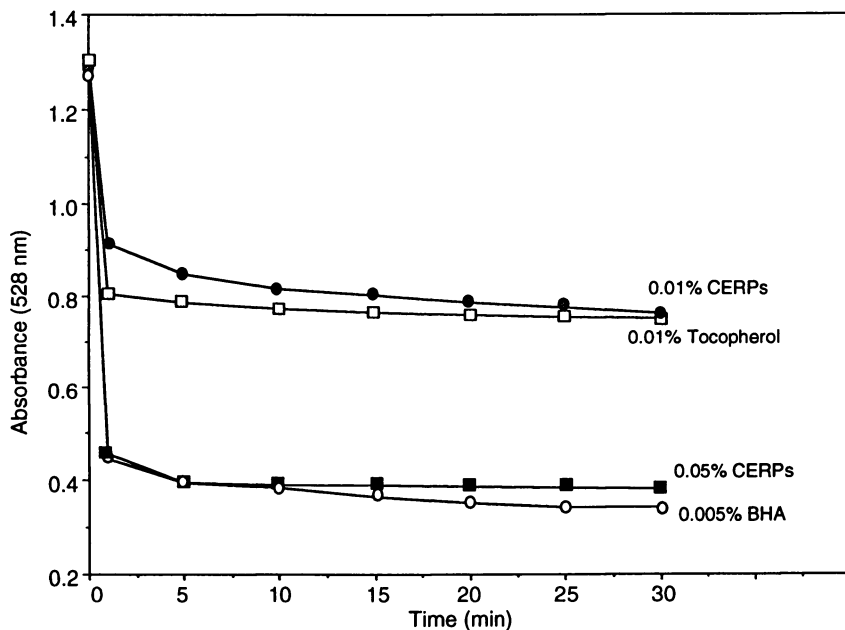
Figure 5. Free radical scavenging activity of CERPs (at 120 min), BHA, and α-tocopherol measured by the DPPH method.
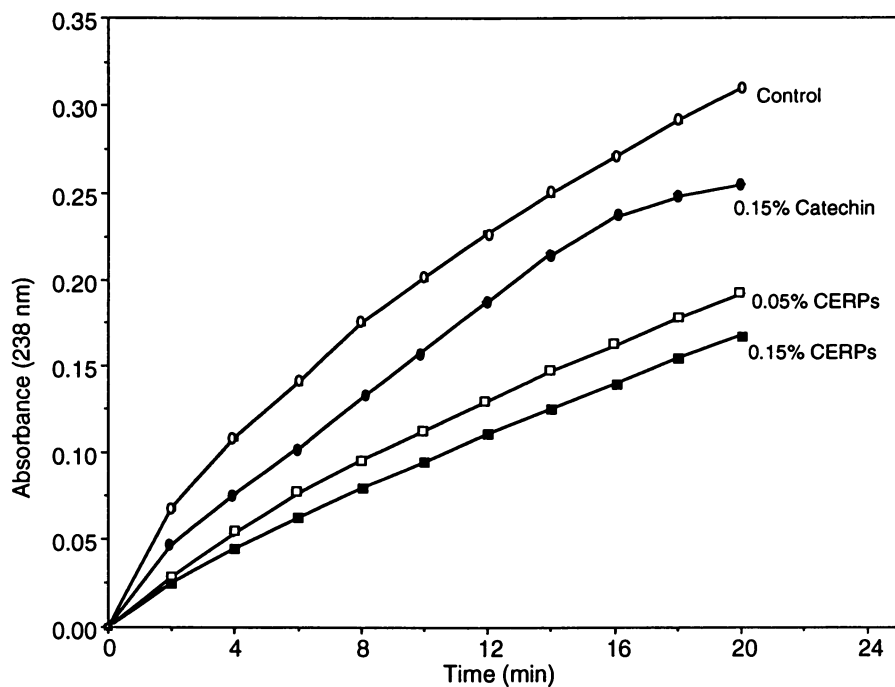
Figure 6. Inhibitory effect of catechin and CERPs (at 120 min) on the linoleic acid oxidation catalyzed by lipoxygenase. Measurement was made on the formed conjugated dienoic acid.

Antioxidant Activity of CERPs as a Lipoxygenase Inhibitor. Lipoxygenase catalyzes oxidation of linoleic acid and related compounds which have a cis, cis-1,4 pentadiene structure and produces a conjugated dienoic acid. This oxidation product, dienoic acid, can be measured at 238 nm. The inhibitory effect of CERPs (120 min reaction time) added to the lipoxygenase-linoleic acid system at the level of 0.05% and 0.15% was higher than that of 0.15% catechin (Figure 6). Again, a higher inhibition effect on lipoxygenase was noticed as the concentration of the added CERPs increased. The inhibitory effect of some phenolic compounds on lipoxygenase has been reported (*31, 32*) and is explained by the coupled oxidation theory or/and conversion of lipoxygenase to an inactive ferrous form (*33, 34*), but there is no report on inhibitory effect of polyphenol oxidation products on lipoxygenase.

Conclusion

The result of this study using catechin-PPO in a model system demonstrated that CERPs at the early stage of the reaction exhibited a high level of antioxidant activity but the activity decreased as the oxidation time increased. Likewise, the early stage reaction products showed a high free radical scavenging activity and high lipoxygenase inhibitory effect. In the tyrosine-enzyme system, a similar pattern of the antioxidant activity was observed. Although the additional antioxidant activity derived from the residual catechin in CERPs cannot be disregarded; it appears that dimers or trimers of catechin, which are the predominant reaction products at the early stage of the enzyme catalyzed browning reactions (*26, 27*), made the most contribution to the antioxidant activity. A similar observation was made on inhibitory effect of CERPs on polygalacturonase (*35*) which dimers and trimers showed a higher inhibitory effect. This antioxidant activity of the CERPs may be explained by their ability to donate a hydrogen atom, free radical scavenging, and lipoxygenase inhibition. However, the exact mechanism of the antioxidant activity of CERPs cannot be explained until their chemical structures are elucidated. Further research in this area is deemed necessary.

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Chapter 17

Mechanisms of Some Reducing Compounds That Inactivate Polyphenol Oxidases

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Action of polyphenol oxidase (PPO) on substrates results in decrease of activity, some substrate reaction inactivation, some loss of copper from the active site of PPO, and modification of one or more of the histidine residues that ligand the copper. Incubation of PPO with ascorbate, especially in presence of added copper, results in decrease in activity and release of copper from the enzyme with some loss of histidine and methionine residues and increase in aspartic acid, glutamic acid, alanine and glycine residues. Incubation of PPO with sodium bisulfite or thiol compounds results in decrease of enzyme activity, with release of copper.

Polyphenol oxidases catalyze the o-hydroxylation of monophenols to o-dihydroxyphenols (Equation 1) and the oxidation of o-dihydroxyphenols to o-benzoquinones (Equation 2). The reactions in Equations 1 and 2 are often

$$p\text{--Cresol} \quad\quad 4\text{--Methylcatechol} \tag{1}$$

$$\text{Catechol} \quad\quad o\text{--Benzoquinone} \tag{2}$$
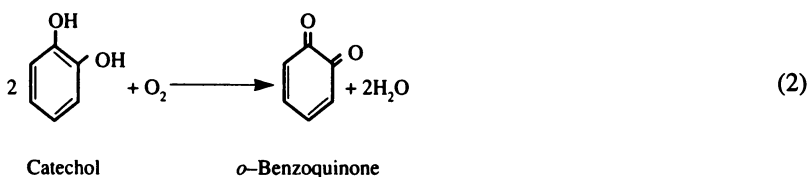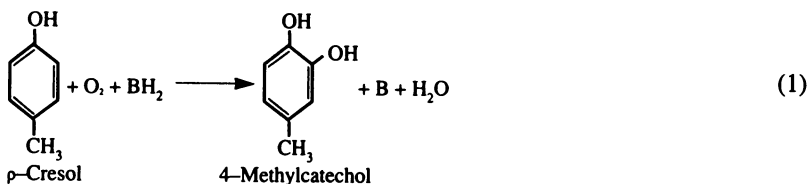
referred to as the cresolase- and catecholase-type reactions, respectively. The rates of the catecholase reactions are generally much faster than the rates of the cresolase activities. Peach (*1*), pear (*2*) and banana (Lerch, K., Givaudan-Roure Research Ltd., Dübendorf, Switzerland, personal communication, 1994) PPOs do not have cresolase activity. Ascorbate, sodium bisulfite and thiol compounds prevent browning of foods caused by polyphenol oxidase (PPO) action on mono- and *o*-dihydroxyphenols. Previous studies have determined that the above compounds prevent initial enzymatic browning by reducing the product, *o*-benzoquinone, back to the original *o*-dihydroxyphenol, thereby preventing brown pigment (melanin) formation until all the reducing compound is oxidized. More recently, it has been demonstrated that these reducing compounds, including substrates, have a direct effect on PPO activity. This paper presents data to substantiate the proposed mechanisms of direct action of the reducing compounds on PPO.

Substrate Conversion to Product and Polyphenol Oxidase Inactivation

Polyphenol oxidases undergo reaction inactivation (*3-8*). The inactivation of mushroom PPO as a result of catalytic oxidation of pyrocatechol is shown in Figure 1 (*8*). The rate of inactivation of PPO is dependent on the concentration of substrate, the specific nature of the substrate (*8*) and the PPO concentration (*9*). Under the reaction conditions used in Figure 1, there is rapid browning of reaction and incorporation of 85 mol of ^{14}C-labeled product (from U-^{14}C-labeled phenol as substrate) per mol of mushroom PPO subunit (32.0 KDa) (*8*). When the same reaction is performed in the presence of 128 mM ascorbate to prevent browning, only 0.04 mol of ^{14}C-labeled product per mol of PPO subunit was covalently bound to the enzyme, even after 24 h (*8*). A similar value was reported by other workers for *Neurospora* PPO reacted under similar conditions (*7*). In addition, there was loss of one histidine residue and one copper in *Neurospora* PPO (*7*); a similar result was found for mushroom PPO (Osuga, D. T., Golan-Goldhirsh, A., Whitaker, J. R., University of California at Davis, unpublished results). Lerch (*10*) concluded that modification of the histidine residue (His 306) in *Neurospora* might be the result of a highly reactive species originating from O_2, perhaps OH·. On the other hand, Golan-Goldhirsh and Whitaker (*8*) suggested that a semibenzoquinone radical, as an intermediate in the normal conversion of *o*-dihydroxyphenol to product, might be responsible for modifying a histidine residue in mushroom PPO (Equation 3). Such an intermediate has not been shown by ESR studies, either because the free radical is

$$2 \quad \text{OH, OH} \quad + 1/2\, O_2 \xrightarrow[H_2O]{PPP} 2 \quad \text{OH, O·} \quad \xrightarrow[H_2O]{PPO; 1/2\, O_2} 2 \quad \text{O, O} \tag{3}$$
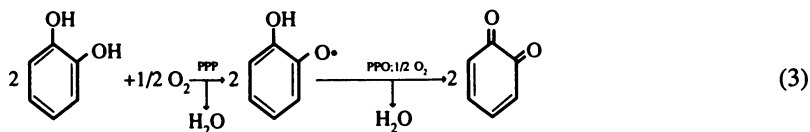
delocalized over the two copper atoms in the active site or because the proposed semibenzoquinone radical is rapidly converted to *o*-benzoquinone and never reaches a detectable level. Trapping experiments are futile since intermediates are formed in the closed active site and are not dumped into the bulk medium.

Spin resonance stabilization NMR techniques have shown that semiquinones are formed during the polyphenol oxidase-catalyzed reaction (*11, 12*). Sugumaran *et al.* (cited in Ref. *13*) have observed free radicals in insect cuticle formation.

Reducing Compounds and Polyphenol Oxidase Inactivation

Ascorbate, sodium bisulfite and thiol compounds slow down or prevent initial browning of foods and model substrate systems containing polyphenol oxidases. Most workers have considered the observed effect of these compounds on rates of PPO-catalyzed reactions to be due to a fast nonenzymatic reduction of the quinones formed back to the colorless o-dihydroxyphenolic substrates (Equation 4) (*14, 15*). However, there is substantial data to show that ascorbate, sodium bisulfite and thiol compounds also have a direct effect in inactivating PPO (*9*).

reductant ———   ——— *o*-quinone ◄———   ———►H$_2$O
(AH$_2$)

          nonenzymic   enzymatic   (4)

oxidant ◄———   ———► *o*-dihydroxyphenol   ——— ½ O$_2$
(A)
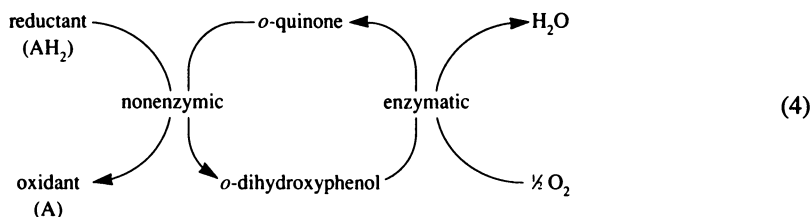
Ascorbate and Polyphenol Oxidase Inactivation.   Polyphenol oxidase is inactivated directly by ascorbate, as shown in Figure 2.   There is a decrease in mushroom PPO activity remaining, as measured by rate of O$_2$ uptake (polarographically) when 5 mM ascorbate and 7.1 μM PPO, at pH 6.5, are incubated together at 25 °C (*8*).   Ascorbate is not as effective as 0.5 mM pyrocatechol in inactivating PPO.   Ascorbate and pyrocatechol together incubated with PPO had even more effect on inactivation of PPO, even though there was no browning. Inactivation of mushroom PPO by substrate and ascorbate combined is quite effective even before there is any browning as shown in Figure 3 (*9*). The lag period is proportional to added ascorbate concentration and the extent and rate of browning after all the ascorbate is used up decreases as a function of the ascorbate concentration.
     It is not necessary to have substrate present in order for ascorbate to inactivate mushroom PPO (Figures 2 and 4C) (*8, 9*). The kinetics of inactivation of PPO by ascorbate is sigmoidal, unlike the effects of dithiothreitol, glutathione and sodium bisulfite (Figure 4) (*9*). This is because dehydroascorbate (formed during incubation, or an intermediate compound) is more effective in inactivating PPO than is ascorbate (see next paragraph). Ascorbate is less effective than dithiothreitol, sodium bisulfite and glutathione in inactivating PPO in the absence of Cu^{2+} (Table I). However, added Cu^{2+} markedly increases the rate of inactivation of PPO (*16*).
     Inactivation of mushroom PPO by ascorbate requires O$_2$ (Table II) (*16*) and is facilitated by Cu^{2+} (*16*). When the reaction is performed under N$_2$ there is no inactivation of PPO after 60 min, in contrast to 72% loss of activity when the same reaction is carried out in the presence of air (Table II). The rate of inactivation of PPO is dependent on Cu^{2+} concentration up to 10 μM, but the rates were identical at 10, 20, 100 and 500 μM Cu^{2+} (*16*). However, there appeared to be no saturation with ascorbate concentration up to 20 mM (*16*).
     Incubation of mushroom PPO with ascorbate in the presence of Cu^{2+} and air at 25 °C for 22 h led to a major decrease in the histidine content (4.2 to 1.1 residues per 32.0 KDa subunit) and some decrease in methionine content (Table III). There is an increase in aspartic acid, glutamic acid, alanine and glycine content of the treated PPO. There is also an increase in urea content (similar to the increase in aspartic
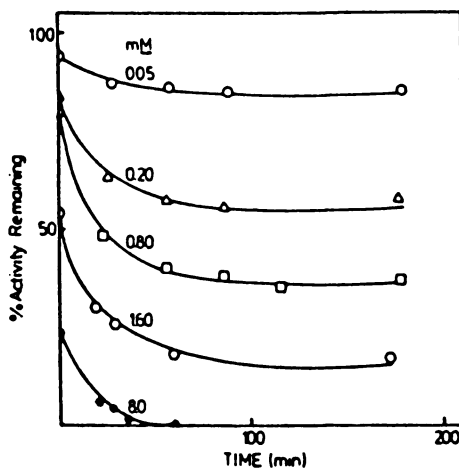
Figure 1. Effect of pyrocatechol concentration on rate and extent of inactivation of mushroom polyphenol oxidase. The reactions, with 7.1 µM enzyme in 0.1 M phosphate buffer, pH 6.5, and pyrocatechol at 25 °C, were followed polarographically. (Reproduced with permission of Ref. *8*. Copyright 1985 Elsevier Sequoia.)
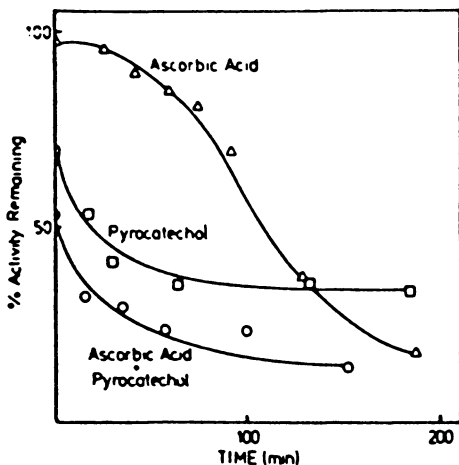
Figure 2. Effect of ascorbate, pyrocatechol and ascorbate + pyrocatechol on rate and extent of inactivation of mushroom polyphenol oxidase. Reaction conditions as in Figure 1 except for △, 5 mM ascorbate (no substrate); □, 0.5 mM pyrocatechol; and ○, 0.5 mM pyrocatechol and 5 mM ascorbate. (Reproduced with permission from Ref. *8*. Copyright 1985 Elsevier Sequoia.)
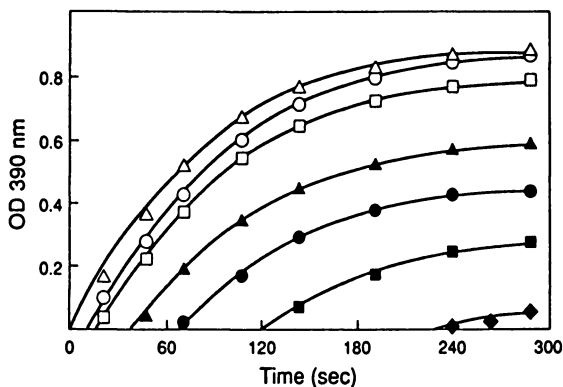
Figure 3. Effect of ascorbate on mushroom polyphenol oxidase oxidation of pyrocatechol. Concentrations of ascorbate (from left to right): 0, 0.1, 0.2, 0.3, 0.5, 0.7 and 0.8 mM. The 1 mL reaction mixture contained 0.1 M sodium phosphate buffer, pH 7.1, 5 mM pyrocatechol, 14 μg PPO and the indicated concentrations of ascorbate, at 23 °C. (Reproduced with permission from Ref. 9. Copyright 1984 American Chemical Society.)

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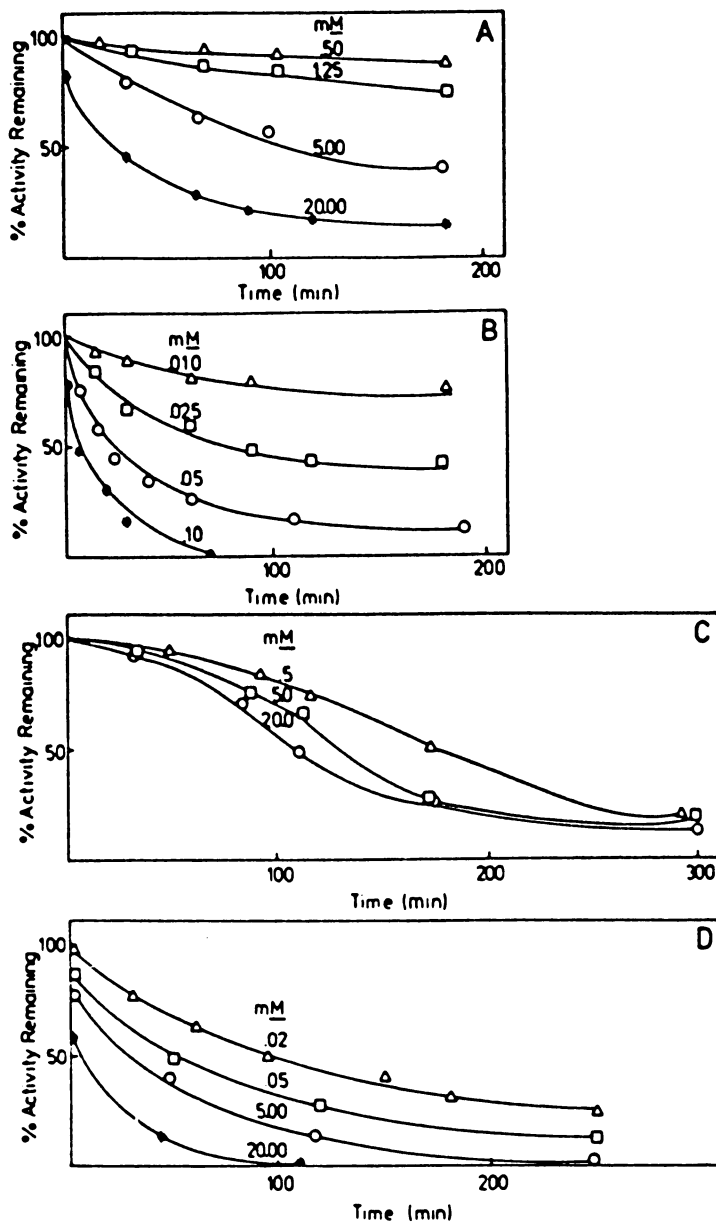
Figure 4. Rate of inactivation of mushroom polyphenol oxidase when incubated alone with reductants in 0.1 M phosphate buffer, pH 6.5, 0.96 mM EDTA and 0.2 µg/ml PPO at 25 °C. A, Glutathione; (reduced); B, dithiothreitol; C, ascorbate; D, sodium bisulfite. (Reproduced with permission from Ref. *9*. Copyright 1984 American Chemical Society.)

Table I. Effect of Reductants on Polyphenol Oxidase (PPO) Inactivation[a]

Reductant	$t_{0.5}$ (min)
Dithiothreitol (0.1 mM)	8
Sodium bisulfite (5 mM)	28
Glutathione (5 mM)	106
Ascorbate (5 mM)	130

[a]Adapted from Ref. 9. Activity loss determined polarographically; 6.3 μM mushroom polyphenol oxidase, in 0.11 M phosphate buffer, pH 6.5, 25 °C.

Table II. Requirement of O_2 for Polyphenol Oxidase (PPO) Inactivation by Ascorbate and Copper

Condition[a]	Incubation time (min)	Activity remaining (%)
Aerobic		
PPO	1	100
PPO, ascorbate/Cu^{2+},[b]	1	100
	60	28
Anaerobic[b,c]		
PPO, ascorbate/Cu^{2+}	1	100
	60	100
	114[d]	30

[a]Adapted from Ref. 61. 7.1 μM Mushroom polyphenol oxidase in 0.11 M phosphate buffer, pH 6.5 and 25 °C.

[b]5 mM Ascorbate, 0.8 mM $CuSO_4 \cdot 5H_2O$.

[c]All solutions were deaerated thoroughly under vacuum before mixing and the system was continually flushed with N_2.

[d]After 60 min under N_2, aerobic conditions restored.

Table III   Amino Acid Composition[a] of Polyphenol Oxidase Incubated with Ascorbate, Cupric Sulfate, or Ascorbate-Cupric Sulfate

Amino acid	Untreated	Ascorbate treated[b]	Cupric sulfate treated[c]	Ascorbate-Cu^{2+} treated[d]
Asx	27.4	27.8	26.9	33.9
Glx	26.9	27.0	26.4	31.2
Gly	19.9	20.9	20.5	23.3
Ala	20.9	21.8	21.6	23.8
Met	1.5	3.1	2.4	0.9
His	4.2	4.0	4.3	1.1

[a]Moles of amino acid residue per subunit (32.0 KDa) of PPO.
[b]19.0 μM protein and 5 mM ascorbic acid in 0.1 M sodium phosphate buffer, pH 6.5, at 25 °C for 22 h in a gyrotory water bath shaker.
[c]19.0 μM protein and 0.8 mM cupric sulfate; other conditions as in "a".
[d]19.0 μM protein, 5 mM ascorbic acid, and 0.8 mM cupric sulfate; other conditions as in "a". Adapted from Ref. *16*.

acid; determined by urease (*16*). Similar products are given by ascorbate or photo-oxidation of histidine derivatives (*17*).

Mechanistic proposals for the effect of ascorbate and Cu^{2+} on degradation of histidine and histidine derivatives include free-radical intermediates (*18, 19*). Based on our own research and other research (*18, 19*), including the ability of histidine derivatives and ascorbate to specifically bind Cu^{2+} (*20-22*), we have proposed (*16*) that the reactive system leading to degradation of histidine residues in proteins is the result of a specific quaternary complex between the imidazole group of histidine, Cu^{2+}, ascorbate and O_2, leading to formation of semidehydroascorbate radical (and possibly other radicals as shown in Figure 5). The products of histidine degradation by a free radical reaction can be accounted for as shown in Figure 6. We have shown by ESR that free radicals are produced in ascorbate/Cu^{2+} systems containing PPO, but we have not identified their specific nature (Osuga, D. T., Whitaker, J. R., University of California at Davis, unpublished results).

Thiol Compounds and Polyphenol Oxidase Inactivation. Dithiothreitol and glutathione in the absence of substrate directly inactivated mushroom PPO when incubated at pH 6.5 and 25 °C (Figure 4; Table I). Dithiothreitol is about 660 times more effective than glutathione in inactivating PPO, due to its dithiol nature, higher redox potential and perhaps less steric hindrance. Incubation of mushroom PPO (5.5 mg) with 2 mM dithioerythritol (DTE) at pH 6.5 in 0.1 M phosphate buffer containing 1 mM EDTA at 25 °C resulted in rapid inactivation of mushroom PPO (Figure 7B; enzyme purified from mushrooms in our laboratory). After 100 min incubation, the activity left was ~10% of the original. A sample of the 100 min-treated PPO was purified from the reaction media on Sephadex G-25 (Figure 7A). The activity of the purified PPO was ~5% of the original activity. No sulfhydryl groups were detected in the PPO by the Ellman method (*23*). The copper content of the DTE treated and purified PPO was 16% of the original PPO, determined by digestion (*24*) of the purified and control PPO and determination of the copper

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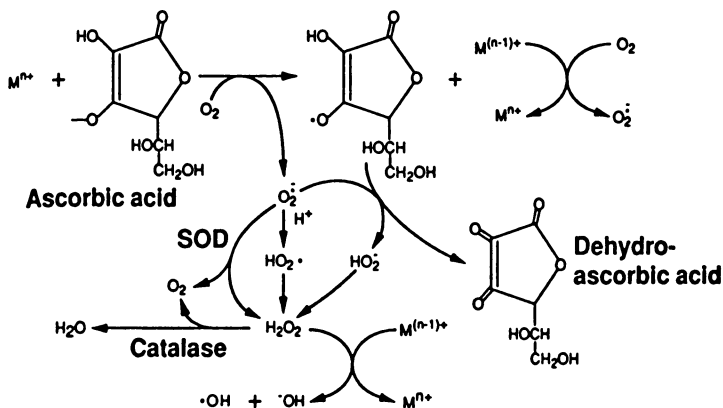
Figure 5. Formation of free radicals during reaction of ascorbate, metal ions and O_2. (Reproduced with permission from Ref. *19*. Copyright 1986 Japan Society for Agricultural Biology and Chemistry.)
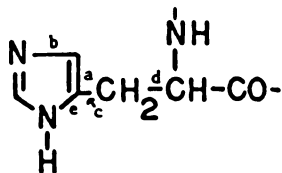
Figure 6. Proposed scission of histidine residues by ascorbate/ Cu^{2+} to give aspartic acid (a-e), glutamic acid (b-e), alanine (c), glycine (d) and urea (a-e).
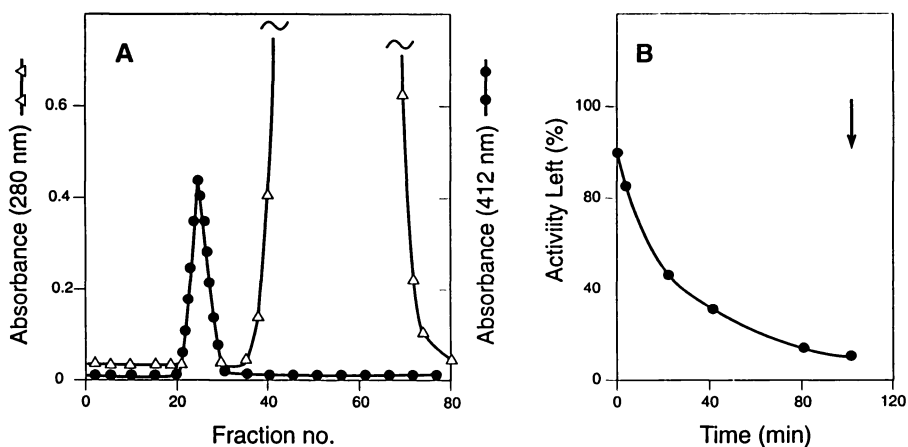
Figure 7. Rate of loss of mushroom polyphenol oxidase activity following treatment with dithioerythritol (B), and purification of the treated PPO on Sephadex G-25 (A). (See text for experimental details.)

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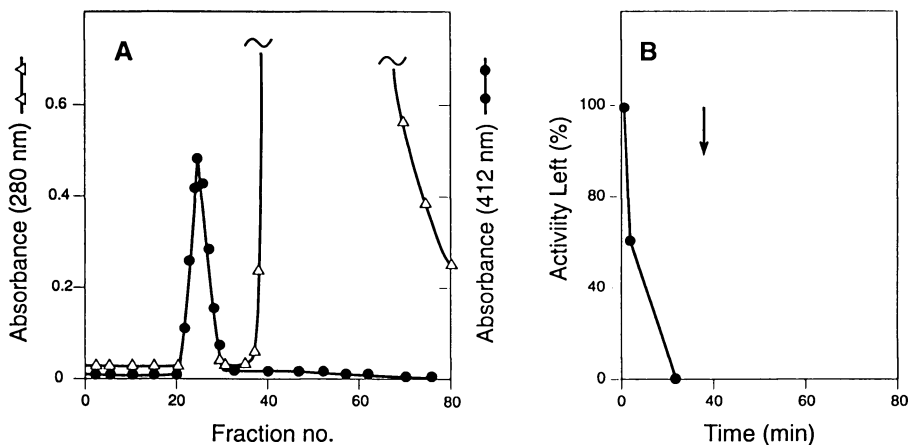
Figure 8. Rate of loss of mushroom polyphenol oxidase activity following treatment with sodium bisulfite (B), and purification of the treated PPO on Sephadex G-25 (A). (See text for experimental details.)
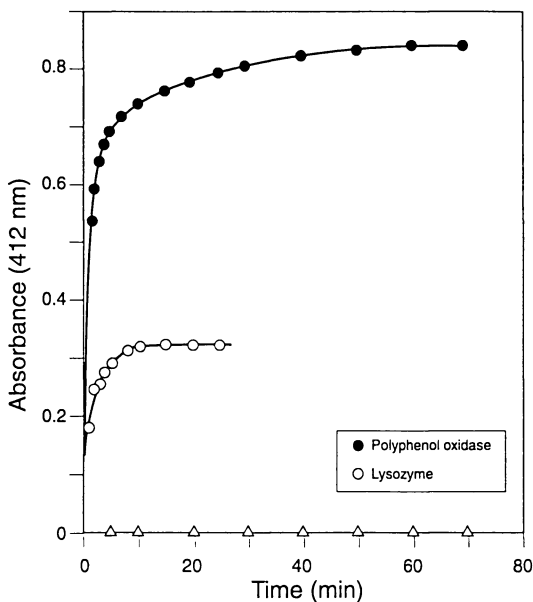
Figure 9. Sulfhydryl groups of sodium bisulfite-treated native mushroom polyphenol oxidase (Δ) and guanidine thiocyanate-denatured PPO (●) and lysozyme (○), following treatment with Ellman's reagent. (See text for experimental details.)

content was by atomic absorbance (*25*). An aliquot of the DTE-treated and purified PPO incubated with ~20 fold excess of $CuSO_4$ for 176 min (*26*). The PPO regained 92% of its original activity.

Treatment of PPO with dithiothreitol (DTT) as described above for DTE gave essentially the same results. Therefore, we conclude that inactivation of PPO by DTE and DTT is due to removal of copper from the enzyme, as shown by restoration of essentially full activity on incubation with $CuSO_4$. No sulfhydryl groups could be detected in the PPO, even when the experiment was carried out under nitrogen, indicating that reduction of disulfide bonds was not responsible for inactivation. Incubation of the treated and purified PPO in air with trace amounts of RSH gave essentially no regain of activity.

Sodium Bisulfite and Polyphenol Oxidase Inactivation. Sodium bisulfite rapidly inactivates mushroom PPO (Figure 4, Table I). To determine the basis of the inactivation, 5.5 mg of mushroom PPO (prepared in our laboratory) was incubated with 20 mM $NaHSO_3$ in 0.1 M phosphate buffer, pH 6.5, at 25 °C. There was a very rapid loss of activity (Figure 8B). The 40 min-treated PPO was purified on Sephadex G-25 (Figure 8A) as described for the DTE-treated PPO (Figure 7A). The treated and purified PPO had ~3% of the original activity. There was no detectable sulfitolysis of the disulfide bonds (Figure 9), as determined by the Thannhauser *et al.* (*27*) method and by the Ellman method (*23*). The disulfide bonds of PPO and of lysozyme (for comparison) could be reduced by DTE and sulfitolyzed by $NaHSO_3$ only after denaturation of the proteins by 2 M guanidine thiocyanate at room temperature (Figure 9).

The $NaHSO_3$-treated and purified PPO had no copper (*25*). Incubation with copper as described above for the DTE-treated sample resulted in a regain of ~9% of the PPO activity. While most of the activity was not restored, in contrast to the case of the DTE-treated PPO, the evidence appears to indicate that loss of PPO activity due to $NaHSO_3$ treatment is due to loss of copper and not due to sulfitolysis or reduction of disulfide bonds of PPO.

Summary

Mushroom polyphenol oxidase is inactivated during conversion of substrates to products and by ascorbate, sodium bisulfite and by dithioerythritol and dithiothreitol. Substrate inactivation of PPO (reaction inactivation; k_{cat} mechanism) leads to loss of histidine residues and copper from the active site. Based on the nature of the products from histidine degradation, it is suggested that a free radical intermediate is produced during the reaction. Ascorbate, especially with low levels (1-10 μM) of copper, aerobically destroys histidine residues of PPO, releasing copper, by a free radical mechanism, giving products similar to those produced by substrate reaction inactivation. Sodium bisulfite and thiol compounds appear to reduce active site Cu^{2+} to Cu^+, which is more readily lost from PPO. One or more of these reactions might be used to irreversibly inactivate PPO, thus permanently preventing browning of food products.

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Chapter 18

Prevention of Enzymatic Browning in Prepeeled Potatoes and Minimally Processed Mushrooms

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Some minimally processed products do not respond well to treatment with browning inhibitors. Peeled potato surfaces are highly reactive because of thermal and mechanical damage during peeling. Digestion with 17% NaOH or 1-2% ascorbic and citric acids at elevated temperatures, followed by treatment with browning inhibitors, can extend potato storage life to 12-14 days. Treatment conditions must be designed to avoid textural defects and nonenzymatic discoloration. Washed mushrooms discolor due to reactions induced by bacterial growth as well as to typical enzymatic browning. Treatment with hydrogen peroxide prior to application of browning inhibitors will suppress bacterial spoilage and improve appearance. Clarified raw apple juice will not brown due to removal of particulate-bound polyphenol oxidase but may become yellow during storage because of nonenzymatic browning of added ascorbic acid. Effective treatment conditions for these products and guidelines for controlling enzymatic browning in other minimally processed commodities are discussed.

One of the major problems that limits the development of minimally processed fruits and vegetables is the occurrence of enzymatic browning at cut and peeled surfaces (1, 2). With many commodities, this problem is especially difficult because of the need to control browning without use of sulfite, which has been banned by the Food and Drug Administration for most fresh applications (3). Our laboratory at the USDA's Eastern Regional Research Center in Philadelphia has conducted research on preservation of minimally processed fruits and vegetables for a number of years. Much of our work has focused on development of methods to control enzymatic browning in apples (4, 5), potatoes (6, 7), mushrooms (8), and fresh juices (9). A wide variety of browning inhibitors, including various polyphenol oxidase inhibitors, reducing agents, acidulants, complexing agents, and proteolytic enzymes have been described at this symposium. In our experience, some products such as sliced apples

respond well to treatment with relatively simple browning inhibitor formulations, i.e., sodium erythorbate in combination with calcium chloride (5). On the other hand, browning of pre-peeled potatoes (6), washed mushrooms (8), and some raw juices (9) cannot be controlled by such treatments because of their unique physical and chemical characteristics, the contribution of other factors such as bacterial growth and non-enzymatic browning to the discoloration process, and the occurrence of treatment-induced defects.

Our experience with these products demonstrates the need to take a broad perspective in dealing with discoloration reactions in minimally processed fruits and vegetables. In this paper, some of the problems we encountered in controlling browning of potatoes, mushrooms, and fresh juices; the product characteristics that interfered with treatment; and approaches that were found to be effective in preventing discoloration will be described. Based on these case studies, some guidelines for treatment development that may be applicable to other commodities will be presented.

Pre-peeled Potatoes

Industry Needs. In many locations throughout the United States, packers provide peeled raw potatoes to restaurants, other food service establishments, producers of french fries and potato chips, and other food processors (10). Without browning inhibitor treatment, pre-peeled raw potatoes would discolor within minutes, turning pink, brown, gray, or black. Use of sulfites to control discoloration, which was considered to be highly effective, was banned by the FDA in 1990 (11). This ban is not now in effect, however, because of successful legal challenges (12). The pre-peeled potato industry would like to be able to control browning for 14 days. Efforts to control browning of peeled surfaces with sulfite substitutes, usually formulations of ascorbic or erythorbic acid with such adjuncts as citric acid, sodium acid pyrophosphate and cysteine, have not yet met this requirement. Published shelf-life data for commercial sulfite substitutes indicate that a shelf-life no greater than 1 week can be expected unless the product is vacuum packed or stored submerged in a preservative solution (13-15). We obtained similar results with a "conventional" formulation containing 4% ascorbic acid, 1% citric acid, and 1% sodium acid pyrophosphate (7). We had no greater success with experimental dips containing ascorbic acid in combination with acidic polyphosphates or cyclodextrins (Sapers, G. M., Eastern Regional Research Center, Philadelphia, unpublished data). Combinations of ascorbic acid-2-monophosphate and ascorbic acid-2-polyphosphates showed some promise (6), but these compounds are expensive and would require FDA approval. Our goal was to control browning in pre-peeled potatoes for two weeks, using only FDA-approved browning inhibitors. To achieve this goal, we would have to overcome the tendency of pre-peeled potatoes to undergo rapid and severe browning.

Basis for Instability of Pre-Peeled Potatoes. Data obtained in 1989 with Russet Burbank potatoes demonstrated that the method of peeling had a large effect on the

extent of browning at the peeled surface (*16*). In this experiment, browning during storage was quantified by measuring the decrease in L-value at the peeled surface with a tristimulus colorimeter. The decrease in L-value was greater with steam or abrasion peeled potatoes than with potatoes peeled with a sharp knife (Table I). We attributed this result to extensive mechanical damage in cell layers at the surface of abrasion peeled potatoes, and to thermal injury that causes membrane leakage in cells at the surface of steam peeled potatoes.

Table I. Effect of Peeling on Browning in Russet Burbank Potatoes During Storage at 4 °C

Peeling Method	ΔL at 6 hr[a]
Knife	-9.7[c]
Steam	-36.2[e]
Abrasion	-18.7[d]
Control[f]	-5.4[bc]

Source: Adapted from ref. 16

[a] $\Delta L = L_{6h} - L_{initial}$ at peeled or control surface.
[b-e] Means within columns, followed by different superscripts, are significantly different at $P < 0.05$ by the Bonfessoni LSD test.
[f] Control is transversely cut surface of same potato plug for which peeled surface is measured.

These results and work done with lye peeled potatoes in the 1950's by Harrington and co-workers at the USDA's Western Regional Research Center (*17*) suggested that removal of unstable tissue at the peeled surface might make pre-peeled potatoes more responsive to treatment with browning inhibitors.

Digestion Treatments for Pre-peeled Potatoes. One of our approaches was to use the low temperature, lye digestion treatment developed by Harrington to remove surface tissues from abrasion, steam or lye peeled potatoes, and then, to treat the new surface with our ascorbic acid-based browning inhibitor dip (*7*). The effectiveness of this two-stage treatment was established by determining the extent of browning with a tristimulus colorimeter and then calculating a parameter we call the percent inhibition from the measured L- and a-values (*18*). Percent inhibition values approaching 100% would indicate that a treatment was highly effective in controlling browning, while values of 50-60% or lower would indicate treatment failure. Percent inhibition data obtained with high pressure steam peeled potatoes (Table II) demonstrated the effectiveness of the experimental lye digestion treatment, used in conjunction with a browning inhibitor dip. Both Russet and round-white

types of potatoes, given this treatment, were still acceptable after 13-15 days at 4 °C, while controls, given only the browning inhibitor dip, failed within 3-6 days. Addition of nonionic or anionic detergents to the digestion solutions did not significantly improve treatment effectiveness. Similar results were obtained with abrasion peeled potatoes (7).

Table II. Effect of Digestion with NaOH and Tween 80 on Browning in High Pressure Steam-Peeled Potatoes During Storage at 4 °C

		Percent Inhibition			
Potato	Treatment[a,b]	3	6	10	13
Russet	None	2[d]	2[d]	-9[d]	-29[d]
	NaOH	90[c]	82[c]	69[c]	51[c]
	NaOH + Tween 80	93[c]	90[c]	79[c]	67[c]
Round-white	None	86[c]	20[d]	6[d]	6[d]
	NaOH	92[c]	89[c]	80[c]	73[c]
	NaOH + Tween 80	94[c]	92[c]	86[c]	81[c]

Source: Adapted from ref. 7

[a] 4 min at 49 °C in 17% NaOH.
[b] Digested samples and undigested controls dipped 5 min in browning inhibitor solution containing 4% ascorbic acid, 1% citric acid, 1% sodium acid pyrophosphate, and 0.2% calcium chloride.
[c-d] Means within columns for each potato type, followed by different superscripts, are significantly different at $P<0.05$ by the Bonferroni LSD test.

The digestion treatment did result in a weight loss of 15-25% because of removal of digested tissue and also created a potential waste disposal problem, namely, disposal of spent lye and digested potato tissue. For these reasons, we investigated alternative digestion treatments that also might remove or, in some way, neutralize unstable tissue at the peeled surface but be less costly and more environmentally benign.

Digestion with Hot Ascorbic Acid/Citric Acid Solutions. Preliminary attempts at enzymatic digestion suggested another approach (Sapers, G. M.; Miller, R. L., Eastern Regional Research Center, Philadelphia, unpublished data). The enzyme treatments themselves were ineffective, but immersion of peeled potatoes in water heated to 55 °C for 15 minutes, followed by application of a browning inhibitor dip, showed some promise. Better results were obtained when ascorbic and

citric acids were added to the hot water (Table III). Apparently, "digestion" with the two acids at an elevated temperature resulted in partial inactivation of polyphenol oxidase as well as leaching of the enzyme and its substrates at the peeled surface. Thus, the peeled surface was less reactive, and browning could be controlled by ascorbic acid and other browning inhibitors, applied in the final dip. Abrasion peeled Russet and round-white potatoes, given both the hot ascorbic/citric acid digestion treatment and conventional ascorbic acid-based browning inhibitor dip, showed significantly less browning than potatoes given only the browning inhibitor dip (Figure 1). A shelf-life of about two weeks was obtained with both Russet and round-white potatoes, given the combination treatment (*19*).

Table III. Effect of Digestion and Browning Inhibitor Treatments on Browning of Abrasion Peeled Russet Potatoes at 4 °C.

Digestion[a]	Browning Inhibitor Dip[b]	ΔL^c			Appearance[g]		
		Days			Days		
		5	9	12	5	9	12
None	No	-22.1[f]	-23.0[f]	-23.6[f]	+++	+++	++++
None	Yes	-2.2[e]	-6.8[e]	-8.3[e]	++	++/+++	+++
1% AA+ 1% CA	Yes	3.0[d]	2.4[d]	1.2[d]	-	-	-/+++
1% AA+ 2% CA	Yes	2.4[d]	1.6[d]	0.0[d]	-	-	-
1% AA+ 2% CA+ 1% SAPP	Yes	0.9[d]	0.8[d]	-1.2[d]	-	-	+

Source: Adapted from ref. 19

[a] 15 min at 55 °C. AA=ascorbic acid; CA = citric acid; SAPP = sodium acid pyrophosphate.
[b] 5 min dip in 4% AA + 1% CA + 1% SAPP + 0.2% $CaCl_2$.
[c] $\Delta L = L_{storage} - L_{initial}$ at peeled surface.
[d-f] Means within columns, followed by different superscripts are significantly different at $P<0.05$ by the Bonferroni LSD test.
[g] Severity of discoloration: ++++, very severe; +++, severe; ++, moderate; +, slight; -, none.

Use of the ascorbic acid/citric acid digestion treatment greatly improved the shelf-life of pre-peeled potatoes but introduced new problems that had to be addressed. One such problem was the occurrence of gray spots during storage of some digested potatoes. Graying appeared to be more prevalent when raw material showed softening, was bruised, or was subjected to overheating during digestion. Less graying was seen when potatoes were stored for several weeks at 20 °C, prior to peeling and treatment, rather than at 4 °C (*19*).
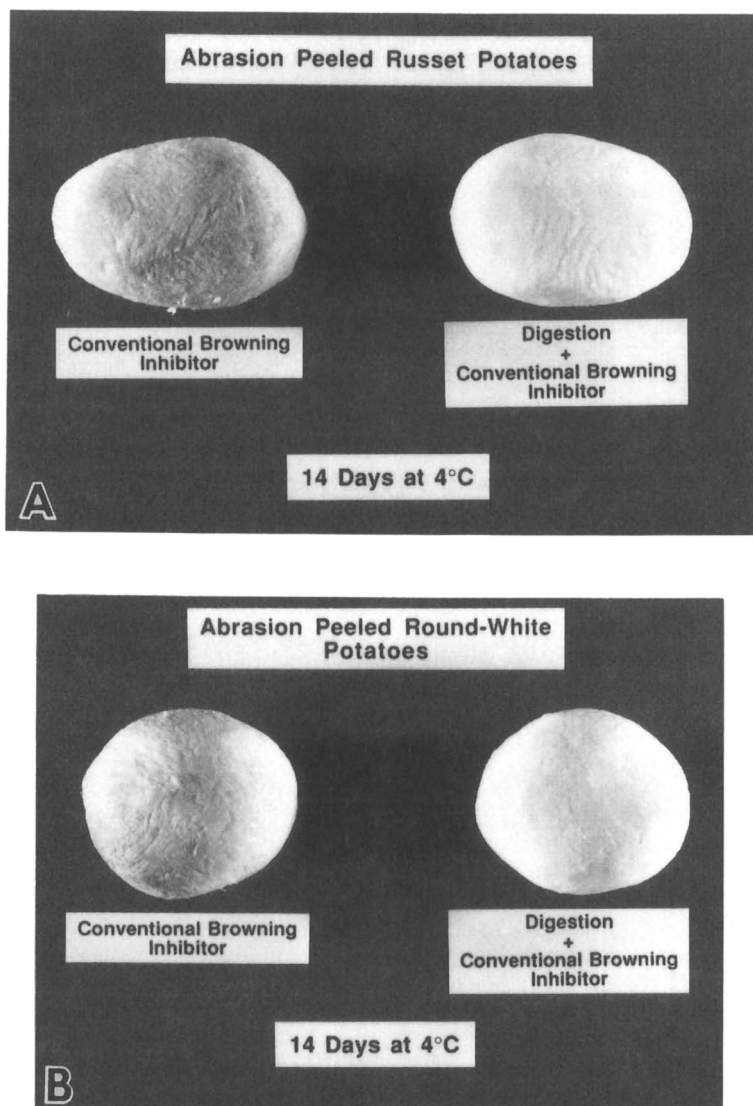
Figure 1. Pre-peeled potatoes digested in 1% ascorbic acid + 2% citric acid for 20 min at 45 °C and/or dipped 5 min in conventional browning inhibitor solution containing 4% ascorbic acid + 1% citric acid + 1% sodium acid pyrophosphate; samples stored at 4 °C for 14 days. A, abrasion peeled Russet; B, abrasion peeled round-white; C, high pressure steam peeled Russet; D, high pressure steam peeled round-white.
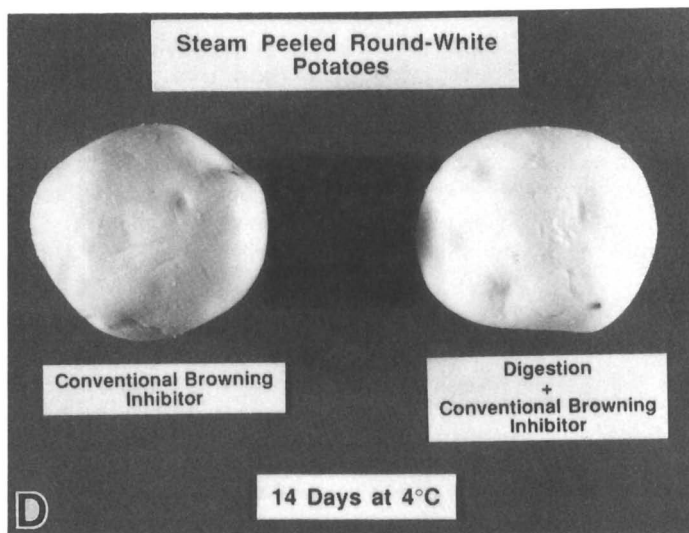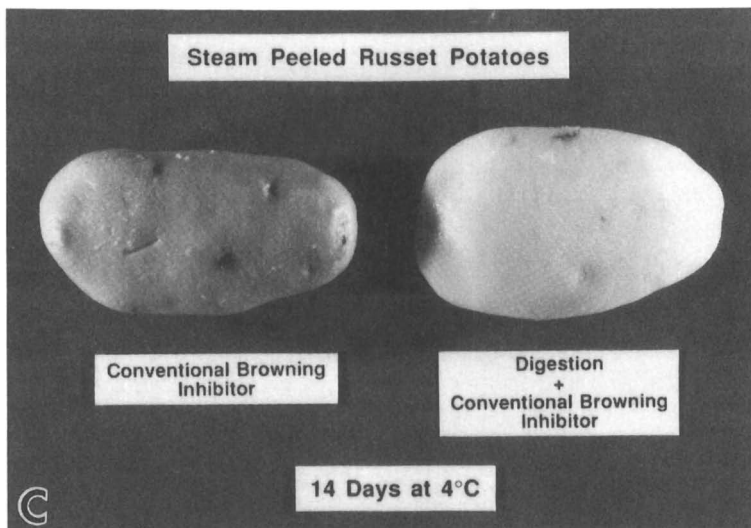
Figure 1. Continued.

A second defect associated with the digestion treatment was surface toughening, which became evident after cooking and sometimes produced a separated layer or interfered with mashing (Figure 2). Surface toughening may have resulted from activation of pectin methylesterase followed by cross-linking of liberated carboxyl groups by endogenous calcium ion (20). This defect was more severe when calcium chloride was added to the browning inhibitor dip and less severe when the digestion temperature and time were decreased and EDTA was added to the digestion solution. These modifications had little effect on product shelf-life (19).

A third defect, sometimes seen in steam peeled potatoes that had been digested in hot ascorbic/citric acid solution, was the development of browning at the peeled surface during or after cooking (Figure 3). This discoloration occurred only in samples that were near the end of their shelf-life, i.e., after storage at 4 °C for two weeks, but still showed little or no browning in the raw state. In experiments carried out to establish the cause of this defect, we found that browning was suppressed by cooling the cooked potatoes in a vacuum or by cooking potatoes in water containing sulfite. Browning could not be induced by application of 2% dehydroascorbic acid to the surface of fresh peeled potatoes prior to cooking. We had speculated that this compound might be involved since it accumulates when added ascorbic acid undergoes oxidation during storage (21) and will undergo nonenzymatic browning (22). Therefore, browning induced by cooking appears to be caused by accumulation of enzymatic browning intermediates during storage and prior to inactivation of polyphenol oxidase by heat. These intermediates probably undergo oxidation and condensation reactions during cooking and cooling to form melanin pigments. Abrasion peeled potatoes were not subject to this defect. This difference may be due to the greater content of polyphenol oxidase and its substrates at the steam peeled surface since these constituents are present in greater concentrations near the potato skin (23).

We believe that the hot ascorbic/citric acid digestion treatment represents a viable alternative to use of sulfites to control browning of pre-peeled potatoes. Further studies to scale up the treatment and establish its economic feasibility are planned. Our experience with potatoes clearly shows the complexity of the browning inhibition problem and the need to take a broad view in developing effective control measures.

Minimally Processed Mushrooms

Defects Induced by Washing. Our experience with minimally processed mushrooms illustrates the need to consider microbiological implications of browning inhibitor treatments as well as the occurrence of discolorations induced by treatment. Fresh mushrooms are subject to severe enzymatic browning during handling and storage but do not respond well to treatment with browning inhibitor dips since washing and application of dips greatly increase product perishability (8, 24). This is due largely to absorption of water during treatment which creates an internal environment favorable to bacterial growth. If mushrooms could be washed, treated with browning inhibitors, and sliced without excessive spoilage, their value would
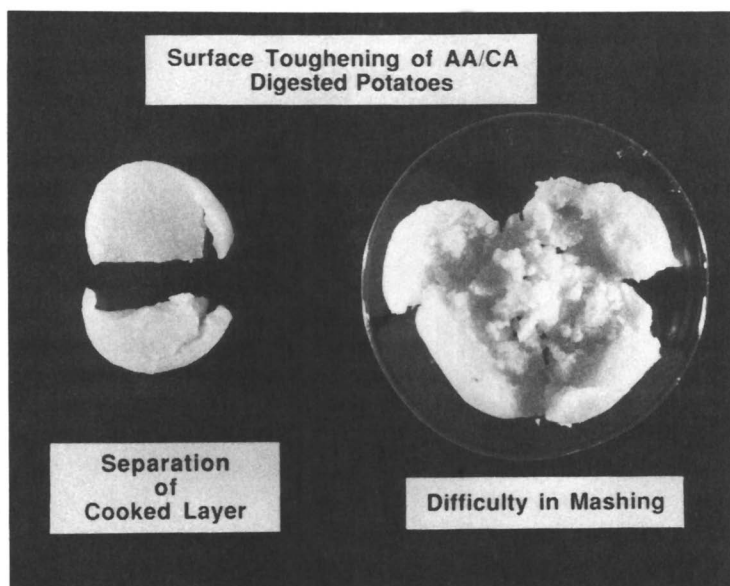
Figure 2. Surface toughening effects in cooked potatoes treated by ascorbic acid/citric acid digestion.
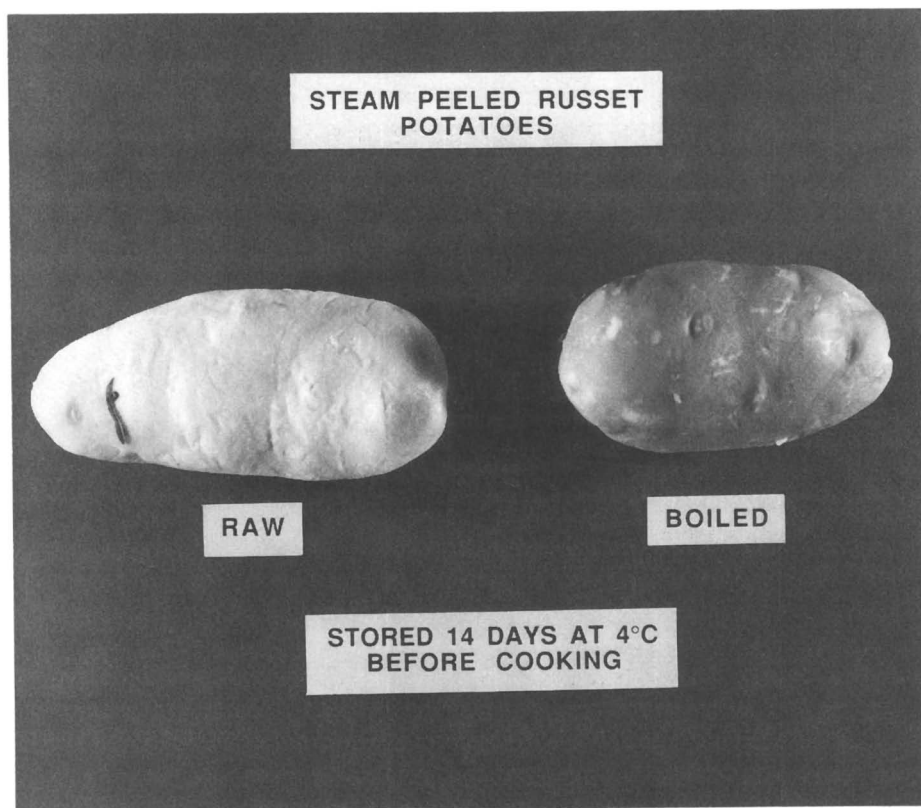
Figure 3. Browning induced by boiling steam peeled Russet potatoes, treated by ascorbic acid/citric acid digestion and stored for 14 days at 4 °C.

be enhanced because they could be used directly in salad bars, sauces, toppings for pizza or burgers, and other food service or consumer product applications.

Several years ago, we became involved with a company that attempted to produce a washed mushroom product and observed purple or gray-colored blotches on mushroom surfaces after 4-6 days of storage. Subsequently, these blotches evolved into sunken brown lesions similar to those produced by the mushroom pathogen, *Pseudomonas tolaasii*. Examination of the lesions by scanning electron microscopy revealed indications of mechanical damage to mushroom hyphae, probably caused by washing, and the presence of large numbers of bacteria (Figure 4). Blotch development could be suppressed by addition of antibiotics such as streptomycin.

The purple discoloration appeared to be related to enzymatic browning since it could be induced by application of a drop of L-DOPA solution to the surface of a washed mushroom. L-DOPA is a well known polyphenol oxidase substrate, found in mushrooms, which turns red and then black when applied to the surface of an unwashed mushroom.

Studies carried out in model systems (25) indicated that a stable purple compound could be produced by reaction of indole-5,6-quinone, a short-lived intermediate in the enzymatic browning of L-DOPA, with a quinone derived from sinapic acid, another phenolic compound found in mushrooms (Figure 5). These reactions might be triggered by injury to mushroom hyphae during washing and/or by metabolic activity of bacteria growing on the damaged surface.

Control of Bacterial Spoilage in Washed Mushrooms. In order to wash or apply browning inhibitor dips to fresh mushrooms without promoting purpling or spoilage, one must suppress the growth of spoilage bacteria. One approach was to reduce water uptake by modifying treatment conditions, for example, by shortening dipping times. A second approach was to reduce the bacterial load on mushroom surfaces. Chlorine, applied as sodium hypochlorite solution, is widely used as a bactericide. However, we found that browning was induced at bactericidal concentrations of chlorine (8). This appears to be due to direct nonenzymatic oxidation of polyphenol oxidase substrates to form enzymatic browning intermediates (26).

Hydrogen peroxide vapor has been used experimentally to control spoilage of table grapes (27). We found that treatment of fresh mushrooms by exposure to hydrogen peroxide vapor or by dipping in a dilute hydrogen peroxide solution could suppress spoilage, even in mushrooms inoculated with *Pseudomonas tolaasii* at levels as high as 3 million bacterial cells per mushroom cap (8, 28). Because mushrooms have a high level of endogenous catalase activity, residual hydrogen peroxide in treated samples was rapidly broken down to oxygen and water. Hydrogen peroxide vapor treatment did induce browning of mushrooms under some treatment conditions. This may have been the result of a peroxidase-catalyzed reaction. However, browning could be controlled by application of a browning inhibitor dip, immediately following the hydrogen peroxide treatment.
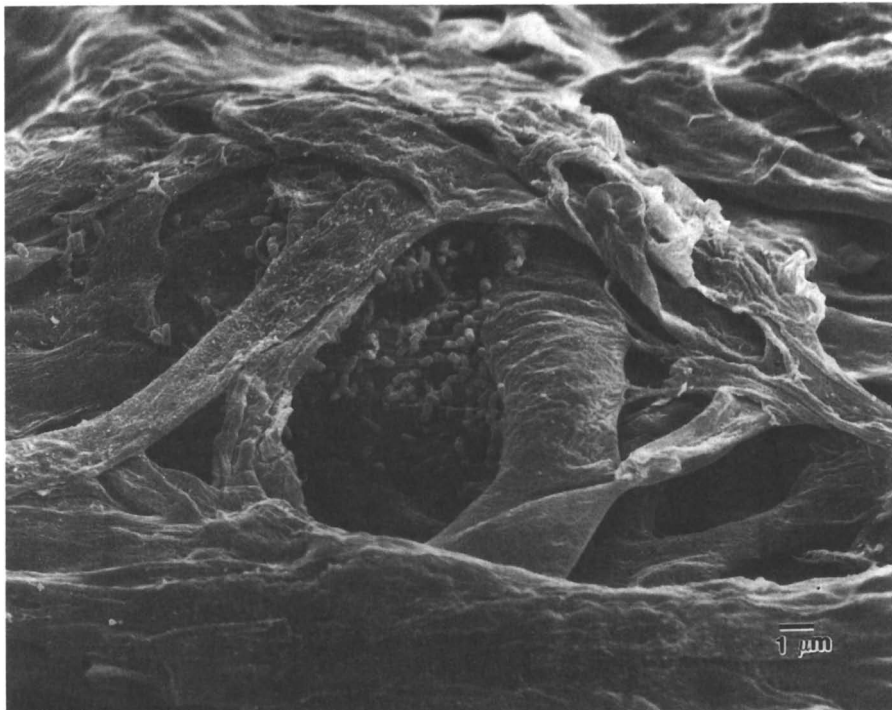
Figure 4.   Scanning electron micrograph of lesion on cap surface of washed
mushroom showing flattened hyphae and attached bacterial cells in interstices.
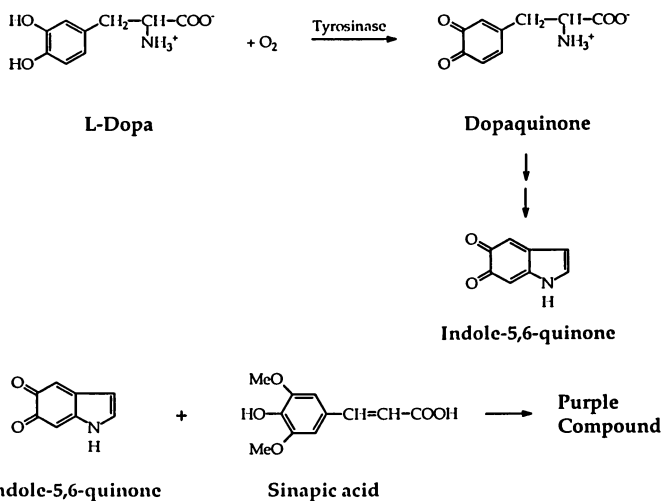
Figure 5.   Reactions involved in formation of purple compound in model system
representing washed mushrooms.

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Control of Enzymatic Browning in Mushrooms. In the course of our research on minimally processed mushrooms, we tested a number of browning inhibitors and combinations of browning inhibitors with antimicrobial compounds (Table IV). Many of these inhibitors were ineffective with mushrooms or had adverse effects on quality (8). Treatment of mushrooms with 4-hexylresorcinol, a compound that is reputed to be highly effective with shrimp and apples (29), actually induced discoloration.   Acidic browning inhibitor dips tended to cause cut edges of mushroom slices to turn yellow.   One of our most effective browning inhibitor formulations was the combination of 4.5% sodium erythorbate, 0.1% cysteine·HCl, and 1000 ppm EDTA (disodium salt), adjusted to pH 5.5 with 10% NaOH. Sodium erythorbate has been used commercially as a browning inhibitor for mushrooms. The other ingredients are classified by the Food and Drug Administration as GRAS (Generally Recognized as Safe) for certain food applications (30). The effectiveness of this formulation in controlling browning of mushroom external and cut surfaces was determined by measuring the change in L-value at the treated surface with a spectrocolorimeter. The decrease in L-value during storage, indicative of browning, was much greater for untreated controls than for treated mushrooms (Figure 6).

Table IV. Browning Inhibitors and Antimicrobials Evaluated for Preservation of Minimally Processed Mushrooms

Browning Inhibitors	Antimicrobials
Ascorbic acid and Na ascorbate	Streptomycin sulfate
Erythorbic acid and Na erythorbate	Hydrogen peroxide
Ascorbic acid-2-phosphate	Sodium hypochlorite
Ascorbic acid-2-triphosphate	Sodium benzoate
Citric acid	Potassium sorbate
Cysteine · HCl	
N-Acetyl cysteine	
4-Hexylresorcinol	
EDTA (disodium salt)	
Sodium acid pyrophosphate	
Sodium hexametaphosphate	
Sporix	
Hydroxypropyl ß-cyclodextrin	
Zinc chloride	
Source: Adapted from ref. 8	

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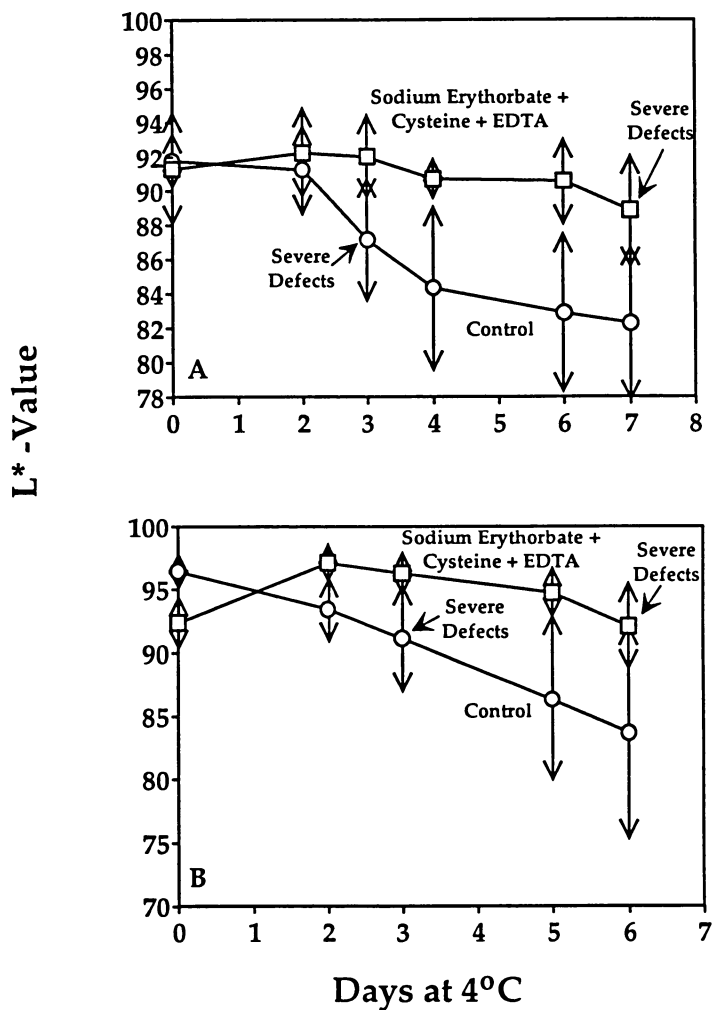
Figure 6. Effectiveness of browning inhibitor treatment in controlling enzymatic browning of mushrooms.   A, External surface of mushroom cap; B, Cross-sectional cut surface of mushroom cap.

In more recent experiments, we have obtained equally good results with a somewhat simpler browning inhibitor dip containing 4.5% sodium erythorbate and 0.1% sodium chloride (*28*). A shelf life of at least one week can be realized for washed mushrooms if they are treated first with a 3-5% hydrogen peroxide solution to control spoilage and then with the new dip formulation to inhibit browning.

Clarified Raw Juices

Our experience with clarified raw apple juice illustrates an important constraint on the use of ascorbate or erythorbate to control browning of minimally processed products. Previously, we found that polyphenol oxidase in raw apple juice was bound to suspended particulates making up the juice cloud (*9*). Removal of this cloud by filtration or centrifugation would eliminate the capacity of the juice to undergo enzymatic browning. However, in order to prevent browning in the freshly prepared raw juice prior to clarification, it was necessary to dip the cut up apples in 1% ascorbic acid solution before juicing. This would result in an ascorbic acid concentration of about 500 ppm in the juice. Lower ascorbic acid concentrations could be used with apples that browned less. With Granny Smith apples, for example, we added about 100 ppm ascorbic acid to the juice.

Clarification was able to prevent browning in refrigerated raw juices stored for several weeks. However, juices stored for longer periods of time sometimes developed a yellow color, which over time became gold and, in some cases, brown. We didn't know whether this discoloration was due to an enzymatic browning reaction resulting from residual polyphenol oxidase in the juice, a nonenzymatic browning reaction, or the metabolic activity of microorganisms in the unpasteurized raw juice.

In a series of experiments designed to establish the cause of this discoloration, we showed that yellowing was not prevented by microfiltration of raw juice through an 0.45 μm membrane. This largely ruled out microbial activity during storage as a source of the discoloration. Yellowing was not prevented by boiling the juice before membrane filtration. This eliminated an enzymatic reaction as the source of the discoloration.

Yellowing was reduced by storage of juice in vacuum and by addition of 100 ppm SO_2 (as sodium bisulfite) or 1% polyvinylpolypyrrolidone to juice. Yellowing was associated with the presence of at least 500 ppm added ascorbic acid. At very low levels of added ascorbic acid, i.e., 100 ppm, juices did not become yellow but tended to brown slowly. Yellowing was greatly enhanced by addition of 1000 ppm dehydroascorbic acid to the juice.

These results suggests that yellowing of raw apple juice, treated with ascorbic acid to prevent enzymatic browning, was due largely to nonenzymatic browning of dehydroascorbic acid which was generated by nonenzymatic oxidation of added ascorbic acid. Nonenzymatic oxidation of phenolic compounds also contributed to this discoloration. Thus, use of ascorbic acid to control enzymatic browning in some minimally processed products may actually result in unforeseen nonenzymatic discolorations. Yellowing of apple juice could be minimized as a problem by

reducing the level of added ascorbic acid and by bottling juice with minimal headspace volume.

Conclusions

The problem of controlling enzymatic browning in minimally processed fruits and vegetables is not a simple case of choosing the best browning inhibitor formulation for each commodity. Our experiences with pre-peeled potatoes, washed mushrooms, and fresh apple juice, demonstrate the importance of taking a broader view in developing treatments to control browning. From these case studies, we can identity several principles or guidelines that may be applicable to other commodities.

First, consider the characteristics of the cut or peeled surface to be treated: the extent of mechanical or thermal injury, occurrence of membrane leakage, and presence of degradative enzymes and their substrates. Second, consider the possibility that secondary nonenzymatic reactions may contribute to product discoloration, if enzymatic browning is controlled. Such reactions even may be a consequence of the browning inhibitor treatment. Third, consider the possibility that product discoloration may be due in part to metabolic activity of microorganisms in the product. Fourth, consider possible adverse effects of browning inhibitor treatments on product flavor and texture. Fifth, consider microbiological implications of browning inhibitor treatments - whether such treatments might actually favor growth of spoilage microorganisms or even human pathogens.

With this holistic approach, we believe that effective and practical solutions can be found for most enzymatic browning problems encountered with minimally processed fruits and vegetables.

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Chapter 19

Inhibition of Apple-Slice Browning by 4-Hexylresorcinol

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The effect of 4-hexylresorcinol (HR) on the browning inhibition of Delicious apple slices during cold storage (0.5 or 4.4 °C) was studied. Significant ($p<0.001$) inhibition in browning of apple slices was obtained with HR solution at concentrations as low as 0.005%. Discoloration was observed on the vascular bundles of the fruit two days after slicing. Combining 0.5% ascorbic acid (AA) with HR eliminated vascular discoloration and synergistically enhanced the browning inhibition. With partial vacuum (20 inch Hg vacuum) packaging and low temperature storage, more than 50 days browning-free storage life of the apple pieces were obtained with 5 min dipping in a solution containing 0.01% HR, 0.5% AA and 0.2% calcium chloride.

Color is an important quality attribute of food. Enzymatic browning reactions produce brown pigments on the surface of sliced fruits and vegetables, rendering the products unacceptable in most cases. Browning is often accompanied by undesirable changes in flavor, texture and loss of nutrients (*1*).

Sulfites are effective anti-browning agents and widely used in food industries (*2-3*). However, the concerns over the allergenic reactions in the asthmatic population (*4*) prompted the Food and Drug Administration (FDA) to limit the use of sulfites in certain food products, i.e. apples and potatoes (*5-6*). The lack of effective browning inhibitors stimulated active search for sulfite alternatives (*7*).

4-Hexylresorcinol (HR) is used as an active ingredient in various medicines for more than 40 years, and no evidence of systematic toxicity is reported (*8-9*). HR was first applied as a browning inhibitor on shrimp and later extended to fruits and vegetables (*10-11*). Dipping shrimps in 0.005% (50 ppm) HR solution for 1 min generated an average residue of 1 ppm (*12*). According to Frankos et al. (*13*), the use of HR as a processing aid on shrimps was generally recognized as safe (GRAS). The allowed daily intake (ADI) for HR was set as 0.053 mg/kg/day (*13*). HR was also

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incorporated with the process of combined methods to inhibit the browning of apple slices without refrigeration (*14*). However, the use of anti-microbial agents, sugar syrup was necessary to preserve the fruits. Tissue softening was also reported. In addition, a 4 hr immersion in 0.02% (200 ppm) HR solution resulted in 100 ppm HR residue in the fruits (Ren, H.; Barbosa-Cánovas, G. V. *Proceedings of International Symposium on Properties of Water*, ISOPOW, Puebla, Mexico, in press).

The objective of this research was to formulate an anti-browning solution containing HR in the absence of preservatives and optimize the dipping and packaging conditions.

Materials and Methods

Preparation of Apple Slices. Delicious apples (*Malus domestica*, Borkh.), received from a commercial warehouse in Yakima, Washington, were stored in a controlled atmosphere (CA) storage room (0.5 °C, 1% O_2 and 96% RH) for subsequent experiments. Uniform, 5 mm thick apple slices were prepared with a mechanical apple peeler (White Mountain Freezer Inc., Wichendon, MA). Apple pieces were obtained by cutting the fruit along the stem-calyx axis with a stainless steel hand slicer. Each fruit generated 8 pieces.

Treating Slices with HR, Ascorbic Acid and Calcium Chloride. Apple slices were dipped in solutions containing HR (OPTA Food Ingredient Inc., Cambridge, MA) at concentrations of 0, 0.005, 0.01, 0.02, 0.03% (w/v) alone or combined with 0.5% ascorbic acid (AA) and/or 0.2% calcium chloride for 10 min. To ensure rapid withdrawal and continuous immersion during dipping, the slices were placed in nylon mesh bags and submerged in the solutions under a perforated plastic plate. Upon withdrawal, the slices were immediately blotted with absorbent paper towels to remove excess solution. The slices were packaged in 2 mil thick plastic pouches (Koch Supplies Inc., Kansas, MO), hermetically sealed and stored at 4.4 °C for subsequent color and texture analyses. A complete factorial design with repeated measures was conducted in triplicate.

Ascorbic Acid Concentrations. Apple slices were dipped in different combinations of AA (0.25, 0.5%, 0.75%) with 0.01% HR and 0.2% calcium chloride solution for 10 min. A complete random design with repeated measures was performed in triplicate.

Packaging. Apple slices dipped in 0.01% HR, 0.5% AA, and 0.2% calcium chloride for 10 min were placed in Koch plastic pouches (AT), Ziploc vegetable bags with freshness vents (Veg) or Freshhold bags (FH) and sealed under ambient atmosphere, partial vacuum (Vac, 20 inch Hg vacuum), or filled with N_2 (N_2). The packaged fruits were stored at 0.5 °C for further analysis. CA treatment was provided by placing the treated slices unpackaged in the CA chambers with O_2 adjusted to a 1% level.

Dipping Time and HR Concentration. Solutions containing the combinations of 0.5% AA, 0.2% calcium chloride and 0.005 to 0.02% HR were prepared. Apple pieces were dipped in the solutions for 2 or 5 min, with or without blotting of the excess

solution. The treated apple pieces were then packaged in the Koch bags, hermetically sealed under partial vacuum and stored at 0.5 °C.

Color and Texture Determination. Colors, expressed by L*, a* and b* values, of apple slices were measured in duplicate through the plastic pouch, with a Minolta CM-2002 Spectrophotometer (Minolta Camera Co., Osaka, Japan). Textures of the slices were assessed by a Food Texture Instrument (Food Technology Corporation Inc., Rockville, MD) on the 13 mm diameter apple discs obtained from the treated apple slices. The measurements were conducted on the two parallel flat sides with a 50% compression of the original thickness at a speed of 60 mm/min. The highest peak force (N) was recorded and designated as the index for the firmness of the apple slices. Analysis of variance (ANOVA) and LSD was conducted using the GLM procedures included in the Statistical Analysis System (SAS) software package (15).

Results and Discussion

Color and Texture of HR, Ascorbic Acid and Calcium Chloride Treated Apple Slices. Gradual decrease in L* and increase in a* were observed on the sliced apples during storage at 4.4 °C (Figure 1a and 1b). As the values of L* and a* represent the lightness and redness of the subject, respectively, and are often used as browning indices (16), the observed decrease in L* and increase in a* indicated progressive browning on the surface of the slices. Dipping the slices in the solutions containing HR significant ($p<0.001$) delayed and reduced the decrease in L* and increase in a*, illustrating that the enzymatic browning of apple slices was inhibited by HR treatments. HR concentrations as low as 0.005% were effective in browning inhibition.

Browning inhibition achieved with HR treatments alone was brief and discoloration was noticed on the flesh vascular bundles two days after the treatment. Treatments consisting of HR alone failed in browning inhibition within 6 days after the slicing, regardless of the HR concentrations used. Combining 0.5% AA with HR solution improved the browning inhibition (comparing Figures 1a and 1b with 2a and 2b). A similar result was reported on apple fruits preserved with combined methods (14). A significant ($p<0.001$) interaction in browning inhibition by AA and HR was observed, suggesting that the enhanced inhibition in browning by AA is synergistic rather than additive. As a reducing agent, AA is a well known browning inhibitor, mainly to reverse the quinones formed during browning reaction back to diphenols (17). Browning occurs on the treated fruits after the depletion of AA (18). HR is a selective inhibitor of polyphenoloxidase (PPO) of shrimp (11) and mushroom tyrosinase isozymes (19). Although the mechanism of PPO inhibition by HR is not clearly explained, an experiment conducted by Dawley and Flurkey (19) suggested that a mixed type of competitive and un-competitive inhibition might be expected. The inhibition in browning of apple slices by HR might be attributed to the inhibition of apple PPO activity (14).

The mechanism of the enhancement of browning inhibition by HR and AA is unknown. It is apparent that the combination of AA and HR prolonged the retention of AA due to the suppression of PPO activity by HR. Consequently, better inhibition in
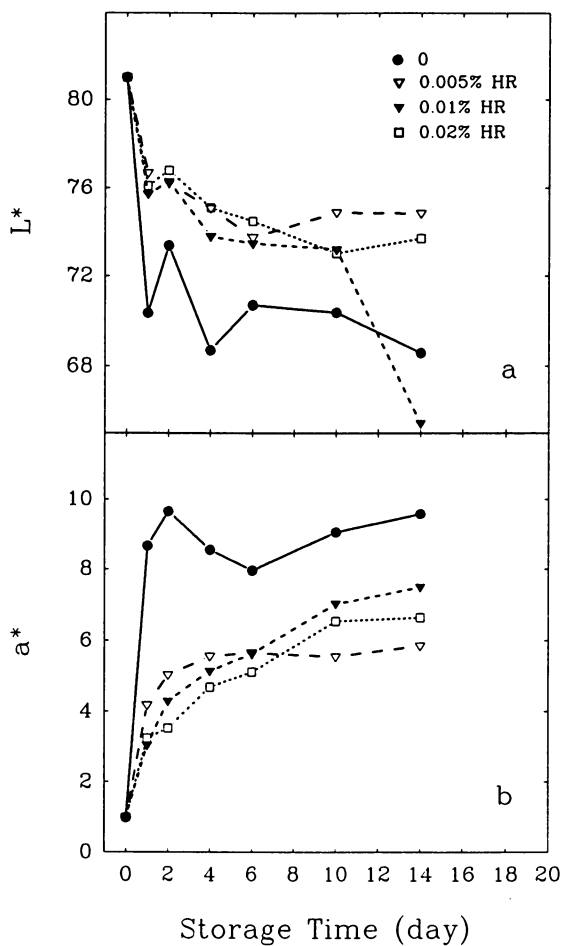
Figure 1. L* (a) and a* (b) values of apple slices dipped in a series HR solutions for 10 min and stored at 4.4 °C.
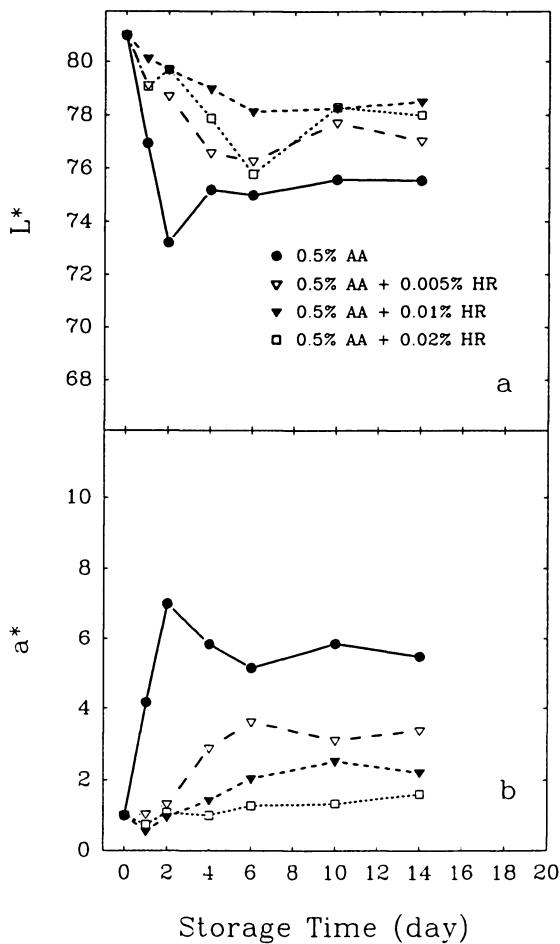
Figure 2. L* (a) and a* (b) values of apple slices dipped in the solutions containing 0.5% AA and a series concentrations of HR for 10 min and stored at 4.4 °C.

browning was achieved due to the presence of AA to reduce the quinones formed during browning reaction catalyzed by any residual PPO activity. Further analyses of PPO activity, the residue of HR and AA are needed to test this hypothesis.

Browning inhibition is related to the concentration of HR in the combination with AA. With high concentrations of HR (0.02%), although the slices maintained the smallest a* value during storage, the L* value decreased. The decrease in L* was probably due to the adverse effect of HR, at high concentration, on the fruit tissue. In fact, tissue softening, leakage and the loss of cell turgor pressure were observed on the fruits treated with 0.02% HR. At a very low concentration of HR (0.005%), although there was no tissue damage, the increase in a* value indicated the inadequate inhibition of browning. Apple slices treated with the combination of 0.5% AA and 0.01% HR maintained stable and large value of L* without a drastic increase in a*, suggesting that 0.01% was the lowest effective concentration of HR tested so far. The treated apple slices were maintained at 4.4 °C for up to 14 days without browning. No excessive tissue softening and leakage were noticed on the slices treated with this combination.

Calcium chloride, 0.2%, inhibited browning for a short period. There were no benefits in browning inhibition by combining calcium chloride with HR or HR and AA except in the case of 0.02% HR and 0.5% AA. However, the texture of the calcium chloride treated slices was firmer than those treated with HR and AA only.

The texture of apple slices was assessed in terms of the highest peak force (N). A gradual decrease in the peak force indicated softening of the slices during storage. Treatments containing HR decreased the peak force, suggesting HR impaired the texture of the fruits (Figure 3). The higher the HR concentration, the smaller the peak force detected, indicating the effect of HR on the texture is concentration dependent. Combining AA with HR exaggerated the loss of the firmness. The higher peak force in the fruits dipped in the solutions containing 0.2% calcium chloride than the fruits treated with HR and AA only indicated that calcium chloride improved firmness of the fruits.

Ascorbic Acid Concentration and Browning Inhibition. Within the range of 0.25 to 0.75%, AA concentration was significantly ($p < 0.001$) related to the effectiveness of browning inhibition (Table I). The higher the concentration, the slower the decrease in L* and the longer the browning protection was. Similar result was reported using AA only (*3*). However, a high concentration of AA (0.75%) imposed an unpleasant sour taste in the fruits. A combination of 0.5% AA and 0.01% HR was chosen for the following experiment.

Table I. L* Value of Apple Slices Dipped in the Solutions Containing HR and AA for 10 min and Stored at 4.4 °C

Treatment	Storage Time (day)			
	0	3	14	21
0.01% HR + 0.25% AA	80.67	77.81	73.72	74.37
0.01% HR + 0.50% AA	80.08	77.42	76.29	75.75
0.01% HR + 0.75% AA	81.16	80.64	77.70	77.40
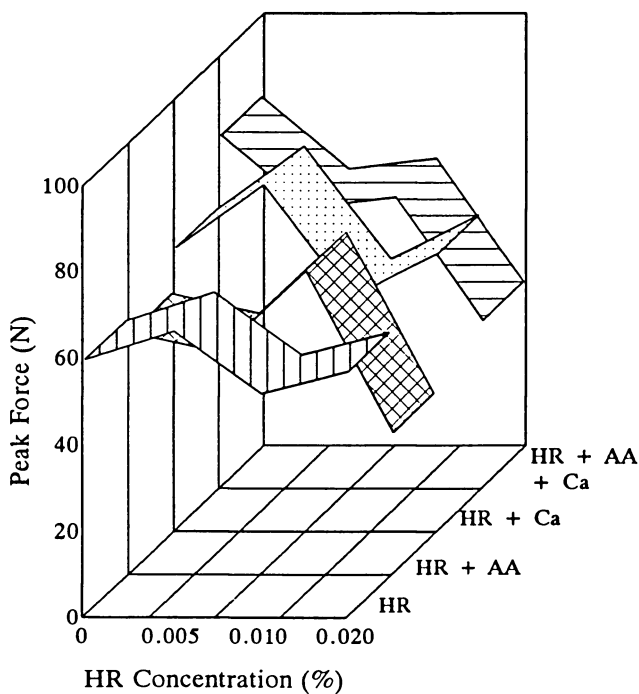
Figure 3. Peak force (N) of sliced apples dipped into HR, 0.5% AA and 0.2% calcium chloride (Ca), alone and their combinations for 10 min and stored at 4.4 °C.

Packaging and Browning Inhibition. Packaging materials and packaging types were significantly ($p<0.001$) related to the effectiveness of browning inhibition achieved by HR combinations (Figure 4). The treated slices packaged in polyethylene bags (AT), with perforations (Veg), or gas permeable Freshhold windows (FH) increased in a* rapidly, suggesting that these packages did not provide additional inhibition in browning. The sliced fruits placed in the CA room with 1% O_2 displayed a slower increase in a* and browning inhibition initially (5 days). However, more browning was observed on the fruit slices in the CA room. Shrinkage was also observed on the fruits, probably due to the moisture loss. Packages with N_2 or partial vacuum significantly ($p<0.001$) inhibited the increase in a*, delayed the browning process and extended the storage life of the treated slices. No browning was observed on the slices packaged with N_2 or under vacuum for 50 days.

The observed difference in browning inhibition due to packaging type is related to the difference in the metabolism of the fruit tissues in the packaging. Unlike thermally processed fruits, the apple slices dipped in the anti-browning solution for 10 min are still alive and respire (*20*). They are highly susceptible to the environmental temperature and gaseous composition (*21-22*). The extended browning inhibition and storage life of apple slices packaged with N_2 can be attributed to the reduced O_2 contents in the packaging, which contributed to the inhibition of PPO activity and reduced respiration (*21*). However, prolonged storage with N_2 would induce anaerobic respiration and produce ethanol, which would in turn impair the storage life of the treated slices. Partial vacuum packaging not only reduced O_2 in the package, but also removed O_2 and ethylene inside the fruits and reduced the respiration and prevented the enzymatic browning further. However, in a similar way with N_2 packaging, apple slices in a vacuum bag may develop anaerobic respiration and produce ethanol if the storage is excessively prolonged. In addition, anaerobic microorganisms, especially *Clostridium botulinum* may grow and produce toxins at the low O_2 condition (*23*). Further experiments are needed to examine the quality and safety of the fruit slices packaged under these conditions.

Dipping Time and HR Concentration. A significant ($p<0.001$) difference in browning inhibition was observed among HR concentrations and dipping time (Figure 5). Among all the concentrations tested, the combination of 0.01% HR, 0.5% AA and 0.2% calcium chloride displayed the smallest a* and largest L* value, indicating the best browning inhibition. For the dipping time examined, 5 min dipping with blotting showed better inhibition than the other treatments. With partial vacuum packaging and low temperature (0.5 °C) storage, apple pieces dipped in the combination of 0.01% HR, 0.5% AA and 0.2% calcium chloride for 5 min with blotting were kept from browning for more than 50 days with acceptable texture.

Conclusion

HR significantly ($p<0.001$) inhibits the browning of apple slices or pieces. AA synergistically enhances browning inhibition. The browning inhibition of apple slices is
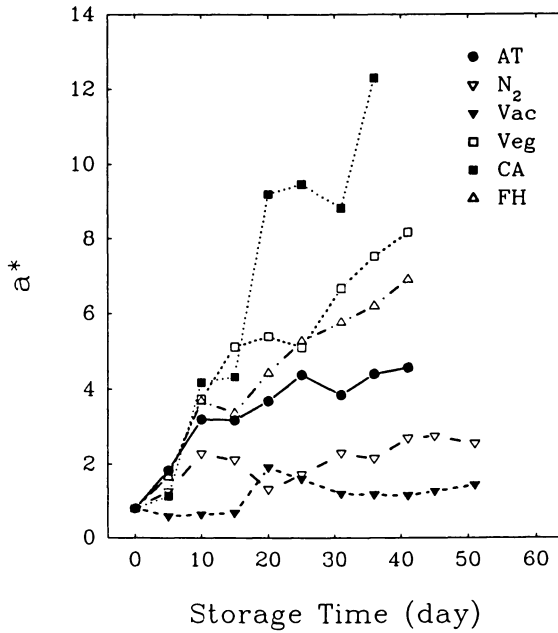
Figure 4. Packaging and a* values of treated apple slices. AT-Koch bag sealed at ambient atmosphere; N_2-Koch bag sealed with N_2; Vac-Koch bag sealed under partial vacuum; Veg-Ziploc vegetable bag; CA-controlled atmosphere room with 1% O_2, 95% relative humidity; FH-Freshhold bag;
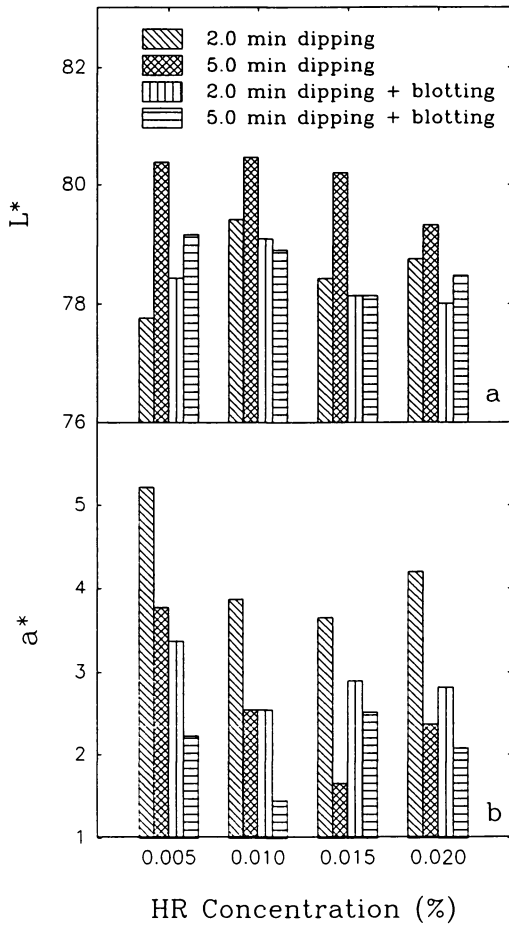
Figure 5. HR concentration and dipping time on browning inhibition in terms of L* (a) and a* (b) of the apple pieces stored at 4.4 °C for 30 days.

improved and extended by vacuum packaging. Apple pieces treated with 0.01% HR, 0.5% AA and 0.2% calcium chloride for 5 min, packaged under partial vacuum were stored at 0.5 °C for 50 days with acceptable color and texture.

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Chapter 20

Polyphenol Oxidase Activity in Japanese Apples

Differences Among Cultivars and Prevention by Heat, Ascorbic Acid, and Reduced Oxygen

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Polyphenol oxidase (PPO) activity in the peel, flesh and core of six Japanese apple cultivars was determined. Influences of temperature, ascorbic acid and oxygen on the inhibition of the PPO activity were investigated. Among the cultivars tested, the highest PPO activity was found in Red Delicious apples and the lowest activity in Ohrin apples. All cultivars had high PPO activity in the core, followed by the flesh and the peel. The enzyme was most active at 30 °C at pH 3.8 but rapidly decreased below 10 °C and above 50 °C. The enzyme from Tsugaru apples was the most heat stable, requiring 2.4 min at 67.5 °C for 90% reduction of the activity. Browning inhibition increased with the addition of ascorbic acid and the decrease of the oxygen concentration. The combination of additional ascorbic acid and oxygen reduction decreased polyphenol oxidase activity and consequently extended the lag period of the browning reaction.

Several distinct forms of apple juices are available on the Japanese market, mainly as clear and cloudy juices, with the latter being sold in the greatest volume. This juice is characterized by having a whitish-yellow color, stable cloudiness and fresh aroma. An essential quality criterion for this type of apple is the bright, whitish-yellow color (1).

Browning of apple juice is mainly initiated by the enzyme polyphenol oxidase (PPO; EC 1.14.18.1) which catalyzes the oxidation of natural phenolic compounds to corresponding quinones (2). These quinones are further polymerized to form browning pigments (3), which are a great concern to apple juice processors.

Factors influencing the rate of the enzymatic browning in apples include PPO activity, polyphenolic components and oxygen. Some researchers (4, 5) indicated that PPO activity was primarily responsible for the intensity of browning, whereas other workers (6, 7) found that it was not related to browning. Harel et al. (8) stated that both PPO activity and phenolic content determined the rate of browning. Coseteng and Lee (9) reported that among seven apple cultivars, PPO activity was directly correlated with the degree of browning in four

cultivars, while in three others, the degree of browning was related to the phenolic concentration.

Differences in the PPO activity due to apple cultivars have been found by several researchers. Mihalyi and Vamos-Vigyazo (*10*), Vamos-Vigyazo et al. (*4, 5*), Amito et al. (*7*), and Janovitz-Klapp (*11*) found considerable variations in PPO activity among apple cultivars and reported that the highest activity was in Red Delicious. Klein (*6*) and Coseteng and Lee (*9*) also reported the highest PPO activity in the Red Delicious cultivar. However, these studies were conducted with European, Australian, or American apple cultivars, whereas there have been no recent studies on the PPO activity of apple cultivars grown specifically in Japan.

PPO is located in the chloroplast thylakoid membranes of plant tissues (*2*). Many researchers have studied the distribution of PPO in various sections of apples and reported contradictory results. Harel et al. (*12*) and Stelzig et al. (*13*) reported that PPO was substantially higher in the peel than in the flesh. Klein (*6*) and Janovitz-Klapp et al. (*11*) found higher PPO activity in the cortex than in the peel. In a recent study using immunostaining, Murata et al. (*14*) demonstrated that in five apple cultivars, chlorogenic acid oxidase was not distributed uniformly but was mainly localized near the core and secondarily near the skin.

Since enzymatic browning is of great organoleptic and nutritional importance in fruit processing, several treatments have been explored to control this reaction (*15*). The most effective method to inactivate PPO is heat treatment. Thermal stability of apple PPO has been investigated by many authors, including Trejo-Gonzalez and Soto-Valdez (*16*) who indicated that PPO activity from Anna apple cultivar was reduced to an undetectable level when treated at 80 °C for 10 min. Murata et al. (*17*) found that the activity of chlorogenic acid oxidase extracted from Fuji apples was reduced to 20% by incubating at 60 °C for 30 min. Zhou et al. (*18*) also reported that PPO activity of Monroe apple peel was stable up to 40 °C but rapidly inactivated above 50 °C. The majority of previous works have concentrated on the thermal instability of PPO at relatively high temperatures. However, there have been no studies on PPO activity at low temperatures, below 20 °C.

A considerable number of chemical compounds have been proposed as PPO inhibitors. Carboxylic acids of the cinnamic series and kojic acid have been demonstrated to be inhibitors for apple PPO (*19, 20*). For fruit and vegetable processors, an important group of compounds are those possessing reducing properties, such as ascorbic acid (*10, 18, 21-23*), cysteine (*18, 23-25*) and sulfite (*26, 27*). These compounds either reduce *o*-quinones, produced by PPO activity, back to their corresponding *o*-diphenols or react with *o*-quinones to form colorless products (*21, 28*). It is well documented that the effectiveness of the reductants in the inhibition of the browning reaction was, in decreasing order, sulfite, cysteine and ascorbic acid (*23*). However, the use of sulfites as food additives becomes more restricted due to its possible damaging effect to health (*29*). Consequently, ascorbic acid has been utilized extensively to prevent the enzymatic browning of cloudy apple juice (*30*). The function of ascorbic acid in controlling enzymatic browning is short-term. It is generally understood that dehydroascorbic acid, which formed through oxidative degradation of ascorbic acid, is involved in the formation of non-enzymatic browning materials (*31*). Therefore, a high dose of ascorbic acid can prevent initial browning, but later accelerates non-enzymatic browning.

For the initiation of enzymatic browning, oxygen must be present as an essential component. It is known that the elimination of oxygen from processing greatly retards the browning reactions (*1, 32*). However, there is little information

about the effect of oxygen concentration on apple PPO activity. Janovitz-Klapp et al. (*33*) reported that apple PPO binds with oxygen before reacting with the phenolic substrate. They also found decreases in PPO activity with decreasing oxygen concentration.

The purpose of this work is to obtain information of apple PPO activities of Japanese cultivars and to describe the kinetics of their thermal inactivation. This paper also investigates the reaction mechanism of PPO in relation to low temperatures, ascorbic acid and oxygen concentrations.

Materials and Methods

Plant Materials. Apple cultivars (*Malus pumila* cv. Red Delicious, Jonathan, Fuji, Golden Delicious, Tsugaru and Ohrin) used in this study were acquired from a local market in Japan. Fuji is the major cultivar grown in Japan, accounting for approximately 50% of the total production. Apples obtained at commercial maturity were immediately utilized for the preparation of acetone powder.

Enzyme Extraction. Approximately 500 g apples were washed, cut and immediately submerged into cold acetone to prepare acetone powder as described by Klein (*6*) and Kahn (*22*). Acetone powder was also prepared from the peel, flesh and core sections of each cultivar. Acetone powder (0.1 g) was suspended in 5 mL cold 0.05 M McIlvaine buffer, pH 5.0, containing 2% Triton X-100, and homogenized at 4 °C. The homogenate was filtered and centrifuged at 20,000 rpm (25,000 g) at 4 °C for 8 min (TL Ultracentrifuge, Beckman). The supernatant was used as the enzyme preparation.

Enzyme Assay. Polyphenol oxidase activity was assayed using 0.05 M catechol as the substrate in both the spectrophotometric and polarographic procedures. The spectrophotometric procedure performed, according to Coseteng and Lee (*9*), was modified as follows; 2.9 mL of 0.10 M McIlvaine buffer at a given pH containing 0.05 M catechol was placed in a 1 cm pathlength cuvette and mixed with 0.1 mL of the enzyme preparation. The change in absorbance at 420 nm at 25 °C was continuously measured using a spectrophotometer (U-2000, Hitachi). Enzyme activity was determined by following the initial increasing rate of absorbance. One unit of enzyme activity was defined as the amount of enzyme that caused a change in absorbance of 0.001/min. The polarographic procedure, that of Janovitz-Klapp et al. (*11*), was performed with a Clark electrode (DO-25A, Horiba) using the same substrate conditions as previously described. Enzyme activity was determined as the initial rate of oxygen uptake and expressed as nanomoles of oxygen consumed per second (nKat). All enzyme preparations and enzyme assays were carried out in triplicate.

Effect of Temperature on PPO Activity. Prior to the thermal stability study, the pH of the enzyme preparation from Fuji apples was adjusted to 3.8 using 0.10 M McIlvaine buffer, pH 3.5. The enzyme solution was incubated at various temperatures, 25 °C - 80 °C, for 5 min and then rapidly cooled in ice water. Residue enzyme activity was assayed spectrophotometrically at pH 5.0 as previously described. To determine the activity at low temperatures, 1 °C - 30 °C, 0.05 mL of the Fuji apple enzyme preparation was incubated with 1.45 mL of 0.10 M McIlvaine buffer, pH 3.8, containing 0.05 M catechol at those temperatures. At given periods, 1.5 mL ethyl alcohol was added to stop enzyme activity. The increase in absorbance of the mixture at 420 nm was measured to determine the PPO activity. All treatments were performed in triplicate.

Heat Treatment. Heat treatments were performed in 2 mL thermal death time (TDT) tubes. Enzyme preparations from the core portion of the six Japanese cultivars were adjusted with 0.10 M McIlvaine buffer (pH 3.5) to a pH of 3.8 and 1 mL portions placed in each TDT tube. Tubes were then placed in an oil bath at various controlled temperatures, removed at selected time intervals, chilled immediately in ice water and assayed for PPO spectrophotometrically at pH 5.0. The D value, or decimal reduction, was defined as heating time required to inactivate 90% of the original activity at various temperatures (34, 35). The Z value, expressing the temperature dependence of thermal inactivation, was obtained from the plots of log D against temperature as the number of degrees required for the thermal inactivation curve to traverse one log cycle (34). Heat treatments at each temperature were made in triplicate.

Effect of Ascorbic Acid. For PPO inhibition studies influenced by ascorbic acid under air-saturated conditions, 2.8 mL of the substrate solution at a given pH and 0.1 mL ascorbic acid solution at various levels were mixed in a cuvette following the addition of the enzyme preparation from the Fuji cultivar, 0.1 mL. The lag period was computed by extrapolation of the linear portion of the color formation to the abscissa axis (28). Residual enzyme activity was determined from changes in absorbance at 420 nm after the lag period. All treatments were performed in triplicate.

Effect of Oxygen and Ascorbic Acid Concentration on PPO Activity. The effect of oxygen concentration on enzyme activity from the Fuji cultivar was studied in a closed reaction vessel in which dissolved oxygen was uniformly distributed by continuous stirring of the substrate solution during the reaction. Various oxygen levels of the substrate solution (60 mL, pH 3.8) were adjusted by bubbling nitrogen gas through the solution at 25 °C before 2 mL of the enzyme preparation was added. To determine the effect of ascorbic acid on the enzyme activity at various oxygen concentrations, 1 mL of the ascorbic acid solution at various levels was added before the addition of the enzyme preparation. PPO activities were assayed by the polarographic and spectrophotometric methods previously described. For the spectrophotometric determination, 0.8 mL aliquots were withdrawn at various intervals and promptly mixed with 2 mL ethyl alcohol to stop enzyme activity. The absorbance at 420 nm was measured to determine PPO activity. Treatments were performed in triplicate.

Statistical Analysis. Statistical evaluations were processed by analysis of variance and the LSD test to identify significant differences among treatments.

Result and Discussion

PPO Distribution in Japanese Apple Cultivars. Six Japanese apple cultivars were analyzed for PPO activity in peel, flesh and core sections as well as total PPO (Table I). The highest total PPO activity was found in Red Delicious (1570 unit/mL). Two cultivars, Jonathan and Fuji, also exhibited relatively high PPO activity ranging from 870 to 780 unit/mL, followed by Golden Delicious and Tsugaru (660 and 655 unit/mL, respectively). Ohrin exhibited the lowest PPO activity (540 unit/mL). These results coincide with several researchers (4, 7, 9-11) who found that PPO activity was the highest in the Red Delicious cultivar grown in Europe, New Zealand and U.S.A. Vamos-Vigyazo et al. (4) and Mihalyi and Vamos-Vigyazo (10) demonstrated that Golden Delicious was low in PPO activity. Recently Klein (6) and Amito et al. (7) also found the lowest

activity in Golden Delicious among the cultivars studied. All six cultivars showed high PPO activity levels in the core (800 to 300 units), followed by the flesh (430 to 126 units) and then the peel (97 to 32 units). Although wide variations in the core PPO activity were found among the cultivars, this activity represented 50% to 75% of the total activity. The level of core PPO activity was 1.3 and 2.1 times higher in Red Delicious than in Fuji and Ohrin, respectively. Red Delicious contained three-fold higher PPO activity in the flesh than the other cultivars except for Golden Delicious which was one-half the content of Red Delicious. The highest PPO activity in the peel was found in Jonathan and the lowest in Fuji and Tsugaru. Harel et al. (*12*) reported that PPO activity was substantially distributed in the peel, while others found higher activity in the cortex rather than the peel (*6, 11*). Among the apple cultivars tested, Janovitz-Klapp (*11*) reported that the PPO activity level in the cortex ranged from 3.1 to 0.6 and 3.0 to 0.3 mKat/Kg in the peel. These differences may be due not only to the different cultivars studied, but also to the extraction and PPO assay procedure applied. The present findings are in partial agreement with the report of Klein (*10*) who demonstrated that peel tissue was lower in PPO activity than cortical tissue of apple cultivars grown in New Zealand. Although there is no available literature on the level of PPO activity in the core of apples, the result of this present study agrees with the report of Murata et al. (*14*) who found that chlorogenic acid oxidase, determined by immunostaining, was localized intensely near apple cores.

Effect of Temperature on PPO Activity. PPO activity of Fuji apples as a function of temperature in the 25 °C - 80 °C range was shown in Figure 1. Maximum activity was exhibited at 30 °C with a relatively broad range of temperature stability. PPO activity was unstable at higher temperatures; for example, the relative PPO activity decreased from 49% to 13% as the temperature increased from 50 °C to 60 °C and then was reduced to an undetectable level at temperatures above 70 °C. This result is in agreement with the thermal stability of apple PPO reported by Trejo-Gonzalez and Soto-Valdez (*16*), Zhou et al. (*18*) and Murata et al. (*17*).

PPO activity from Fugi apples was measured at relatively low temperatures, from 1 °C to 30 °C, at pH 3.8. As shown in Figure 2, the activity was substantially decreased at the lower temperatures. When the reaction was performed at 10 °C, 5 °C and 1 °C, the relative activity was reduced 30, 20 and 13%, respectively. This indicated that PPO activity was largely suppressed at temperatures below 10 °C and supported the observation that apple juice processed at a low temperature is light brown in color.

Inactivation of Polyphenol Oxidase by Heat. The PPO enzymes obtained from the cores of the six Japanese cultivars were heated at temperatures ranging from 55 °C to 67.5 °C at pH 3.8 for various periods of time. It is common when working on thermostability of food enzymes to express enzyme inactivation in terms of the pertinent parameters of the inactivation plots, D and Z. When the logarithms of the residual activity were plotted against heating times, the enzyme showed linear inactivation kinetic curves. Small D values, indicating less time required to inactivate 90% of the initial enzyme activity, represent an enzyme with low heat stability. Variations of the thermal stability of PPO among the apple cultivars examined were shown in Table II. At 55 °C, the enzyme from Tsugaru apples was the most stable at 30.6 min, while the enzyme from Golden Delicious, at 20.8 min, showed the least heat stability with the other cultivars ranging from 21.3 to 26.3 min. At 67.5 °C, the highest heat resistance was again found with the Tsugaru cultivar and the lowest with Golden Delicious. The other

Table I. Distribution of Polyphenol Oxidase Activity in Peel, Flesh, Core of Six Japanese Cultivars

Cultivar	Peel	PPO Activity (unit/mL preparation)		
		Flesh	Core	Total
Red Delicious	78[b]	430[a]	800[a]	1570[a]
Jonathan	97[a]	130[de]	648[b]	870[b]
Fuji	32[e]	146[c]	606[b]	780[c]
Golden Delicious	67[c]	271[b]	350[d]	660[d]
Tsugaru	33[e]	126[e]	500[c]	655[d]
Ohrin	54[d]	135[d]	383[d]	540[e]

[a-e]Superscripts for each column are significantly different at 1% level
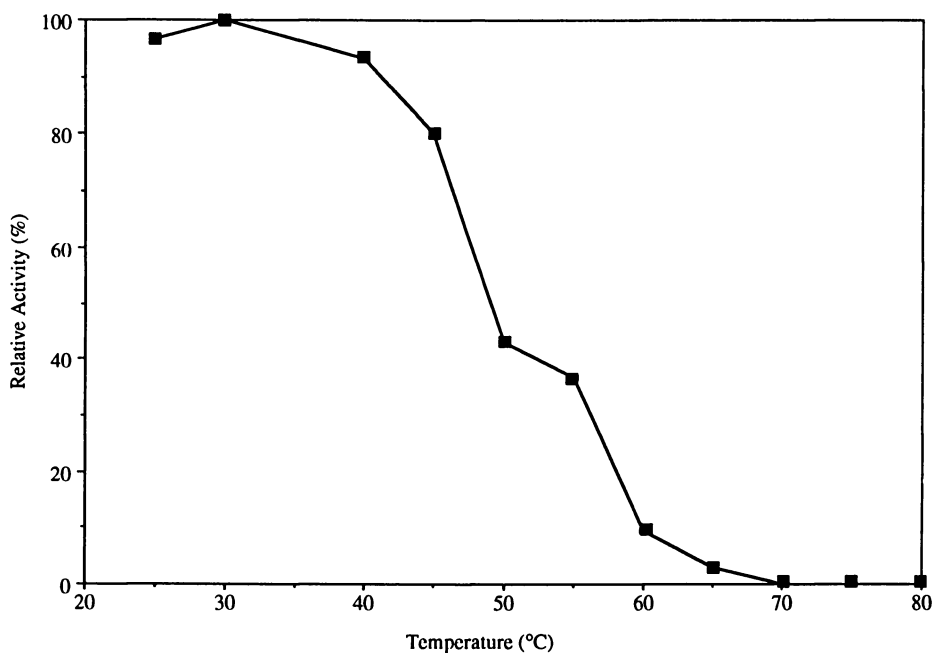
Figure 1. Thermal stability of polyphenol oxidase from Fuji cultivar.
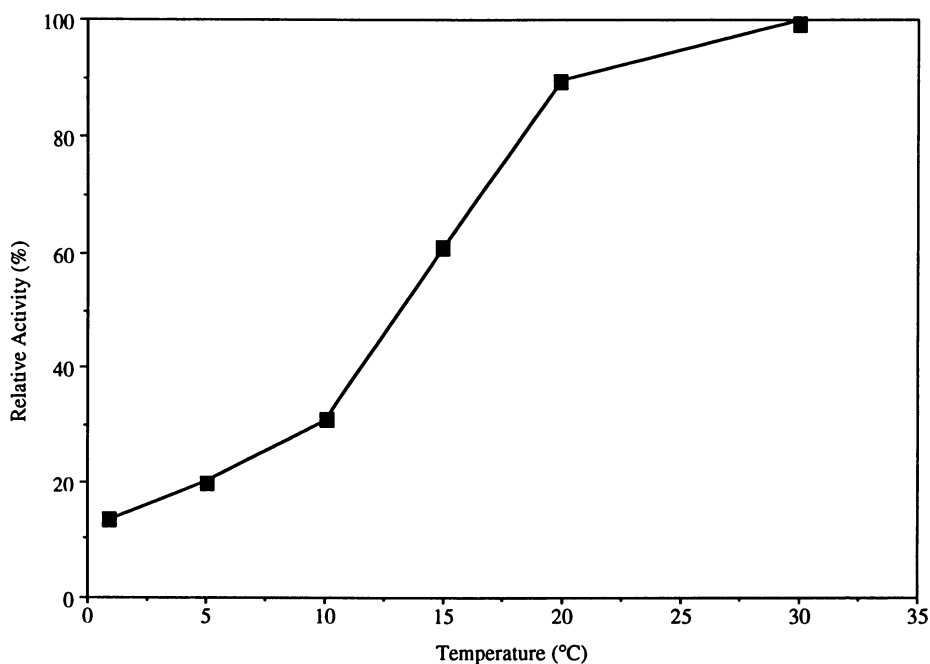
Figure 2. Effect of temperature on PPO activity from Fuji cultivar.

Table II. Thermal Inactivation of PPO from the Core of Six Japanese Cultivars

Cultivar	D-value (min)				Z Value (°C)
	D_{55}	D_{60}	D_{65}	$D_{67.5}$	
Tsugaru	30.6[a]	15.1[a]	5.0[a]	2.4[a]	11.4[b]
Ohrin	26.3[b]	8.2[d]	4.3[c]	1.8[c]	11.4[b]
Red Delicious	25.7[b]	8.6[cd]	3.5[d]	1.5[e]	10.5[c]
Fuji	22.9[c]	9.2[c]	4.6[b]	1.7[d]	11.8[b]
Jonathan	21.3[c]	10.7[b]	5.3[a]	2.0[b]	13.0[a]
Golden Delicious	20.8[c]	7.1[e]	4.5[bc]	1.4[f]	11.8[b]

[a-f]Superscripts for each column are significantly different at 5% level

cultivars had approximately the same level of D values, between 1.5 min and 2.0 min. Several researchers have conducted kinetic studies of thermal inactivation for PPOs extracted from various origins; avocado (22), pear (36), mango (37), grape (38, 39) and mushroom (34) and have found that large differences in D values exist for PPOs over a wide pH range. Limited information is available on the kinetics of thermal inactivation for apple PPO. Since polyphenol oxidase has been considered as one of the indicator enzymes for adequate heat treatment of fruits (40), the results of this present work would be useful for process evaluation of apple juice production.

As shown in Table II, the largest Z value was in the PPO from Jonathan apples (13.0 °C) and the smallest from Red Delicious (10.5 °C). The range of Z values for the other cultivars was between 11.4 °C and 11.8 °C.

Inhibition of Polyphenol Oxidase by Ascorbic Acid. Under air-saturated conditions, the effects of various concentrations of ascorbic acid on PPO activity extracted from Fuji apples were studied spectrophotometrically at two pHs, 3.8 and 5.0. Many researchers have demonstrated that PPO, in the presence of ascorbic acid, develops a lag period prior to any change in absorbance (10, 18, 21-23). As shown in Table III, for both pH values tested, as the concentration of ascorbic acid increased, the lag period was prolonged. This result indicated that ascorbic acid was effective in retarding the development of browning pigments. The presence of the lag periods found in this study agrees with the results of previous works (10, 18, 21-23). At the same ascorbic acid level, the lag period was longer with pH 3.8 than with pH 5.0. As depicted with the 0.17 mM ascorbic acid concentration, no browning developed for 960 sec with pH 3.8 while the lag period was 640 sec in duration in the reaction at pH 5.0. Kahn (22) reported that avocado PPO was essentially inhibited by 1.0 mM ascorbic acid for at least 1 min. Hsu et al. (21) also found that the addition of 0.5 mM ascorbic acid to mushroom PPO completely suppressed the development of brown pigments for several hours. These differences in the duration of the lag period may be due to the characteristics of PPOs, the quantity of activity and reaction conditions used for the experiments. Ascorbic acid is generally more effective when used in an acidic solution (31). This explains the longer lag period found with pH 3.8 than pH 5.0.

Ascorbic acid appears to inhibit browning by its ability to reduce o-quinones back to their corresponding o-diphenols or by its direct effect on the enzyme. The inhibitory effect of ascorbic acid in the browning of apples (10, 18, 23, 41), avocados (22), grapes (39) and mushrooms (21) is well documented. By increasing the concentration of ascorbic acid, the residual activity following the lag period was lowered at both pHs as shown in Table IV. The residual activity was less than 20% of the original in the presence of 0.142 mM ascorbic acid at both pHs. This data concurs with the results reported by Janovitz-Klapp et al. (23) and Zhou et al. (18), who demonstrated that for apple PPO, the higher the ascorbic acid concentration, the lower the residual activity after the lag period.

Inhibition of Polyphenol Oxidase Activity by the Reduction of Oxygen Concentration in Combination with Ascorbic Acid. Oxygen is an essential component for PPO activity. The oxygen dependence of PPO activity extracted from Fuji apples was investigated both polarographically and spectrophotometrically at the oxygen range of 0.01 mM - 0.25 mM at pH 3.8. As shown in Figure 3, at low concentrations of oxygen, PPO activities were also low. At an oxygen level between 0.05 mM and 0.15 mM, similar suppression was found. Even with an oxygen concentration of 0.01 mM, PPO activity assayed by polarography was apparent. However, the usual lag period observed by

Table III. Effect of Ascorbic Acid Concentration on the Lag Period at pH 3.8 and 5.0

Ascorbic Acid (mM)	Lag period (sec)	
	pH 5.0	pH 3.8
0	0	0
0.028	37	53
0.057	60	124
0.085	141	186
0.114	193	293
0.142	395	466
0.170	640	960

Table IV. Effect of Ascorbic Acid Concentration on Fuji Apple PPO at pH 3.8 and 5.0

Ascorbic Acid (mM)	Residual activity (%)	
	pH 5.0	pH 3.8
0	100.0	100.0
0.028	64.0	87.5
0.057	57.2	68.5
0.085	44.0	62.6
0.114	32.3	22.8
0.142	19.5	9.0
0.170	14.1	2.4

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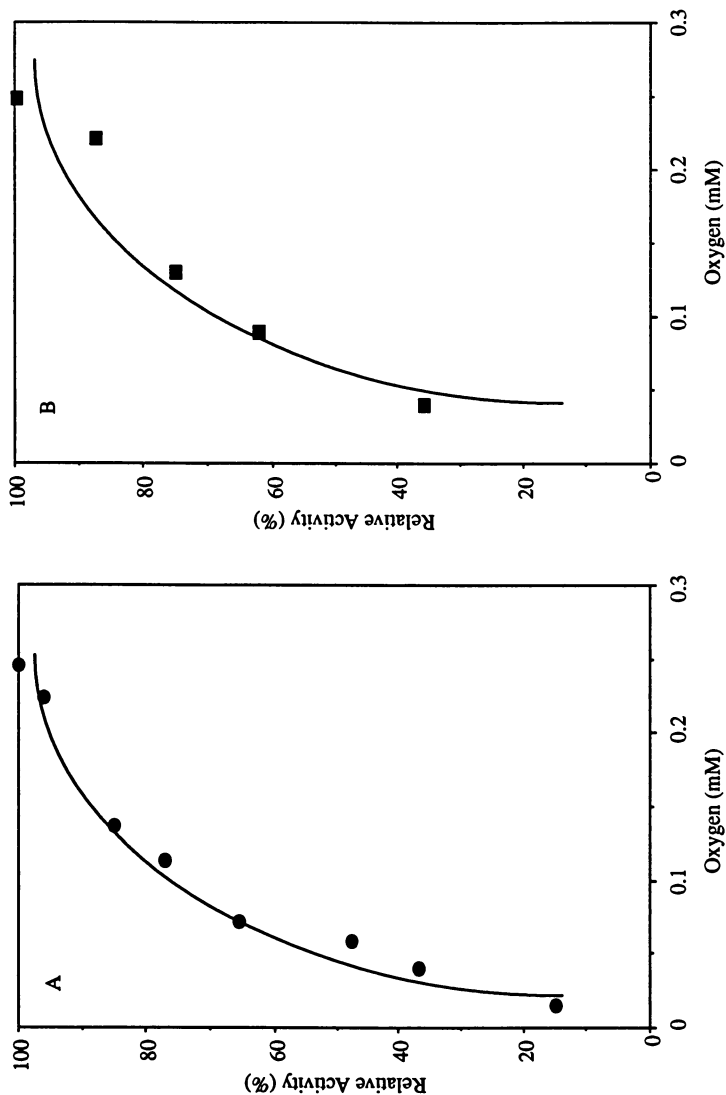
Figure 3. Effect of oxygen concentrations on Fuji apple PPO assayed by polarography (A) and spectrophotometry (B).

spectrophotometry for PPO in the presence of reducing compounds, was missing (*10, 18, 21-23*). Therefore, it was clear that limiting the amount of oxygen resulted in suppressing PPO activity as well as decreasing the rate and formation of brown pigments. These results of the present study are consistent with the findings of Janovitz-Klapp et al. (*33*).

The combined effects of ascorbic acid addition and dissolved oxygen reduction on PPO activity from Fuji apples were studied at pH 3.8 by both assay methods. By polarography, in spite of the presence of ascorbic acid, the oxygen uptake was prompt (data not shown). PPO activity in the absence and presence of ascorbic acid (0.028 mM or 0.057 mM) decreased correspondingly with reduced oxygen concentration (Figure 4). This implies that ascorbic acid is non-competitive with oxygen. This result agrees with the inhibition mode of benzoic acid demonstrated by Janovitz-Klapp et al. (*23*).

As shown in Figure 5, the duration of the lag period lengthened with increasing amounts of ascorbic acid and decreasing concentration of dissolved oxygen, while the oxygen uptake was immediate (data not shown). In the presence of 0.028 mM ascorbic acid, the length of the lag period was 2.5 times longer in 0.097 mM dissolved oxygen than in 0.248 mM dissolved oxygen. After the lag period preceding the development of browning, residual activity decreased with increased amounts of ascorbic acid and decreased oxygen concentration. For instance, in the presence of 0.057 mM ascorbic acid, residual PPO activity in 0.101 mM and 0.069 mM dissolved oxygen was reduced to 45% and 30% of the activity found in 0.259 mM dissolved oxygen, respectively. This result clearly indicated that apple PPO is strongly suppressed by the addition of ascorbic acid and reduced levels of dissolved oxygen, thus delaying the development of browning pigments. Since the level of ascorbic acid in apples is reported as 2 -10 mg/100g edible portion, eliminating oxygen prior to heat pasteurization will prevent the enzymatic browning in the production of cloudy apple juice without adding ascorbic acid or other reducing compounds.

Conclusion

Red Delicious apples, among the six Japanese apple cultivars investigated, exhibited the highest total PPO activity and Ohrin apples the lowest. For all the cultivars studied, the level of PPO activity was highest in the core, followed by the flesh and the peel. The enzyme activity was greatly reduced when the reaction was carried out at temperatures in the range of 1 ˚C - 5 ˚C. Maximum temperature for PPO was observed at 30 ˚C. Significant differences in thermal inactivation of PPO among the cultivars were found. At 67.5 ˚C, the largest D value was found in Tsugaru and the lowest in Golden Delicious. In the presence of ascorbic acid, oxygen uptake was immediate while, spectrophotometrically, the lag period which preceded visual browning was observed. Decreasing the oxygen level strongly suppressed enzyme activity. Apple PPO activity was greatly inhibited by the combination of ascorbic acid addition and oxygen reduction, resulting in decreasing residual activity and delaying the development of browning pigments.

Acknowledgment

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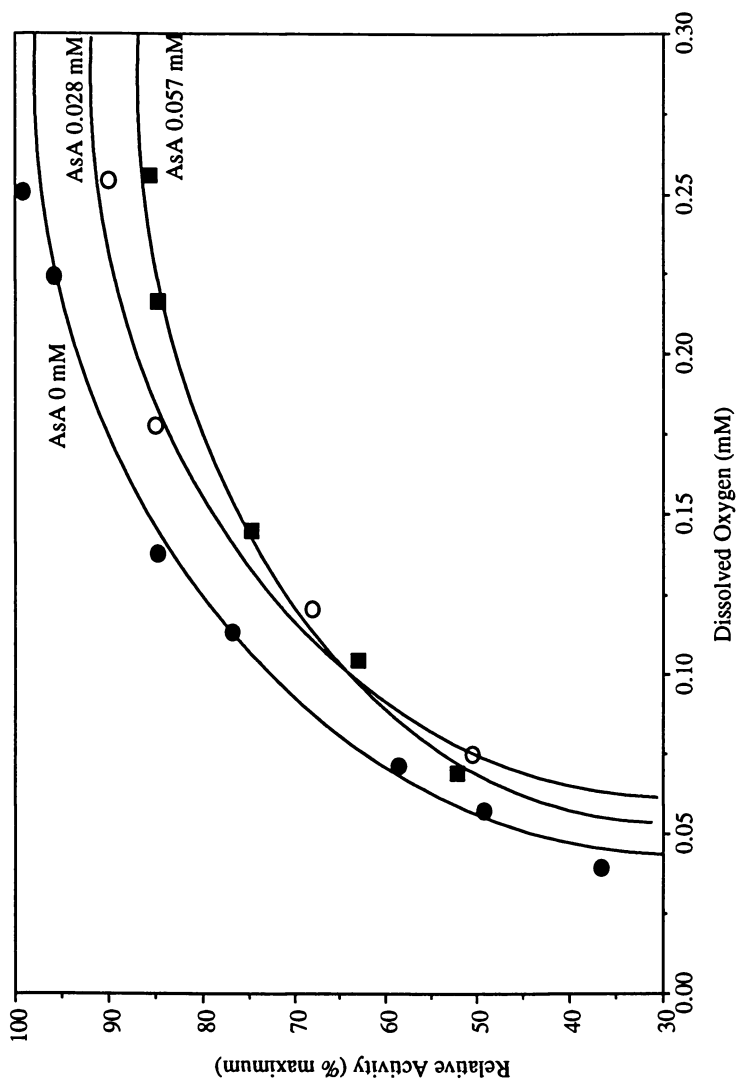
Figure 4. Effect of ascorbic acid (AsA) on Fuji apple PPO under various oxygen concentrations assayed by polarography.

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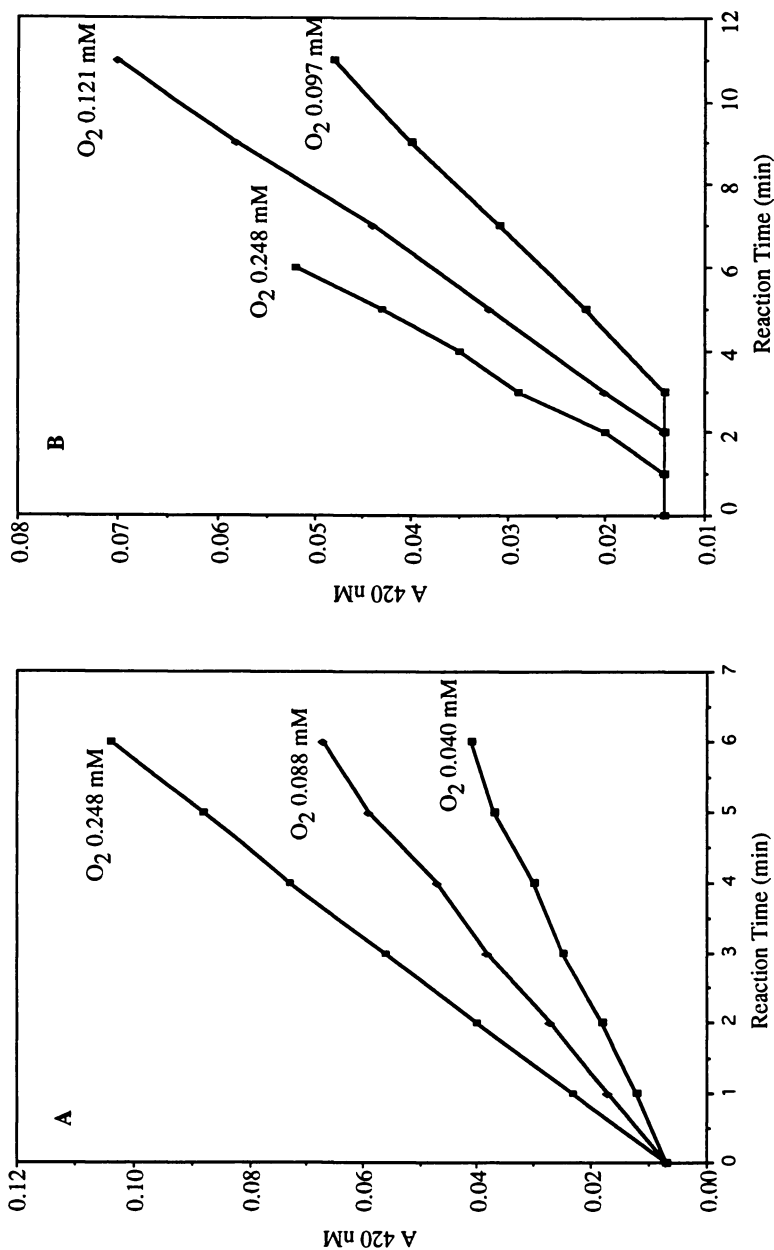
Figure 5. Reaction rate of Fuji apple PPO activity in the absence and presence of ascorbic acid under various oxygen concentrations assayed spectrophotometrically [(A) 0 mM; (B) 0.028 mM; (C) 0.057 mM; (D) 0.101 mM ascorbic acid].

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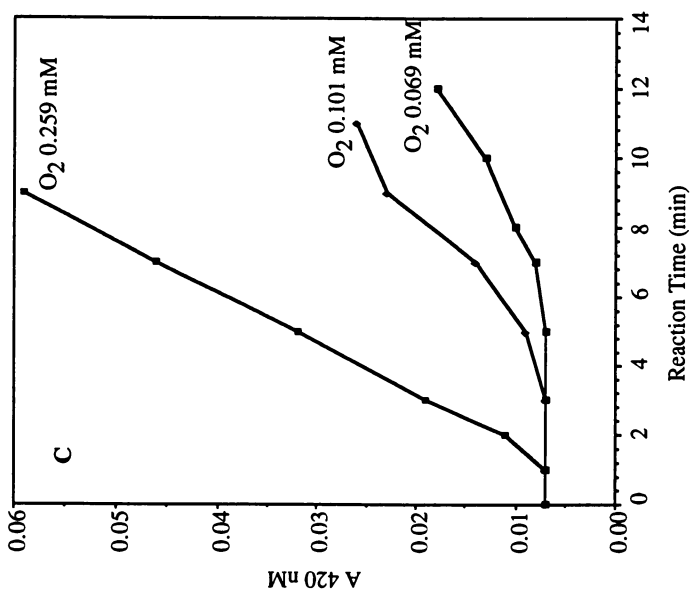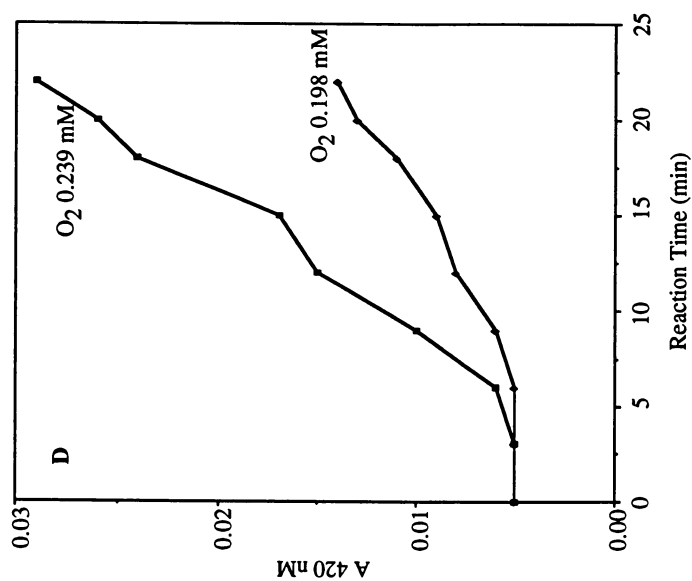
Figure 5. Continued.

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Chapter 21

Extraction, Partial Characterization, and Inhibition Patterns of Polyphenol Oxidase from Burdock (*Arctium lappa*)

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Enzymatic browning of burdock (*Arctium lappa*) and its inhibition were studied. Polyphenol oxidase was isolated by ammonium sulfate precipitation, and using gel-filtration and ion-exchange chromatography, resulting in a 160-fold purification of the crude extract. The optimum condition for the maximum enzyme activity was obtained at 30 °C and at pH 7. Heat inactivation studies showed that heating for about 10 min at 60 °C caused a 50% loss in enzyme activity. Inhibition of the enzyme reaction was studied with a combination of 4-hexylresorcinol, ascorbic acid, and citric acid.

Enzymatic browning in fruits and vegetables is the discoloration arising from a result of tissue injury or senescence. Polyphenol oxidase (EC 1.10.3.1) (PPO) is involved in the oxidation of endogenous phenolic compounds into quinones, in the presence of oxygen, which spontaneously react with other phenolic compounds, amino acids, etc., to produce brown pigments (*1*). PPO has been the subject of many reviews (*2-6*). Isolation and characterization of PPO from numerous fruits and vegetables are reported (*6*).

Burdock (*Arctium lappa*) is grown mainly as a vegetable in northeastern Asia. Browning develops very rapidly in finely ground forms or peeled forms of edible burdock roots. The browning discoloration not only limits the shelf-life of many minimally processed vegetables but causes a loss in the nutritional value (*7*). Since bisulfites are banned in the USA for use in raw fruits and most vegetables (*8*), due to a safety reason (*9*), many studies have been devoted to find potential alternatives to sulfites. 4-Hexylresorcinol (4-HR) was one browning inhibitor reported to be effective and safe (*10*). In the present work, we describe the extraction and partial characterization of PPO from burdock. The preliminary inhibition patterns of 4-HR, ascorbic acid, and citric acid on the PPO activity are also analyzed.

Experimental

Materials. Burdock roots from a commercial market were washed, kept for 1 day in the cold room (4 °C), and used as an enzyme source. Sephadex G-100 and DEAE-Sepharose CL-6B were obtained from Pharmacia. Insoluble polyvinylpolypyrrolidone (PVPP) was purchased from Sigma and acid-washed as suggested (11). All other analytical grade chemicals were purchased from Sigma, Bio-Rad, or Junsei, and used without further purification.

Preparation of Enzyme Extract. All enzyme extraction and purification procedures were performed at 4 °C, unless otherwise stated. 250 g of burdock roots were homogenized in 250 mL of 10 mM sodium phosphate buffer (pH 7.0), containing 1% Triton X-100, 1% ascorbic acid and 1% insoluble PVPP, using a pre-chilled blender. The crude homogenate was filtered through four layers of cheese-cloth and the filtrate was centrifuged at 25,000 g for 30 min at 4 °C. The supernatant was used as the crude enzyme preparation.

Enzyme Purification. The crude supernatant was brought to 25% saturation with solid ammonium sulfate and allowed to precipitate for 30 min. The precipitate was removed by centrifugation at 25,000 g for 15 min. The supernatant was then brought to 75% saturation with additional solid ammonium sulfate, slowly with stirring over a period of 1 hr. The pellet was collected after centrifugation at 25,000 g for 15 min. It was dissolved in 20 mL of 10 mM sodium phosphate buffer (pH 7.0) containing 1% ascorbic acid and dialyzed with three buffer changes against the same buffer overnight. After centrifugation at 25,000 g for 15 min, the supernatant of the dialysate was applied to a Sephadex G-100 column (2 x 90 cm) equilibrated in 10 mM phosphate buffer. Fractions containing PPO activity were pooled and applied to a DEAE-Sepharose CL-6B column (2.5 x 24 cm) equilibrated in the phosphate buffer. After elution of unbound proteins with 100 mL of the phosphate buffer and subsequently with 50 mL of the phosphate buffer plus 0.3 M NaCl, the PPO activity was eluted using the phosphate buffer containing 0.5 M NaCl. The fractions with PPO activity were pooled separately and dialyzed against the phosphate buffer (pH 7.0).

Protein Quantitation and Enzyme Purity Determination. The protein content of plant extracts and enzyme fractions was quantitated by the Bradford method using bovine serum albumin as a protein standard (12). Enzyme purity was examined by carrying out SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) based on the method of Laemmli (13). 10% polyacrylamide gels were run in mini-gel electrophoresis units at room temperature and stained with Coomassie Blue R-250.

Enzyme Assay. Enzyme activity was determined by measuring the rate of change in absorbance at 480 nm at 30 °C. The reaction mixture in 10 mM sodium phosphate buffer (pH 7.0) included 8 mM DL-DOPA for catecholase activity. One unit of enzyme activity was defined as the change in absorbance of 0.001 min^{-1} under the conditions of assay.

Effects of pH and Temperature. The standard assay procedure was used for the determination of the relative PPO activity at several pH values, with 0.1 M acetate buffer (pH 4 to 5), 0.1 M bis–Tris buffer (pH 6 to 7), and 0.1 M Tris buffer (pH 8). Optimal temperature was determined by assaying the relative enzyme activity at various reaction temperatures (20 °C to 50 °C). The thermostability was also studied at 40 °C, 50 °C, 60 °C, and 70 °C. For the study, enzyme samples were incubated at the required temperature for fixed time intervals. After cooling the sample on ice, the residual activity was determined.

Enzyme Kinetics Study. Kinetic parameters including Michaelis constant, K_m, for burdock PPO were calculated using the equations of Lineweaver and Burk (*14*) and Dixon (*15*). DL-DOPA was used in a concentration between 0.625 mM and 10 mM in 10 mM sodium phosphate buffer (pH 7.0). The inhibitory effects of 4–HR, ascorbic acid, and citric acid on the oxidation of DL–DOPA by burdock PPO were also investigated. Prior to addition of the substrate, the mixture containing the enzyme–inhibitor complex was incubated at 30 °C for 5 min. After equilibration, DL–DOPA was added and the enzyme activity was monitored as previously described.

Results and Discussion

Extraction and Purification of PPO. One major problem encountered during the extraction of the enzyme from burdock was the rapid oxidation of natural phenolic compounds, resulting in browning and even blackening of the enzyme extract. There have been reports that browning causes partial inactivation (*6*) and apparent increases in multiplicity of enzymes isolated (*16*). Initially, insoluble PVPP, due to its ability to remove phenolics (*11*), was used as a pre–column to the Sephadex G–100 column. However, it caused a significantly reduced flow rate of the Sephadex column even after the applied sample was filtered through Millex–GV 0.22 μm membrane. The use of insoluble PVPP during homogenization was instead optimized at 1.0% (w/v) in the grinding buffer, which did not result in browning of the extract on standing. The extracted enzyme activity was found to increase up to 1.0% (w/v) Triton X–100, probably due to released membrane–bound PPO enzymes (*17,18*), and then to reach a plateau.

The results of a typical purification of burdock PPO are shown in Table I. It is noted that the most active fraction eluted from the DEAE–Sepharose chromatography was purified 160–fold, with 5% of the activity of the original crude extract. The purity of the enzyme preparation was monitored by SDS–PAGE. Two peaks with PPO activity were eluted in the ion–exchange chromatography (Figure 1). The major peak, eluted at higher ionic strength buffer, had a higher activity.

Characterization of Burdock PPO. Figure 2 shows the effect of pH on the oxidation of DL–DOPA by the purified burdock PPO. The optimum pH for PPO activity varies with source of the enzyme and with different substrates in a relatively wide range of pH (*19*). The pH curve obtained from burdock PPO shows a maximum at pH 7.0.

A typical temperature dependence of the purified enzyme is shown in Figure 3. The enzyme had the maximum activity at 30 °C, with a precipitous
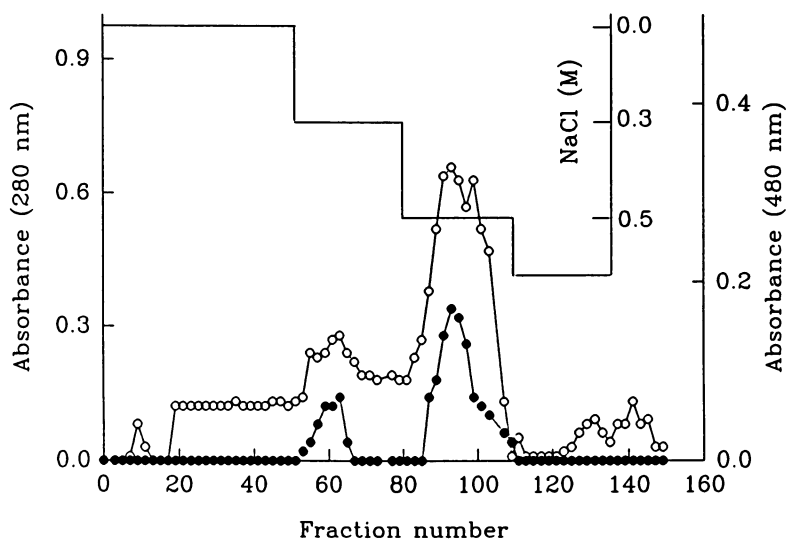
Figure 1. An ion-exchange chromatography elution profile of burdock PPO on DEAE-Sepharose CL-6B.   PPO activity (•) was measured under the standard assay conditions at 480 nm and protein absorbance (o) was measured at 280 nm.
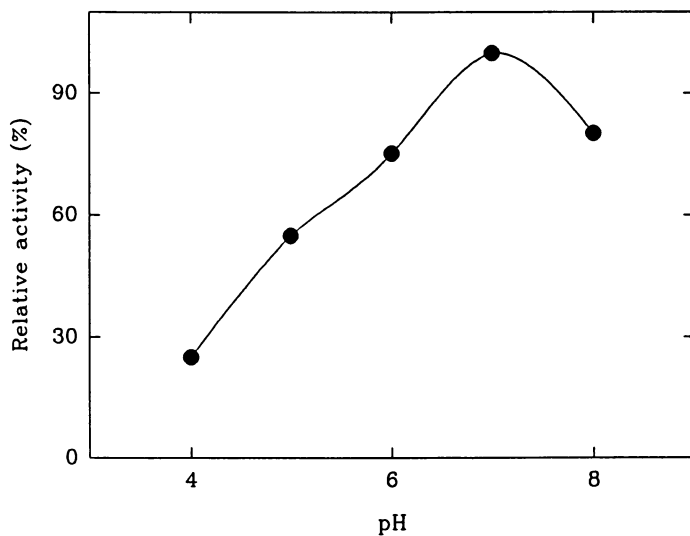
Figure 2. Effect of pH on the activity of burdock PPO.

Table I. Results obtained during purification of PPO from burdock (Arctium lappa)

step	Total volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield %	purification factor
Crude extract	500	1210	164	0.14	100	1.0
Ammonium sulfate (25-75%)	220	370	69	0.19	42	1.4
Gel filtration	42	6.3	31	4.92	19	35.1
Ion exchange	16	0.4	9	22.5	5	161

drop in activity at both sides, which is much lower than that (60 °C) reported by Murao et al. (*20*). The activities at 20 °C and 40 °C were decreased much less than half the maximum activity at 30 °C. Figure 4 shows a thermostability profile of PPO. Heating for 30 min at 40 °C did not cause a significant loss of enzyme activity. The time required for 50% inactivation of activity at 60 °C, however, was found to be about 10 min. There has been a report that PPO from guava (*21*) was relatively less thermostable than other fruits such as grape (*22*), banana (*23*), avocado (*24*), pear (*25*), and mango (*26*). The purified PPO from burdock was also relatively less stable, losing about half of its original activity after 7 days, if maintained at 4 °C. However, held at room temperature, half of the original activity was lost overnight at pH 7.0.

Inhibition Effects of 4-HR, Ascorbic Acid, and Citric Acid. The effects of increasing amounts of 4-HR, ascorbic acid, and citric acid were studied on the PPO activity in oxidizing DL-DOPA. All these compounds showed a concentration dependent inhibitory effect on the oxidation of DL-DOPA and Figure 5 illustrates the case of 4-HR.

The Michaelis constant for the oxidation of DL-DOPA was 4.2 mM, deduced from Lineweaver-Burk double reciprocal plot (Figure 6). The calculated K_m was close to that of white shrimp (3.2 mM) but was higher than the value of mushroom (0.29 mM) and of apple (0.04 mM) for the oxidation of DL-DOPA (*27*). The type of inhibition was also deduced from double reciprocal plots and a competitive inhibition was observed for 4-HR (Figure 7). Citric acid was also found to behave as a competitive-type inhibitor (Figure 8). In Table II a preliminary study of the inhibitory effects of 4-HR, ascorbic acid, and citric acid is described. The addition of 4-HR (about 100 μM) to the assay system containing DL-DOPA caused the inhibition of PPO by 50%. However, 4-HR was found to be effective at a lower concentration (up to 1 μM), compared to other inhibitors used. Combined uses of these inhibitors showed a synergistic effect at a low concentration. The inhibition studies are to be more thoroughly investigated.
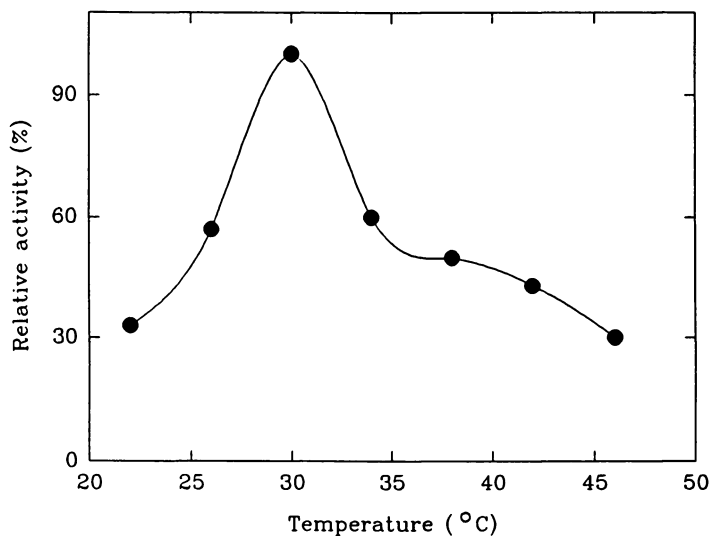
Figure 3. Effect of temperature on the activity of burdock PPO.
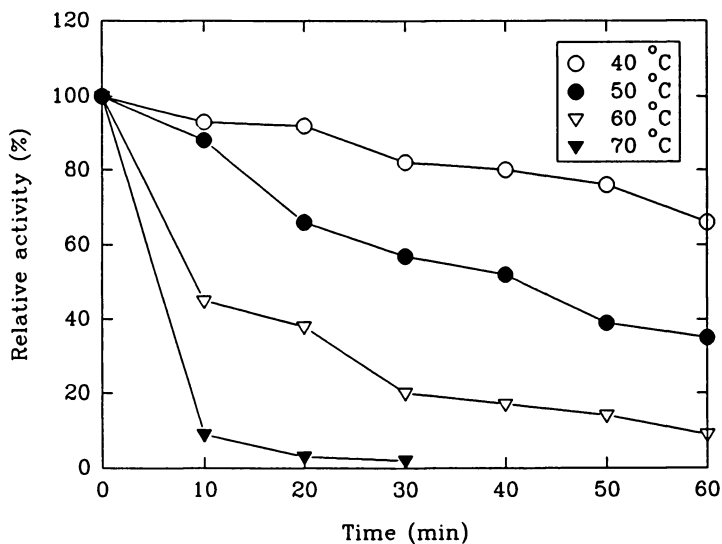
Figure 4. Thermostability of burdock PPO at various temperature.
o 40 °C; • 50 °C; ▽ 60 °C; ▼ 70 °C.
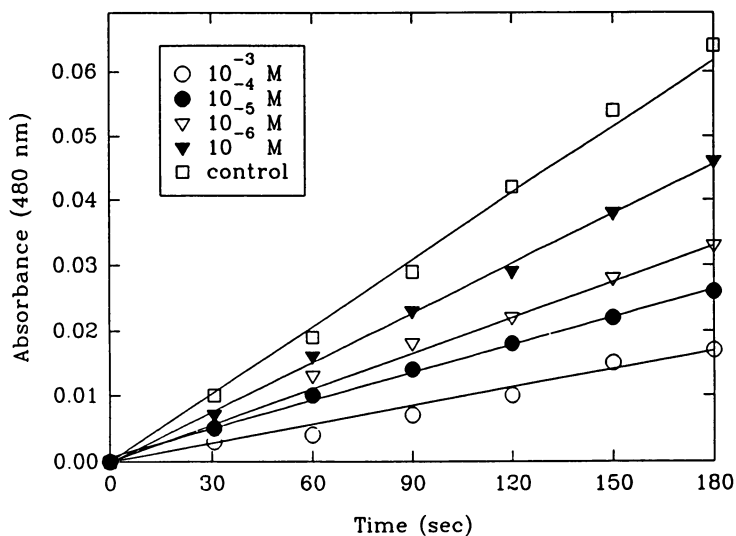
Figure 5. Effect of 4-hexylresorcinol on the rate of DL-DOPA oxidation by burdock PPO.
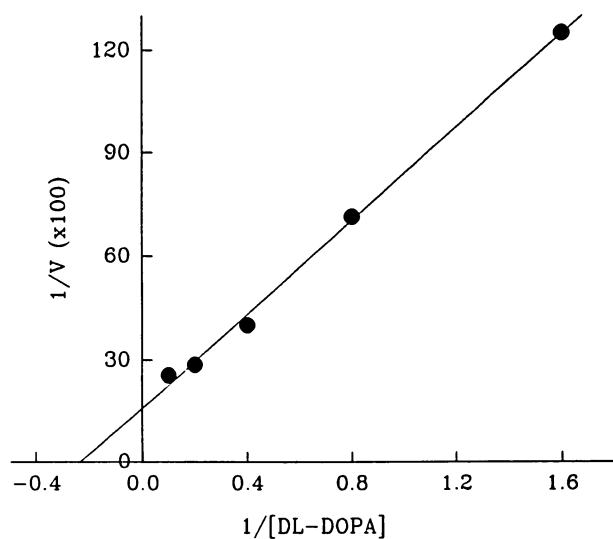
Figure 6. Lineweaver-Burk plot of DL-DOPA oxidation activity of burdock PPO.
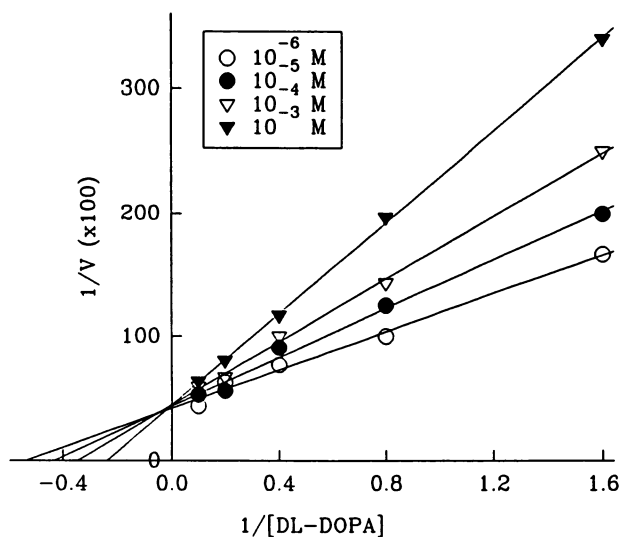
Figure 7. Inhibition of burdock PPO by 4-hexylresorcinol.
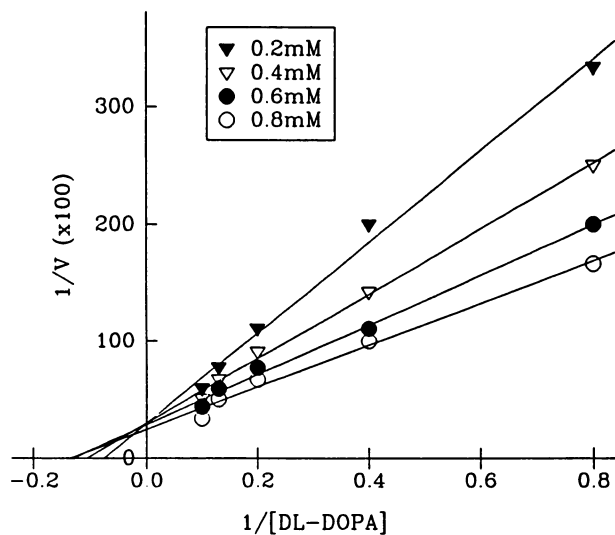
Figure 8. Inhibition of burdock PPO by citric acid.

Table II. Effect of inhibitors on the burdock PPO activity

Concentration	4-HR	Ascorbic Acid	Citric Acid	4-HR/ Ascorbic	4-HR/ Citric	Citric/ Ascorbic
			(% Inhibition)			
None	0	0	0	0	0	0
1 μM	32	3	5	62	59	46
10 μM	45	38	48	68	68	57
100 μM	56	54	55	70	73	60
1000 μM	62	–	70	–	–	–

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Chapter 22

Multiple Effects of Maltol and Kojic Acid on Enzymatic Browning

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Maltol (3-hydroxy-2-methyl-4H-pyran-4-one) inhibits the rate of oxidation of different *o*-dihydroxyphenols by tyrosinase when assayed spectrophotometrically but not when assayed polarographically. This finding, as well as spectral data obtained, suggest that maltol does not inhibit tyrosinase activity *per se* but only gives an apparent inhibition probably due to its ability to conjugate with *o*-quinones. Kojic acid (5-hydroxy-2 (hydroxymethyl) - 4H-pyran-4-one), a γ-pyrone closely related to maltol, is a very effective inhibitor of tyrosinase as judged by its effect on the rate of pigmented products formation and on the rate of oxygen uptake when different *o*-dihydroxyphenols are oxidized by the enzyme. In addition to the ability of kojic acid to inhibit the enzyme *per se*, the data show that kojic acid can change the spectrum of some pigmented products formed in its absence, probably due to the ability of some o-quinones, formed enzymatically, to oxidize kojic acid to a yellow product(s). This possibility is supported by the finding that kojic acid is oxidized to a yellow product(s) by the horseradish peroxidase/H_2O_2 (HRP/H_2O_2) system as well as by $NaIO_4$, Ag_2O and $KMnO_4$.

Enzymatic browning is caused mainly by the action of the enzyme tyrosinase, also known as phenolase, polyphenoloxidase, catecholase and cresolase. Tyrosinase is a copper-containing enzyme that can carry out two different biochemical reactions:

A. monohydroxyphenol + O_2 + AH_2 → *o*-dihydroxyphenol + H_2O + A

B. *o*-dihydroxyphenol + ½O_2 → *o*-quinone + H_2O
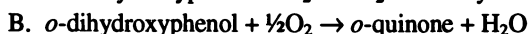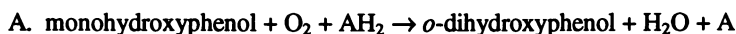
where AH_2 represents a hydrogen donor. The *o*-quinones formed polymerize very rapidly to pigmented product(s).

Chelators that can bind copper at the active site of tyrosinase are effective inhibitors of the enzyme. This is true, for example, with mimosine (2-amino-3-hydroxy-4-oxo-1 (4H)-pyridine propionic acid (*1*) and with tropolone (2, 4, 6-cycloheptatrien-1-one) (*2*). In the course of our search for means to prevent enzymatic browning in food, we noted that γ-pyrone derivatives like maltol (3-hydroxy-2-methyl-4H-pyran-4-one)   and   kojic   acid   (5-hydroxy-2(hydroxymethyl)-4H-pyran-4-one) as well as mimosine and tropolone all have an α, β-unsaturated keto-enol as a common denominator in their structure, a feature wich makes them good copper chelators.

Since maltol is used commercially as a food flavorant (*3*), and since kojic acid contributes to the special taste, color and flavor of food material such as miso, soy sauce and sake (*4*), it was decided to examine the effect of maltol and of kojic acid on tyrosinase activity considering their potential use as chemicals that might prevent undesirable enzymatic browning in food. Our studies were conducted using mushroom tyrosinase as the enzyme source.

Effect of Maltol on Tyrosinase

We found that maltol, a γ-pyrone derivative, is a relatively poor inhibitor of the rate of formation of pigmented products when different *o*-dihydroxyphenols are oxidized by tyrosinase and that it does not inhibit at all the rate of oxygen uptake during the reaction (*5*). Furthermore, it was observed that the spectrum of pigmented products formed during the oxidation of different *o*-dihydroxyphenols by tyrosinase in the presence of maltol was different than that formed in its absence (*5*). The inhibitory effect of maltol on the rate of oxidation of various *o*-dihydroxyphenols to pigmented products but not on the rate of oxygen uptake during the reaction, along with the spectral data obtained, suggested that maltol exerts its inhibitory effect on tyrosinase by forming an adduct with the *o*-quinone formed during the reaction (*5*).

Based on the data obtained, it was concluded that maltol, in spite of its being a copper chelator, does not inhibit tyrosinase *per se* by binding the copper at the active site of the enzyme (or by any other mechanism), but rather gives an inhibition of pigmented products formation (*5*).

Effect of Kojic Acid on Tyrosinase

Kojic acid is a γ-pyrone derivative structurally related to maltol and, like maltol, a good chelator of transition metal ions such as Fe (III) and Cu (II) (*6, 7*). Kojic acid was reported to inhibit effectively the activity of tyrosinase from various sources including fungi, plants and animal tissues (*8-13*). It was of interest to examine whether kojic acid inhibits tyrosinase activity *per se* or gives only an apparent inhibition of pigmented product(s) formation, as maltol does, by conjugating with *o*-quinones.

Kojic acid was found to inhibit very effectively the rate of oxidation of *o*-dihydroxyphenols like catechol, 4-methyl catechol, 4-t-butyl catechol (t-BC), protocatechuic acid (3,4-DBA), caffeic acid and 3,4-dihydroxyphenylpropionic

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acid when assayed spectrophotometrically or polarographically. The effect of kojic acid on the rate of pigmented products formation and on the rate of oxygen uptake, when 3,4-dihydroxyphenylpropionic acid is oxidized by tyrosinase is illustrated, as an example, in Figure 1, and Figure 2, respectively.

The inhibition exerted by kojic acid on the rate of oxidation of different *o*-dihydroxyphenols by tyrosinase when assayed spectrophotometrically and polarographically can be taken as evidence that kojic acid, unlike maltol, inhibits tyrosinase *per se*.

The possibility that kojic acid inactivates tyrosinase, namely, causes an irreversible loss of activity of the enzyme, was examined. Tyrosinase in 47 mM sodium phosphate buffer (pH 6.5) was preincubated in the absence (control) or the presence (experimental) of 3.3-13.3 mM kojic acid for 60 min, dialyzed separately overnight against 0.05M sodium phosphate buffer (pH 6.5) and then aliquots of each of the dialyzed samples were assayed for tyrosinase activity using DL-DOPA as the substrate. Identical activities were detected in the control and in the experimental samples. It was therefore concluded that kojic acid does not inactivate tyrosinase, in agreement with the finding of Chen *et al.* (8) who reported that the inhibition exerted by kojic acid on tyrosinase was reversible by dialysis or by chromatography on Sephadex G-100.

The effect of kojic acid on the rate of oxidation of different trihydroxyphenols by tyrosinase was also examined. It was found that kojic acid inhibited effectively the rate of oxidation of pyrogallol, 2,3,4-trihydroxyacetophenone (2,3,4-THAP), 2,4,5-trihydroxybutyrophenone (2,4,5-THBP), inhibited to a limited extent the rate of gallic acid oxidation while it greatly stimulated the rate of oxidation of methyl gallate and n-propyl gallate to pigmented products. The data obtained with methyl gallate as the substrate are illustrated in Figure 3 as an example.

On the other hand, kojic acid inhibited the rate of oxygen uptake during the oxidation of each of the above trihydroxyphenols by tyrosinase; the higher the concentration of kojic acid, the more pronounced was the inhibition. Kojic acid was a more effective inhibitor of the rate of oxygen uptake when methyl gallate and n-propyl gallate were the substrates compared with its effect on the rate of pyrogallol and gallic acid oxidation. The data obtained using methyl gallate as the substrate is shown in Figure 4.

The finding that kojic acid inhibits the rate of oxygen uptake when each of the above trihydroxyphenols was oxidized by tyrosinase indicates, as was the case when *o*-dihydroxyphenols were used as the substrates, that kojic acid inhibits tyrosinase *per se*, perhaps by binding copper at the active site of the enzyme. However, the stimulation exerted by kojic acid on the rate of pigmented products formation when methyl gallate (Figure 3) and n-propyl gallate were oxidized by tyrosinase suggests that kojic acid also has an effect on the pigmented products formed during the enzymatic reaction.

Such an interaction was indeed found to occur. The data in Figure 5 demonstrate that when 6.6 mM methyl gallate was acted upon by 100 µg tyrosinase and kojic acid was added 4 min after the initiation of the reaction (indicated by an arrow), a time when the rate of pigmented products formation (λ=360 nm) was almost at the plateau phase, the addition of kojic acid resulted in an increase in the rate of pigmented products formation. However, the
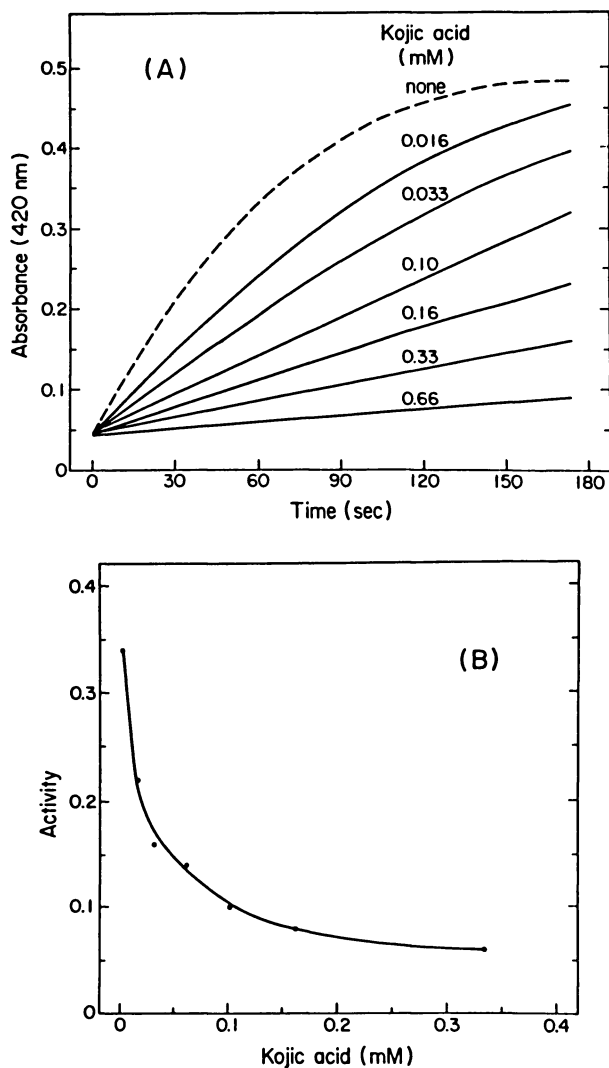
Figure 1.   Effect   of   kojic   acid   on   the   rate   of 3,4-dihydroxyphenylpropionic   acid   oxidation   by   tyrosinase   to pigmented products.
The reaction mixture included, in a total volume of 3 ml: 6.7 mM 3,4-dihydroxyphenylpropionic acid, 47 mM sodium phosphate buffer (pH 6.5), 10 μg tyrosinase (added last), and kojic acid as indicated. Activity (ΔOD 420 nm/min) was estimated from the initial portions of the kinetic curves presented in part A and was plotted as a function of various concentrations of kojic acid in part B.
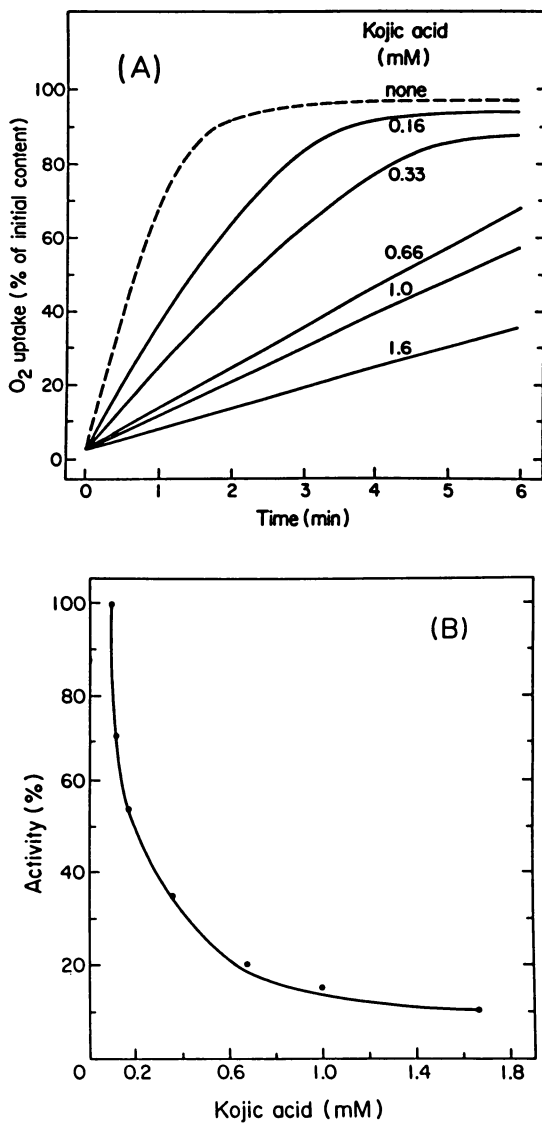
Figure 2.   Effect of kojic acid on the rate of oxygen uptake when 3,4-dihydroxyphenylpropionic acid is oxidized by tyrosinase.

The reaction mixture included, in a total volume of 3 ml: 6.6 mM 3,4-dihydroxyphenylpropionic acid, 47 mM sodium phosphate buffer (pH 6.5), 10 µg tyrosinase (added last), and kojic acid as indicated. Activity (% O_2 uptake/min) was estimated from the initial portions of the kinetic curves presented in part A and was plotted as a function of various concentrations of kojic acid in part B.
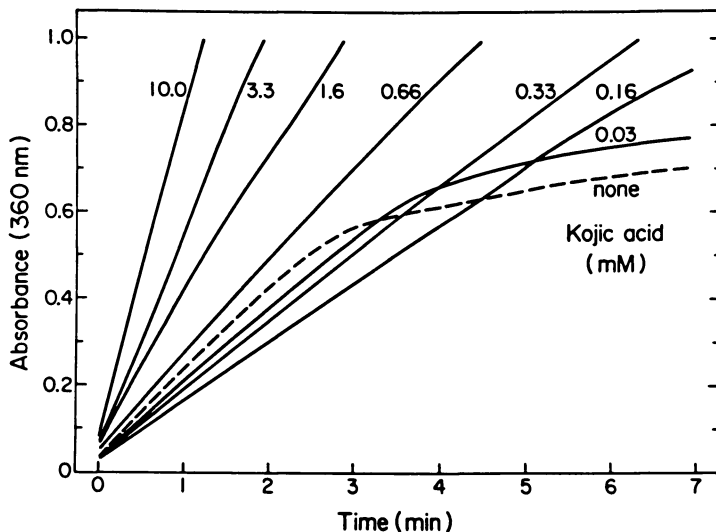
Figure 3. Effect of kojic acid on the rate of methyl gallate oxidation by tyrosinase to pigmented products

The reaction mixture included, in a total volume of 3 ml: 6.6 mM methyl gallate, 47 mM sodium phosphate buffer (pH 6.5), kojic acid as indicated and 100 µg tyrosinase (added last).

increase was less pronounced compared to when kojic acid, at the same concentration, was added at time zero (Figure 5, curves c vs. b, respectively). Additional data presented in Figure 6 show that the increase in the rate of formation of pigmented products absorbing at 360 nm was dependent on the concentration of kojic acid added at the plateau phase during the incubation of methyl gallate with tyrosinase. In the range tested (0.066-1.6 mM), the higher the concentration of kojic acid added at the plateau phase, the more pronounced was the increase in absorbance at 360 nm.

The finding that the addition of kojic acid at the plateau phase of methyl gallate oxidation by tyrosinase stimulated the rate of pigmented products formation in the 360-380 nm region (Figure 5) and the observation that the stimulation was dependent on kojic acid concentration (Figure 6) suggest the following: (a) that the o-quinone of methyl gallate conjugates with kojic acid to yield product(s) with much higher extinction coefficient (in the 360-380 nm region) than that of the o-quinone alone or (b) that the o-quinone of methyl gallate can oxidize kojic acid to a yellow product absorbing at the 360-380 nm region.

Further studies revealed that the spectrum of products obtained when certain o-dihydroxyphenols and certain trihydroxyphenols are acted upon by tyrosinase in the presence of kojic acid is different than that obtained in its absence. The data obtained with 3,4-dihydroxyphenylpropionic acid as the substrate is shown below as an example (Figure 7).
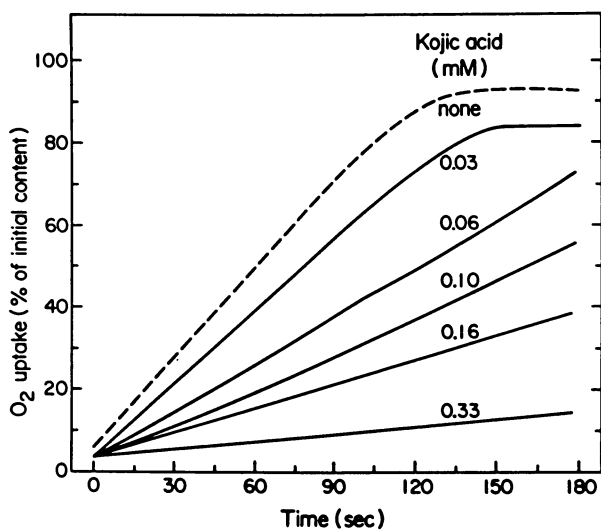
Figure 4. Effect of kojic acid on the rate of oxygen uptake when methyl gallate is oxidized by tyrosinase.
The reaction mixture included, in a total volume of 3 ml: 6.6 mM methyl gallate, 47 mM sodium phosphate buffer (pH 6.5), kojic acid as indicated and 100 μg tyrosinase (added last).
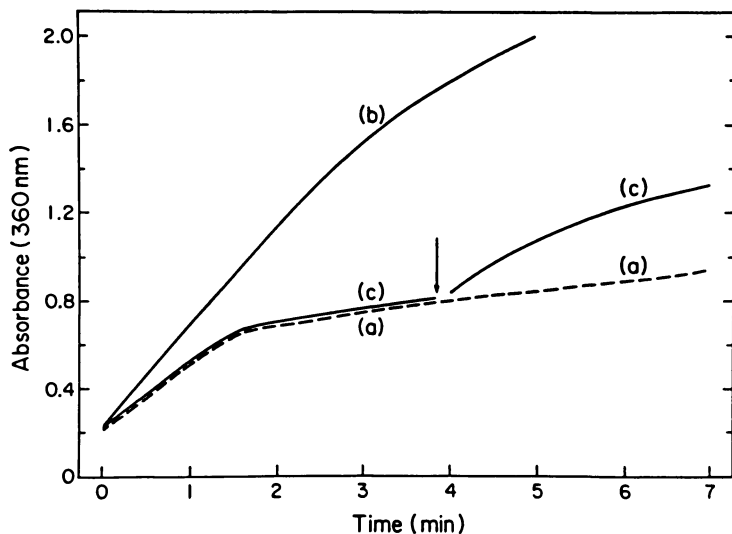
Figure 5.   Effect of time of addition of kojic acid on the rate of pigmented products formation when methyl gallate is oxidized by tyrosinase

Reaction mixture (a) included, in a total volume of 3 ml: 6.6 mM methyl gallate, 47 mM sodium phosphate buffer and 100 μg tyrosinase (added last).   Reaction mixture (b) was identical to (a) except that it contained 0.66 mM kojic acid from time zero.   Reaction mixture (c) was identical to (a) except that at the time indicated by the arrow, 0.1 ml containing 2 μmoles kojic acid was added.
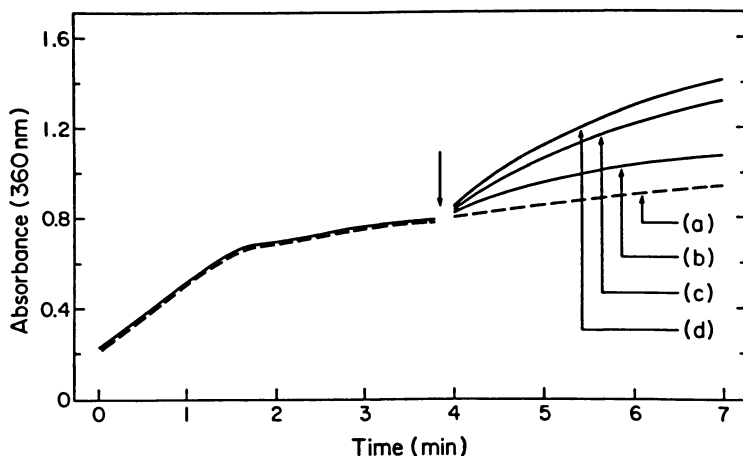
Figure 6. Effect of various concentrations of kojic acid added at the plateau phase of methyl gallate oxidation by tyrosinase on the rate of formation of pigmented products.

The reaction mixture included, in a total volume of 3 ml: 6.6 mM methyl gallate, 47 mM sodium phosphate buffer (pH 6.5), and 100 μg tyrosinase (added last). At the times indicated by the arrows, 0.1 ml aliquots containing 0, 0.2, 2, and 5 μmoles kojic acid were added to reactions (a), (b), (c), and (d), respectively, yielding fiinal kojic acid concentrations of 0, 0.066, 0.66, and 1.6 mM, respectively.

Controls showed that an aqueous solution of kojic acid is colorless and its spectrum is characterized by a relatively high peak at 225 nm and a low peak at 275 nm. Controls also showed that incubation of 3.3-33.3 mM kojic acid in the presence of 47 mM sodium phosphate buffer (pH 6.5) with 100-300 μg tyrosinase (in a total volume of 3 ml) for 20, 40, 60 min or 20 h, did not yield any pigmented products absorbing in the visible range of the spectrum.

The spectra of the final products obtained when 3,4-dihydroxyphenylpropionic acid was acted upon by tyrosinase in the absence or presence of kojic acid are different, as illustrated in Figure 7; whereas in the absence of kojic acid the sample was characterized by a shoulder at 440-510 nm, that in the presence of kojic acid was characterized by a peak at 340 nm. These results support the idea that 3,4-dihydroxyphenylpropionic acid-*o*-quinone (i.e., 4-carbxyethyl-*o*-benzoquinone) interacts with kojic acid to yield the yellow product(s) characterized by a peak at 340 nm.

In order to fully prove the possibility that 4-carboxyethyl-*o*-benzoquinone interacts with kojic acid, the following experiment was conducted: *o*-dihydroxyphenols are oxidized by Ag_2O to the corresponding *o*-quinones. Therefore, a solution of 3,4-dihydroxyphenylpropionic acid in the presence of sodium phosphate buffer (pH 6.5) was mixed with a few grains of Ag_2O for about 30 sec and immediately filtered through 4 layers of Whatman No. 41 filter
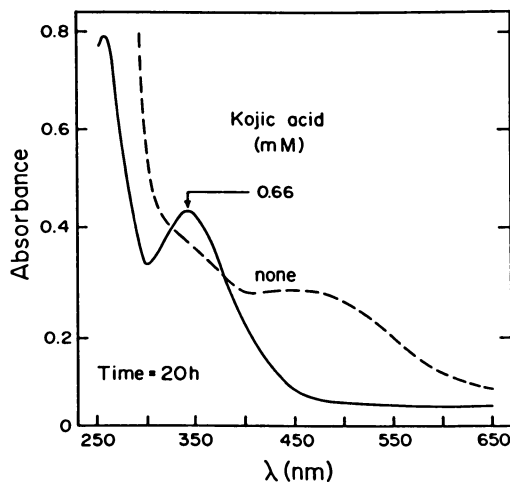
Figure 7. Effect of kojic acid on the spectrum of the final products obtained when 3,4-dihydroxyphenylpropionic acid is oxidized by tyrosinase.

The reaction mixture included, in a total volume of 3 ml: 0.16 mM 3,4-dihydroxyphenylpropionic acid, 47 mM sodium phosphate buffer (pH 6.5), 100 μg tyrosinase (added last), and kojic acid as indicated. The samples were incubated for 20 h before scanning. The sample containing kojic acid (—) was diluted 1:6 with water before scanning while that without kojic acid (---) was scanned as such.

paper. Equal aliquots of the filtrate, containing 4-carboxyethyl-*o*-benzoquinone were immediately mixed with either water (control) or with kojic acid (experimental) (each in a total volume of 3 ml) and the samples were incubated for 20 h. At that time, the control sample was reddish and the experimental sample was light yellow. The samples were diluted 1:3 and 1:108 and their spectrum scanned. At 1:3 dilution, the control sample was characterized by a peak at 490 nm and a flat shoulder at 320-380 nm while that of the experimental sample had an undefined spectrum (Figure 8). At 1:108 dilution, the control sample had a peak at 275 nm while the experimental sample had a shoulder at the 320-380 nm region (Figure 8).

It can thus be concluded that the spectrum of products obtained when 3,4-dihydroxyphenylpropionic acid was oxidized by Ag_2O was different than that obtained when kojic acid was added to a solution of Ag_2O-oxidized 3,4-dihydroxyphenylpropionic acid. These results can be taken as proof that a chemical interaction occurs between the *o*-quinone of 3,4-dihydroxyphenylpropionic acid (4-carboxyethyl-*o*-benzoquinone) and kojic acid.

Additional studies showed that a yellow product(s) absorbing in the 320-360 nm region was formed not only when 3,4-dihydroxyphenylpropionic

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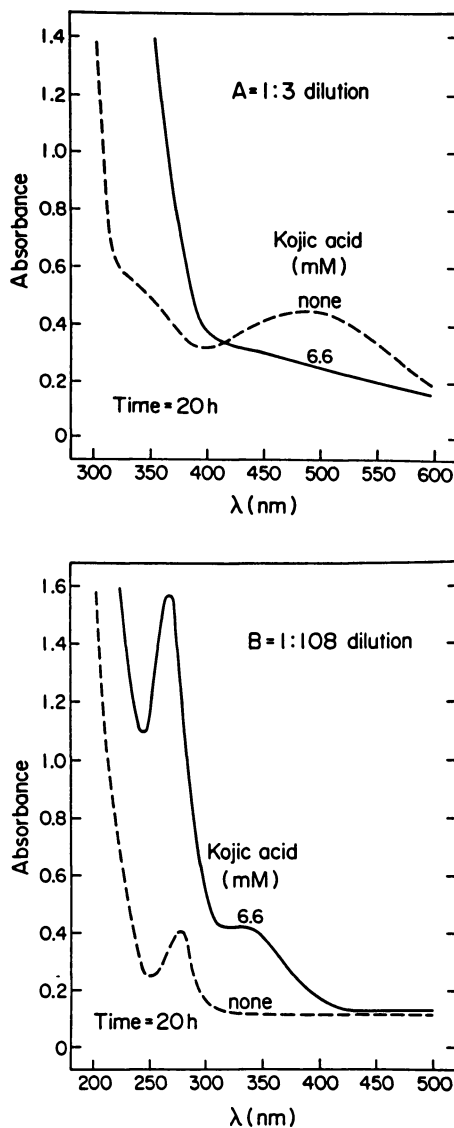
Figure 8. Spectrum of product(s) formed by an interaction of 3,4-dihydroxyphenylpropionic acid-*o*-quinone with kojic acid. A reaction mixture of 6 ml containing 16.6 mM 3,4-dihydroxyphenylpropionic acid and 47 mM sodium phosphate buffer (pH 6.5) was mixed with a few grains of Ag_2O for a few seconds and immediately filtered. 1 ml of the filtrate was mixed with either 2 ml water (---) (control) or 2 ml 0.01 M kojic acid (—) (experimental) yielding in the latter case 6.6 mM as the final concentration of kojic acid. The samples were incubated for 20 h and then diluted 1:3 (A) or 1:108 (B) with water before scanning.

acid was the substrate but also when 3,4-DBA, t-BC, methyl gallate and n-propyl gallate were each acted upon by tyrosinase in the presence of kojic acid but not in its absence, while no yellow product(s) was formed when gallic acid, pyrogallol, 2,3,4-THAP and 2,4,5-THBP were each serving as the substrate. The finding that similar products are formed by the interaction of kojic acid with different o-quinones leads to the idea that certain o-quinones, depending on their oxidation-reduction potential, can oxidize kojic acid to a yellow product(s).

This possibility was fully supported by the finding that kojic acid was oxidized to a yellow product(s) by the horseradish peroxidase/H_2O_2 (HRP/H_2O_2) system as well as by $NaIO_4$, Ag_2O, and $KMnO_4$. Data obtained with the HRP/H_2O_2 system and with $NaIO_4$ are illustrated below.

Interaction of Kojic Acid with the HRP/H_2O_2 System

Peroxidase, a haem containing enzyme, in the presence of H_2O_2, can also oxidize o-dihydroxyphenols to pigmented products, thus contributing to the enzymatic browning of food. The effect of kojic acid on horseradish peroxidase (HRP) was therefore examined. It was found that when HRP and kojic acid were incubated in the presence of H_2O_2 but not in its absence, a bright yellow product(s) characterized by a peak at 375 nm was formed (Figure 9). The relationships between, and effects of, various concentrations of HRP, kojic acid and H_2O_2 on the rate of kojic acid oxidation to the yellow product(s) were studied. The oxidation of kojic acid to the yellow product(s) was found to occur best in the presence of very low concentrations of H_2O_2 relative to that of kojic acid (Figure 9), suggesting that kojic acid, relative to H_2O_2, is a poor hydrogen donor (AH_2) for HRP.

Interaction of Kojic Acid with $NaIO_4$, $KMnO_4$, and Ag_2O

In view of the finding that a yellow product(s) was formed by the interaction of kojic acid with certain o-quinones as well as by the HRP/H_2O_2 system, suggesting that the yellow product(s) is an oxidized form of kojic acid, we examined the response of kojic acid to oxidizing agents such as $NaIO_4$, $KMnO_4$, and Ag_2O. Some of the results obtained when kojic acid interacts with $NaIO_4$ are shown below as an example.

When kojic acid was acted upon by $NaIO_4$ in the presence of sodium phosphate buffer (pH 6.5), a yellow product(s) was formed and its intensity increased as a function of time (Figure 10). The yellow product(s) was also stable in the presence of sodium acetate or sodium maleate buffers (pH 6.5).

As seen in Figure 11 part A, the spectrum of the yellow product(s) obtained when kojic acid was acted upon by $NaIO_4$ for 20 h in 1:0.25, 1:0.5 or 1:1 ratios in the presence of sodium phosphate buffer (pH 6.5) is characterized by a peak at 375-380 nm. The yellow product(s) formed after incubation for 20 h or more is referred to as the "final yellow product(s)". A plot of the maximum absorbancy at 375 nm of the "final yellow product(s)" formed when 3.3 mM kojic acid was acted upon by various concentrations of $NaIO_4$ was plotted in Figure 11 part B.
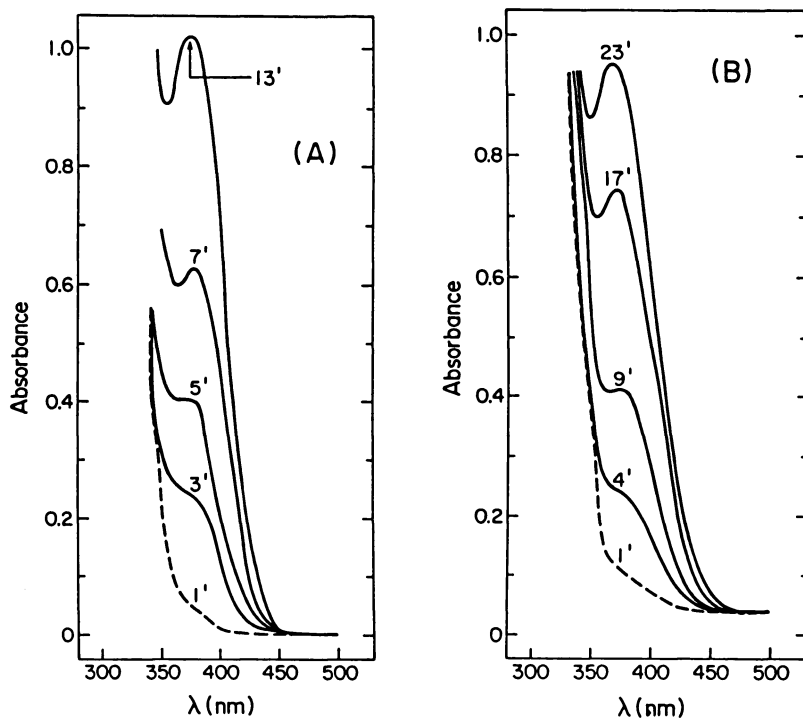
Figure 9. Changes with time in the spectrum of products formed when kojic acid is acted upon by the HRP/H_2O_2 system: Effect of H_2O_2 concentration.

The reaction mixture included, in a total volume of 3 ml: 6.6 mM kojic acid, 47 mM sodium phosphate buffer (pH 6.5), 10 µg HRP (added last), and H_2O_2 as follows: A = 3 µM; B = 33 µM; C = 1000 µM.

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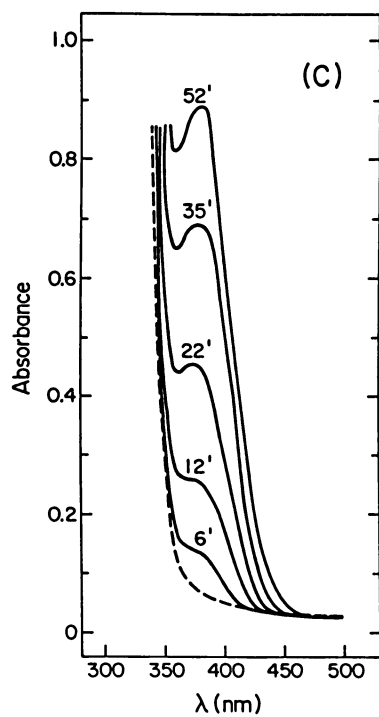
Figure 9. Continued.
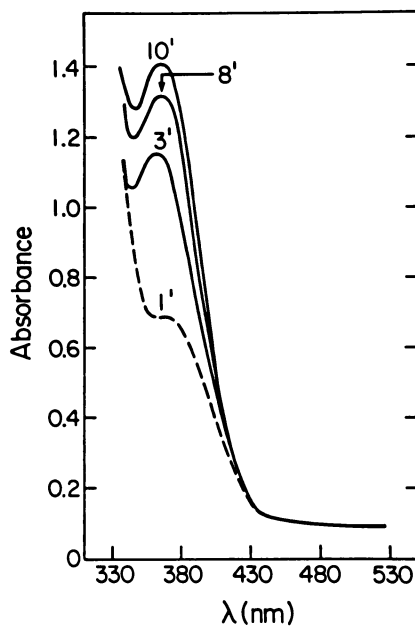
Figure 10. Changes with time in the spectrum of products obtained when kojic acid is acted upon by NaIO$_4$ in 1:0.5 ratio in the presence of sodium phosphate buffer (pH 6.5).

The reaction mixture included, in a total volume of 3.5 ml: 3.3 mM kojic acid, 1.6 mM NaIO$_4$ (added last), and 47 mM sodium phosphate buffer (pH 6.5).
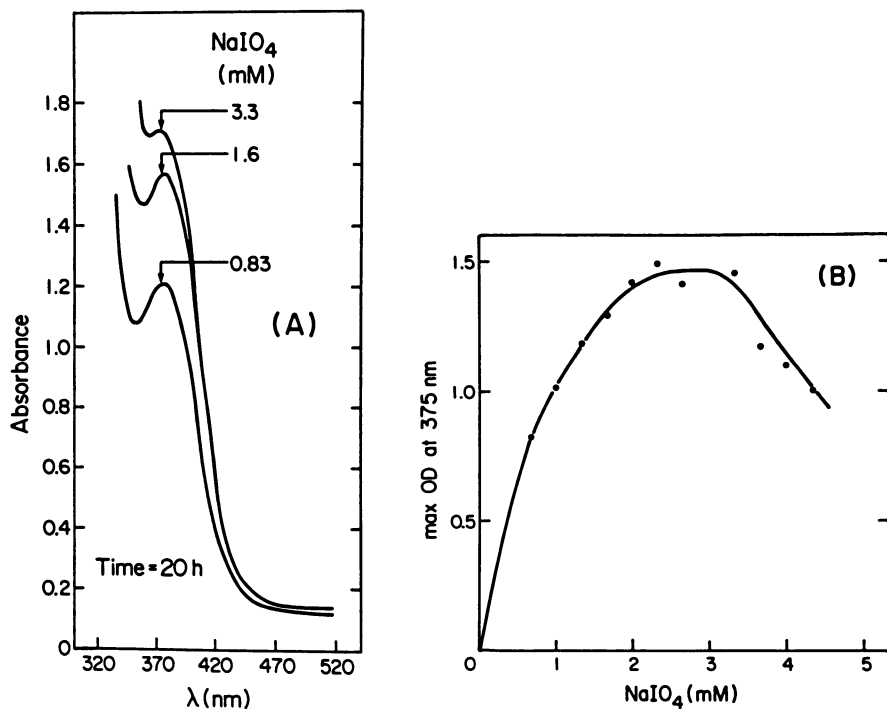
Figure 11. Spectrum of the "final yellow product(s)" obtained when kojic acid is acted upon by NaIO₄. Relationship between maximum absorbance at 375 nm and NaIO₄ concentration.

The reaction mixture included, in a total volume of 3 ml: 3.3 mM kojic acid, 47 mM sodium phosphate buffer (pH 6.5), and NaIO₄ in concentrations up to 4.3 mM. The spectrum was scanned after 20 h incubation (only some of the data is shown).   The maximum absorbance at 375 nm of the "final yellow product(s)" is plotted in part B as a function of NaIO₄ concentration.

At concentrations of $NaIO_4$ up to 2.3-3.3 mM, the higher the $NaIO_4$ concentration, the higher was the maximum absorbancy at 375 nm, but at $NaIO_4$ concentrations above 3.3 mM the extent of the absorbancy of the "final yellow product(s)" formed declined (Figure 11 part B). These results suggest that one molecule of $NaIO_4$ oxidizes one molecule of kojic acid while higher concentrations of $NaIO_4$ bleach the yellow product(s) formed.

Polarographic data showed that molecular oxygen is consumed during the oxidation of kojic acid by $NaIO_4$ to the yellow product(s). A yellow product(s) characterized by a peak at 370-380 nm, was also formed when kojic acid was oxidized by $KMnO_4$ or Ag_2O.

Conclusions

Our data show that kojic acid inhibits very effectively the rate of pigmented products formation as well as the rate of oxygen uptake when various o-dihydroxy- and trihydroxyphenols are oxidized by tyrosinase. These results suggest that kojic acid inhibits tyrosinase *per se*, probably due to its ability to bind to copper at the active site of the enzyme since kojic acid is a good chelator of Cu (II) (7). It is important to remember that the inhibition exerted by kojic acid on tyrosinase activity was reversed by dialysis indicating that kojic acid does not inactivate the enzyme (by removing copper from the active site of the enzyme or by another mechanism) but only inhibits its activity. The results that we obtained on the effect of kojic acid on tyrosinase are different than those that we have obtained with maltol, a γ-pyrone derivative closely related to kojic acid. Maltol did not inhibit the rate of oxygen uptake but only the rate of pigmented products formation when different o-hydroxyphenols were acted upon by tyrosinase, this being probably due to the ability of maltol to conjugate with o-quinones but not to inhibit the enzyme (5).

We do not have an explanation as to the chemical differences between kojic acid and maltol that could account for the differences in the response of tyrosinase to these related γ-pyrone derivatives nor to the ability of maltol to conjugate with o-quinone and the ability of kojic acid to be oxidized by certain o-quinones. Clues to these differences could help design very effective inhibitors of tyrosinase. We do not have, as yet, data as to the structure of the yellow product(s) formed when kojic acid is oxidized by certain o-quinones, by the HRP-H_2O_2 system, by $NaIO_4$, $KMnO_4$, or Ag_2O.

Kojic acid was found to be a much more efficient inhibitor of tyrosinase than maltol, making kojic acid a potentially better inhibitor of enzymatic browning in food. Several conclusions, of interest to food biochemists, that can be drawn from the finding shown above, relate to the potential use of kojic acid for the prevention of browning that results from tyrosinase activity (8-10). Kojic acid would produce a yellow pigment(s) if added to tissue which, besides tyrosinase, posseses peroxidase activity as well. In such tissues, kojic acid would inhibit browning resulting from tyrosinase activity (the extent of the inhibition being dependent on the type and level of endogenous o-dihydroxy- and

trihydroxyphenols in the tissue); but the yellow product(s) formed by the action of peroxidase on kojic acid in the presence of very low concentrations of H_2O_2 would contribute an undesirable yellow pigment(s) to the treated tissue.

In addition, trihydroxyphenols such as gallic acid, methyl gallate and n-propyl gallate are often used in the food industry as antioxidants (14, 15). The finding that an interaction of kojic acid with o-quinones of n-propyl gallate and of methyl gallate result in the formation of a yellow product(s) leads to the conclusion that the use of kojic acid to inhibit enzymatic browning in processed food to which methyl gallate or n-propyl gallate were added as an antioxidant is not to be recommended in the case of samples that are not yellow originally.

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Chapter 23

Effect of Cyclodextrins on Polyphenol Oxidation Catalyzed by Apple Polyphenol Oxidase

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In order to find nonsulfite antibrowning agents for the food industry, cyclodextrins (CD) have been tested. CD are able to form inclusion compounds with polyphenols and inhibition properties have been studied in model solutions containing one or two phenols and purified apple polyphenol oxidase (PPO). The dissociation constants (K_D) of a series of phenolic compounds with α-CD, β-CD and hydroxypropyl-β-CD have been determined both by enzymatic kinetic analysis and NMR experiments in solution with one phenol. Large differences have been found among the phenols tested resulting in wide variations in the inhibitory properties of CD. In the mixtures of two phenolic compounds, addition of β-CD can result either in a large inhibition, a slight inhibition, or even a slight activation of oxygen uptake, depending on the kinetic constants and K_D of the phenols present in the solution. Moreover, since β-CD induced a modification of the balance of free substrates in a multiphenolic solution, the colors obtained after enzymatic oxidation catalyzed by PPO can also be modified in different ways by addition of β-CD as demonstrated on the 3 types of mixtures of two phenolic compounds that we have tested, namely chlorogenic acid / (-)-epicatechin, chlorogenic acid / (+)-catechin and 4-methylcatechol / caffeic acid.

Enzymatic browning which occurs during handling, storage and processing of fruits and vegetables is mainly initiated by the enzyme polyphenol oxidase (*o*-diphenol : oxygen oxidoreductase EC 1.10.3.1) or PPO (*1, 2*). The loss of cell integrity results in the decompartmentation of phenolic substrates and enzymes and then, in the presence of molecular oxygen, the oxidative production of colored quinones (*3*). Brown pigmentation following this enzymatic reaction and subsequent non enzymatic

reactions, is generally considered to be detrimental to food quality from both the organoleptic and nutritional points of view (4). Therefore, the control of enzymatic browning has always been a challenge to food scientists owing to the losses that it causes in many food products (5). Sulfur dioxide (and its derivatives) is by far the most effective inhibitor of browning. It acts at very low concentrations and is inexpensive. However, because of the potential health hazards, the tendency of national and international regulations is to reduce or even ban its use in the fruit and vegetables industry (6). Thus, an active field of research is currently under development to find non-sulfite antibrowning agents for the food industry (7).

Enzymatic browning can be controlled by different ways (8). Besides heat and acidification treatments (9,10), several chemical inhibitors have been examined including ascorbic acid and its derivatives (11-14), cysteine (15,16), halide ions (17,18), aromatic carboxylic acids (18), sulfated polysaccharides (19), kojic acid (20), resorcinol derivatives (21), polyphosphates (22) and extracts from honey (23) and ficin (24). These compounds mainly affect the enzyme, polyphenolic substrate, or reaction products, although, in some cases, two or three targets can be affected at the same time. Recently, cyclodextrins (CD) have also been proposed for control of the enzymatic browning of apple products (22, 26) and use of CD have been patented (25).

CD are macrocyclic torus-shaped non-reducing oligosaccharides of six or more D-glucopyranose residues. The most extensively studied CD are the α-, β-, and γ-CD forms which have respectively six, seven and eight glucose units per macrocycle linked by α (1-4) glycosidic bonds. The central cavity is hydrophobic whilst the outside parts are hydrophilic, due to the location of the primary and secondary hydroxyls at the narrow and wide bases, respectively (27, 28). In aqueous solution, the hydrophobic cavity can accomodate a wide range of guest molecules forming a stable complex. Although the host : guest ratio is usually of 1 : 1, one, two or three CD molecules can contain one or more guest molecules. For phenolic compounds, β-CD offers the most suitable cavity size since its dimensions are comparable to those of substituted phenyl groups (29). This, coupled with the fact that β-CD is the cheapest to obtain commercially, explains why it is by far the most extensively studied CD (27, 28). However, β-CD has a very low water solubility (1.85 g / 100 mL at 25 °C) compared with α- and γ-CD (14.5 and 23.3 g / 100 mL respectively). Since this low solubility is mainly due to intra-molecular hydrogen bonding, various derivatives have been proposed with enhanced water solubility (30, 31). Among them, hydroxypropyl-β-CD (HP-β-CD) derivatives are highly water soluble (> 50 g / 100 mL at 25 °C) and more stable in acidic conditions and in the presence of α-amylase than β-CD (32).

Only three studies have been carried out on the effects of CD on enzymatic browning of apple (22, 25, 26). The first one showed that β-CD, but not α- or γ-CD, was an effective inhibitor in Granny Smith juice browning (22). The others indicated that insoluble β-CD and derivatized-β-CD gave similar results to that of β-CD but the percentage of inhibition which increased with the CD concentration was dependent on the apple variety (25, 26). The latter authors proposed an effect of the phenolic composition.

Few studies have been devoted to the interactions of the main phenolic substrates of apple enzymatic browning, i.e. chlorogenic acid and flavan 3-ol derivatives (8), with CD. These works were concerned with the complexation of the

flavan 3-ol derivatives by α- and β-CD (*33, 34*) on the one hand and that of chlorogenic acid by α-, β-and γ-CD and polymerized β-CD (*35*) on the other hand. These studies, mainly using NMR spectroscopy and UV spectrophotometry, showed that the dissociation constants of the phenol-CD complex (assuming a 1:1 stoichiometry) widely varied according to the nature of the phenol and of the CD. In the latter study, the authors concluded that the enzymatic browning control was mainly due to the sequestration of PPO substrates by CD (*35*).

The purpose of this work was mainly focused on the effect of CD on the enzymatic phenolic oxidation catalyzed by purified apple PPO in model solutions. Dissociation constants of the phenol-CD complex have been obtained and compared to NMR data. Since in natural products, PPO acts on a phenol mixture, the enzymatic study has been extended to solutions containing two phenolic compounds. In the latter case, both oxygen uptake and color formation have been studied.

Materials and Methods

Materials. Apples from the variety Red Delicious picked at commercial maturity were used as an enzyme source. The PPO was 120-fold purified from the cortex in three steps : extraction, fractional precipitation by ammonium sulfate and hydrophobic chromatography with Phenyl Sepharose (Pharmacia) according to the method of Janovitz-Klapp et al. (*36*). The different phenolic substrates were reagent grade from Sigma (St.Louis) and used without further purification. Native α-CD, β-CD and a HP-β-CD derivative were kindly provided by Roquette (Lestrem, France). Their specifications were as follows : α-CD > 99.4 %, β-CD 99 % min and HP-β-CD (non substituted β-CD < 1 %) with a molar substitution between 0.55 and 0.65. All CD were used as received.

Assay of PPO Activity. PPO activity was routinely assayed by polarography at 30 °C according to the method of Janovitz-Klapp et al. (*37*) using 4-methylcatechol (20 mM) as substrate in a McIlvaine buffer solution at pH 4.5. Activity was expressed in nmol of oxygen consumed per second (nkat) in the assay conditions.

Inhibition of PPO Activity by CD. The phenolic substrate concentrations ranged from 0.5 to 40 mM (the limits were dependent on the Km values) in the control and with two concentrations of the different CD. The CD concentrations ranged from 2 to 20 mM depending on the phenol studied. A constant amount of 30 nkat of purified apple PPO was added to 2 mL of the air saturated phenolic solution. Some inhibition experiments were carried out on binary mixtures of phenols, namely 4-methylcatechol (1 to 20 mM) and caffeic acid (0.1 to 1 mM) or chlorogenic acid (0.5 to 5 mM) and catechin (0.5 to 5 mM), using β-CD (5 or 10 mM). In the latter case, the two isomers of catechin, (+)-catechin and (-)-epicatechin, were tested.

Calculation of the K_D (dissociation constant) of the CD-Phenol Complex from Kinetic Experiments. Assuming a 1 : 1 stoichiometry

$$K_D = \{[CD] \, [S]\} / \{[CD.S]\}$$

The mass balance equations for S and CD are

$$[S]_t = [CD.S] + [S] \quad \text{and} \quad [CD]_t = [CD.S] + [CD]$$

where $[S]_t$ and $[CD]_t$ are the total amounts of phenol and CD in the solution, respectively.
Hence,

$$K_D = \{([CD]_t - [S]_t + [S]) [S]\} / \{[S]_t - [S]\}$$

After rearranging

$$[S]^2 + ([CD]_t - [S]_t + K_D) [S] - K_D [S]_t = 0$$

The negative solution can be ignored because [S] must be positive

$$[S] = \{ - ([CD]_t - [S]_t + K_D) + \{([CD]_t - [S]_t + K_D)^2 + 4 K_D [S]_t\}^{1/2} \} / 2 \qquad \textbf{(A)}$$

This expression representing the amount of free phenol available for PPO may be substituted in the velocity equation assuming that PPO is inactive on the [CD.S] complex

$$v = Vm [S] / (Km + [S]) \qquad \textbf{(B)}$$

Firstly, the apparent Km and Vm values were determined from the control experiments by using a non linear regression data analysis program developed by Leatherbarrow (38). Secondly, the K_D values were obtained using an homemade program by fitting experimental and calculated velocities in the presence of CD.

¹H NMR Experiments. All studies were carried out at room temperature with a Bruker AC-200 E spectrometer operated at 200 MHz. The free induction decays were accumulated in 16 K data points over a 2.4 KHz spectral width, with a 3 μs pulse width (ca 40° flip angle) and operating at LB = - 0.5 and GB = 0.3. The number of scans ranged from 100 to 300. ¹H NMR spectra were recorded in 99.8 atom-% D_2O solutions (SDS, France). Chemical shifts were assigned relative to water (4.74 ppm) using 3-(trimethylsilyl)-propionic acid, sodium salt in D_2O as external reference. For the determination of the apparent K_D value, the β-CD concentration was maintained at 5 mM in D_2O while the phenol concentration varied between 0.5 and 40 mM (10 different concentrations have been tested for each phenol). Spectra were recorded at least one hour after each addition and the values of chemical shift changes $\Delta\delta$ ($\Delta\delta = \delta_{free} - \delta_{complexed}$) which occur for the H-3 and/or H-5 β-CD protons were used for the estimation of K_D (33, 39).

L*, a*, b* Measurements. The effect of β-CD on the color variations during the PPO catalyzed oxidation of binary phenolic mixtures was followed using tristimulus colorimetry. Five mL of air saturated phenolic substrates with or without β-CD (4 mM), were placed in small beakers. As soon as 8 nkat of purified apple PPO was added, the L*, a*, b* values of the solution were recorded every minute for 30 minutes using a Minolta CR 300 chromameter. Three mixtures have been tested, namely 4-methylcatechol / caffeic acid, chlorogenic acid / (+)-catechin and chlorogenic acid / (-)-epicatechin.

Results and Discussion

Effect of CD on PPO Activity in a Single Phenolic Solution. When a constant amount of CD was added to solution containing variable amounts of phenolic substrate, an inhibition of oxygen uptake catalyzed by purified apple PPO was observed. This inhibition decreased as the phenolic concentration increased resulting in a sigmoidal shape for the velocity curve versus substrate concentration. Two typical curves are given in Figure 1 for chlorogenic acid and (+)-catechin in the presence of β-CD. CD are well-known for their ability to form inclusion complexes with phenols (*34*) resulting in substrate depletion for PPO (*26, 35*). Assuming a 1 : 1 stoichiometry between CD and phenol (*33, 35*), an equation containing the dissociation constant (K_D) of the complex CD / Phenol, which gives the amount of free substrate available for the enzyme has been developed (Equation A). The K_D value can be obtained by fitting the experimental and calculated velocities. For the above experiments with β-CD, the K_D values obtained were 2.2 and 0.19 mM for chlorogenic acid and (+)-catechin respectively. With these values, the experimental points were in close agreement with the calculated velocities (dashed and dotted lines in Figure 1). A similar treatment of data was carried out for a series of phenolic compounds in the presence of α-CD, β-CD or HP-β-CD. The K_D values obtained for these different complexes are given in Table I. With β-CD, the K_D values ranged from 0.2 to more than 30 mM meaning that the binding strength of β-CD widely varied from one phenol to another. Obviously, for low substrate concentrations, high K_D values resulted in slight inhibition of PPO activity and conversely, low K_D values in strong inhibition. Among the phenols tested, β-CD bound weakly the smallest ones, i.e. pyrocatechol, 4-methylcatechol and *o*-dihydroxyphenylacetic acid whereas the largest ones, i.e. chlorogenic acid and catechins which are also the most important natural substrates for apple PPO, were bound more strongly. Moreover, the K_D values of (+)-catechin was more than 10 fold lower than that of (-)-epicatechin, meaning that β-CD was stereoselective between these two isomers of catechin. HP-β-CD exhibited a similar behaviour than β-CD since the K_D values were of the same order of magnitude. Owing to its higher water solubility and since in almost all cases the K_D values were slightly lower, HP-β-CD could be considered as a better inhibitor of apple enzymatic browning than β-CD. α-CD was tested for smaller phenols because of its reduced inner cavity (ca 5 Å). However, compared to β-CD or HP-β-CD, its binding capacity of pyrocatechol and 4-methylcatechol was not improved. Therefore, α-CD cannot be substituted for β-CD (or HP-β-CD) for inhibition of PPO when the enzyme acted on phenolic substrates of small size. Previous workers have shown that α-CD was not an effective inhibitor of Granny Smith apple juice browning (*25, 35*).

The K_D values were obtained at pH 4.5, which corresponds to the optimum pH of apple PPO activity (*36*). Since pH is an important factor of apple enzymatic browning both for the enzyme activity and for the inhibition by aromatic carboxylic acids and halide salts (*18*), we have examined the pH influence on the complex β-CD / phenol. For the phenols tested, i.e. hydrocaffeic, *o*-dihydroxyphenylacetic and chlorogenic acids, the K_D values did not change between pH 3.5 and 5.5 (data not shown). Therefore, the inhibition properties of β-CD were not affected by pH in the
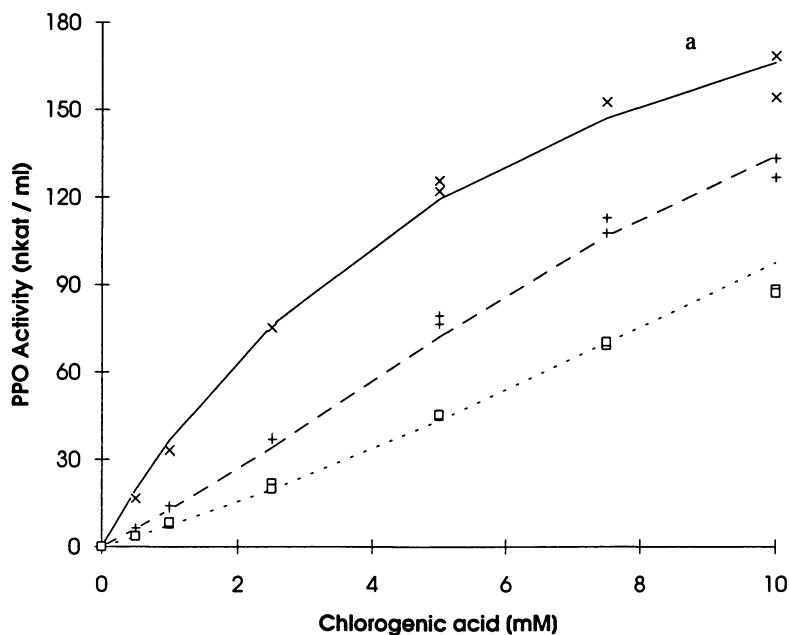
Figure 1. Effect of β-CD on phenolic oxidation catalyzed by purified apple PPO.
1a. With chlorogenic acid as phenolic substrate
1b. With (+)-catechin as phenolic substrate
(X) Control without β-CD; (+) β-CD = 5 mM (1a) or 2 mM (1b); (□) β-CD = 10 mM (1a) or 4 mM (1b). Full, dashed and dotted lines corresponded to the calculated values using equations A and B with Km = 5.6 mM, Vm = 260 nkat.mL^{-1} and K_D = 2.2 mM for chlorogenic acid and Km = 7.4 mM, Vm = 140 nkat.mL^{-1} and K_D = 0.19 mM for (+)-catechin.
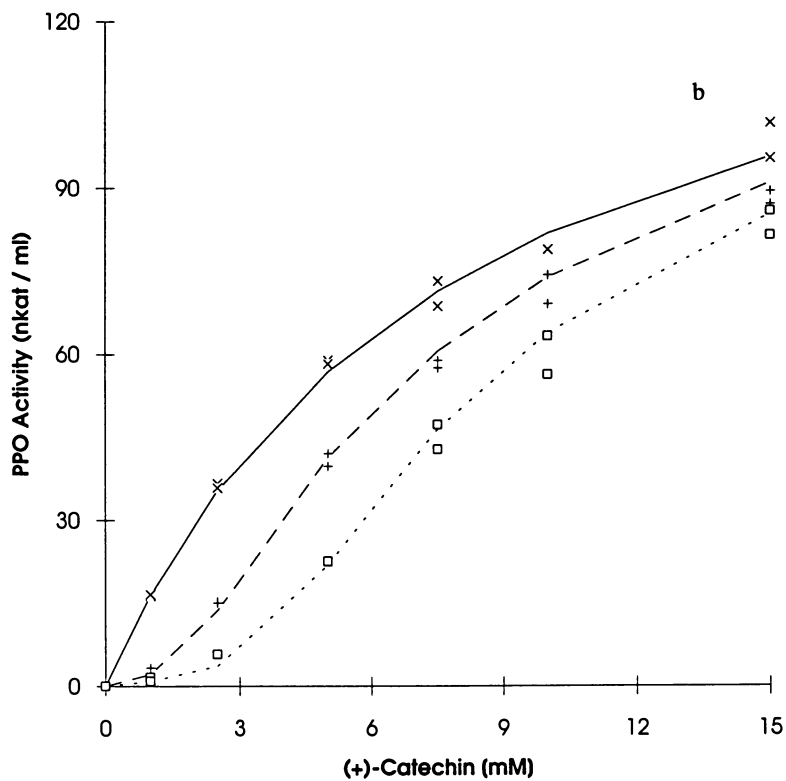
Figure 1. Continued.

range of this study. These statements are in agreement with previous data showing that K_D for chlorogenic acid was relatively insensitive to pH in the range 3.5 to 5.5 (*35*).

Table I. Dissociation Constants (K_D) of the Complex CD / Phenol for different CD and Phenolic Compounds determined either by Enzymatic Kinetic Analysis or by ^{1}H NMR Spectroscopy in D_2O

Phenolic Compound	K_D [mM]			
	α-CD	β-CD		HP-β-CD
	Kinetic*	Kinetic*	^{1}H NMR**	Kinetic*
Pyrocatechol	27	36	24	16.2
4-Methylcatechol	27	17.5	17	8.9
Caffeic acid		7.7		
o-Dihydrophenylacetic acid		16	11	
Hydrocaffeic acid		5.8	7	5.6
Chlorogenic acid		2.2	5.2	1.5
(-)-Epicatechin		2.7	4	1.4
(+)-Catechin		0.19	0.20	0.22

* Determined kinetically by inhibition of PPO activity in air-saturated McIlvaine buffer solution at pH 4.5 and 30 °C.
** Determined by ^{1}H NMR spectroscopy (200 MHz) in D_2O at room temperature using chemical shift changes for H-3 and/or H-5 in β-CD.

In order to confirm the K_D values found by enzymatic kinetic analysis, the complex β-CD / phenol was also analyzed by ^{1}H NMR spectroscopy in D_2O. For the calculations from the chemical shift changes involving the H-3 and/or H-5 protons of β-CD, two assumptions have been made. Firstly, the β-CD / phenol complex kinetics are fast on the NMR time scale and secondly, the complex stoichiometry is 1 : 1. Previous workers have shown that these two assumptions were feasible at least for chlorogenic acid and catechins (*33, 35*). The K_D values obtained with β-CD are given in Table I. Although slight differences were observed between the values given by NMR spectroscopy and kinetic analysis, the two methods gave reasonably similar results. The discrepancies could be due to some differences in the medium used, D_2O in the first case and McIlvaine buffer water solution at pH 4.5 in the second case. Moreover, our results are similar to those obtained by other workers, namely 1.9 mM for both chlorogenic acid and (-)-epicatechin (*35*) and 2.2 and 0.3 mM for (-)-epicatechin and (+)-catechin, respectively (*33*).

Effect of CD on PPO Activity in a Binary Mixture of Phenolic Compounds. It has been shown that in the presence of a two phenolic compound mixture, PPO acted independently on both phenolic substrates, S_1 and S_2 (*37*). Since the amount of oxygen consumed per mole of oxidized phenol is the same for S_1 and S_2, the total oxygen uptake is given by

$$v = \{(Vm_1 [S_1] / Km_1) + (Vm_2 [S_2] / Km_2)\} / \{1 + ([S_1] / Km_1) + ([S_2] / Km_2)\} \quad (C)$$

where Vm_1, Km_1 and Vm_2, Km_2 are the maximum velocity and the Michaelis constant of S_1 and S_2, respectively.

The part of oxygen consumed for the S_1 oxidation is represented by

$$\{Vm_1 [S_1] / Km_1\} / \{1 + ([S_1] / Km_1) + ([S_2] / Km_2)\}$$

and the other part, i.e.

$$\{Vm_2 [S_2] / Km_2\} / \{1 + ([S_1] / Km_1) + ([S_2] / Km_2)\}$$

corresponds to the oxygen consumed for the S_2 oxidation.

Following the nature of the phenol S_2 (i.e. the value of its kinetic constants), its addition to a substrate solution containing the other phenol S_1 could result in a decrease ("inhibition") or an increase ("activation") of the total oxygen uptake. The first case was apparent when both $Vm_2 \ll Vm_1$ and $Km_2 \ll Km_1$ as shown for the caffeic acid / 4-methylcatechol mixture with purified apple PPO by Janovitz-Klapp et al. *(37)*.

When β-CD was added to a binary mixture of phenols, this compound bound both phenols. Obviously, the amounts of free forms S_1 and S_2 were dependent on the K_D values of the respective complexes $[CD.S_1]$ and $[CD.S_2]$. The concentrations $[S_1]$ and $[S_2]$, available for PPO and which are useable for equation C, can be calculated by solving the following set of 5 equations representing mass balances and equilibrium constants of the system :

$$[CD]_t = [CD] + [CD.S_1] + [CD.S_2]$$
$$[S_1]_t = [S_1] + [CD.S_1]$$
$$[S_2]_t = [S_2] + [CD.S_2]$$
$$K_{D1} = ([S_1] [CD]) / [CD.S_1]$$
$$K_{D2} = ([S_2] [CD]) / [CD.S_2]$$

where $[CD]_t$, $[S_1]_t$ and $[S_2]_t$ represent the total amounts of the β-CD, the first and the second phenolic substrate, respectively.
By eliminating $[CD]$, $[CD.S_1]$ and $[CD.S_2]$, two equations are obtained

$$[S_1]^3 \{(K_{D2} / K_{D1}) - 1\} + [S_1]^2 \{(K_{D2} / K_{D1}) ([CD]_t - [S_1]_t) + K_{D2} - K_{D1} \cdot [CD]_t + 2 [S_1]_t + [S_2]_t\} + [S_1] [S_1]_t \{2 K_{D1} - K_{D2} + [CD]_t - [S_1]_t - [S_2]_t\} - K_{D1} [S_1]_t^2 = 0 \quad (D)$$

and

$$[S_2] = [S_2]_t / \{(1 + (K_{D1} / K_{D2}) ([S_1]_t - [S_1]) / [S_1]\} \quad (E)$$

The equation D can be solved with a computer and gives the $[S_1]$ value. This latter concentration can be used in the equation E to determine the $[S_2]$ value. With these two values of $[S_1]$ and $[S_2]$ in equation C, we are now able to predict the oxygen uptake in a two phenolic compound mixture in the presence of β-CD.

Three types of binary mixtures have been studied, namely 4-methylcatechol / caffeic acid, chlorogenic acid / (-)-epicatechin and chlorogenic acid / (+)-catechin owing to the large differences in their kinetic constants (Km and Vm) with apple PPO and dissociation constants (K_D) with β-CD (Table II).

Table II. Kinetic and Dissociation Constants of the Phenolic Compounds used in the Two Phenolic Compound Mixtures

Phenolic compound	Vm* (%)	Km [mM]	K_D [mM]
4-Methylcatechol	100	4.8	17.5
Caffeic acid	4.8	0.15	7.7
Chlorogenic acid	93	5.6	2.2
(-)-Epicatechin	50	7.4	2.7
(+)-Catechin	54	7.4	0.19

* Values of Vm are given in % of the value obtained for 4-methylcatechol.

All the results are expressed in residual activity, i.e. the ratio of PPO activity found in the presence of β-CD to the PPO activity without β-CD. An excellent correlation has been found between the experimental and the calculated velocities for the 3 binary mixtures studied (Figure 2).

For the chlorogenic acid / (-)-epicatechin mixture (Figure 2a), the residual activity steadily increased with both concentrations of the two phenolics. Since both phenols exhibited close K_D values, addition of β-CD resulted in a similar decrease of the free amounts of the two substrates. Thus, the decreases in oxygen uptake for the S_1 and S_2 oxidations are approximately the same and the effect of β-CD is similar to the one observed with one phenol.

For the chlorogenic acid / (+)-catechin mixture (Figure 2b), the behaviour was different. For the low concentrations of chlorogenic acid, the residual activity first decreased and then increased as the (+)-catechin concentration increased. The catechin concentration corresponding to the minimum in residual activity decreased as the chlorogenic acid concentration increased. In this case, owing to the large difference in the K_D values (Table II), β-CD bound preferentially (+)-catechin. Therefore, for low chlorogenic acid concentrations and medium (+)-catechin concentrations, addition of β-CD resulted in a large decrease of free substrate (mainly (+)-catechin) leading to a decrease in the residual activity. For high chlorogenic acid concentrations, the influence of (+)-catechin concentration on PPO activity was almost nihil (37), thus in these solutions the effect of β-CD is comparable to its effect on pure chlorogenic acid solution.

Lastly, another behaviour was observed for the 4-methylcatechol / caffeic acid mixture (Figure 2c). In the presence of caffeic acid, increasing concentrations of 4-methylcatechol between 0 and 5 mM resulted in a rapid increase of the residual activity followed by a plateau for the low caffeic acid concentrations (0.11 and 0.25 mM) or a slight decrease after 10 mM in 4-methylcatechol with 0.5 and 1 mM in caffeic acid. For these caffeic acid concentrations, the maximum residual activity was higher than 1, meaning that the β-CD addition resulted in an activation of the oxygen uptake. This surprising result can be explained by the large difference in the kinetic constants of the two phenols. Since both Vm and Km of caffeic acid are largely lower than that of 4-methylcatechol (Table II), its addition results in a large decrease of oxygen uptake as demonstrated by Janovitz-Klapp et al. (37). The addition of β-CD decreased both phenol concentrations. However, for the oxygen uptake, the decrease
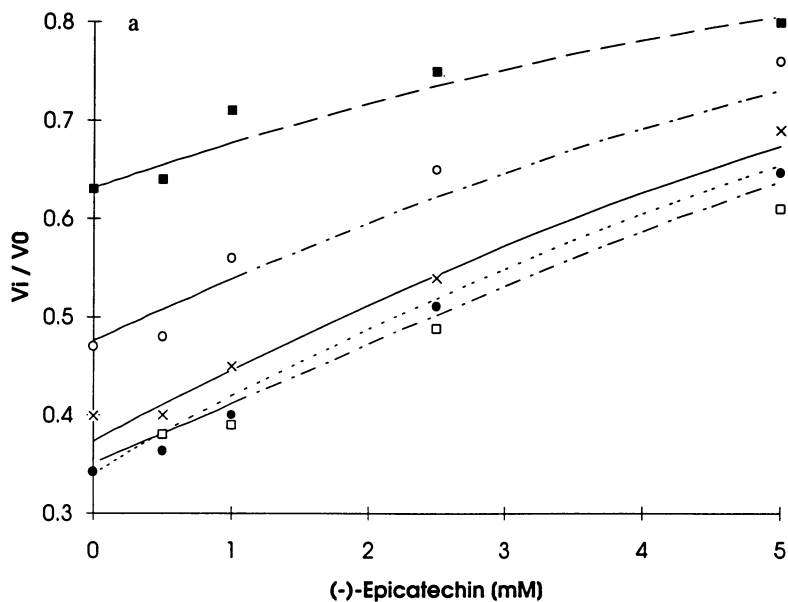
Figure 2. Effect of β-CD on the oxidation of a two phenolic compound mixture catalyzed by purified apple PPO.

2a. Chlorogenic acid / (-)-Epicatechin with β-CD = 5 mM

2b. Chlorogenic acid / (+)-Catechin with β-CD = 5 mM

2c. 4-Methylcatechol / Caffeic acid with β-CD = 10 mM

(□) chlorogenic acid 0 mM (2a and 2b), caffeic acid 0 mM (2c); (●) chlorogenic acid 0.5 mM (2a and 2b), caffeic acid 0.1 mM (2c); (X) chlorogenic acid 1 mM (2a and 2b), caffeic acid 0.25 mM (2c); (O) chlorogenic acid 2.5 mM (2a and 2b), caffeic acid 0.5 mM (2c); (■) chlorogenic acid 5 mM (2a and 2b), caffeic acid 1mM (2c). The different lines corresponded to the calculated values using equations C, D and E with the Km, Vm and K_D values given in Table II. *Continued on next page*
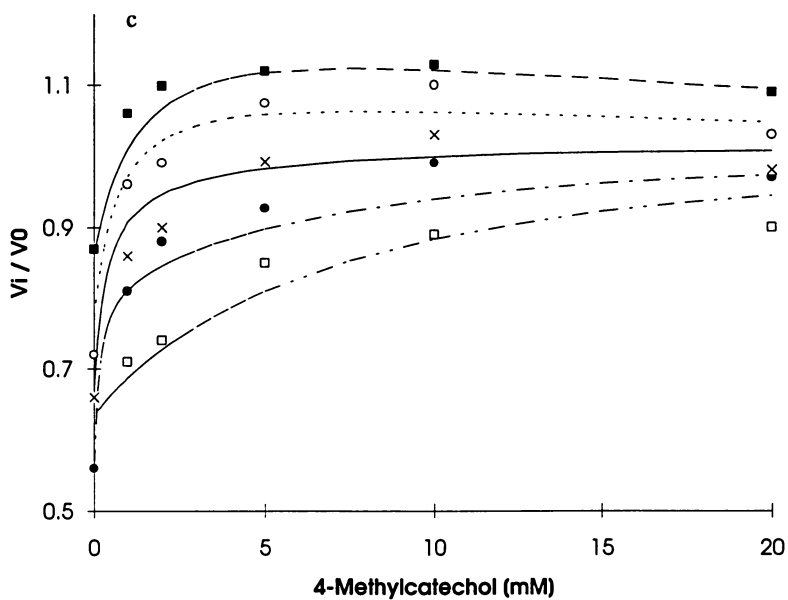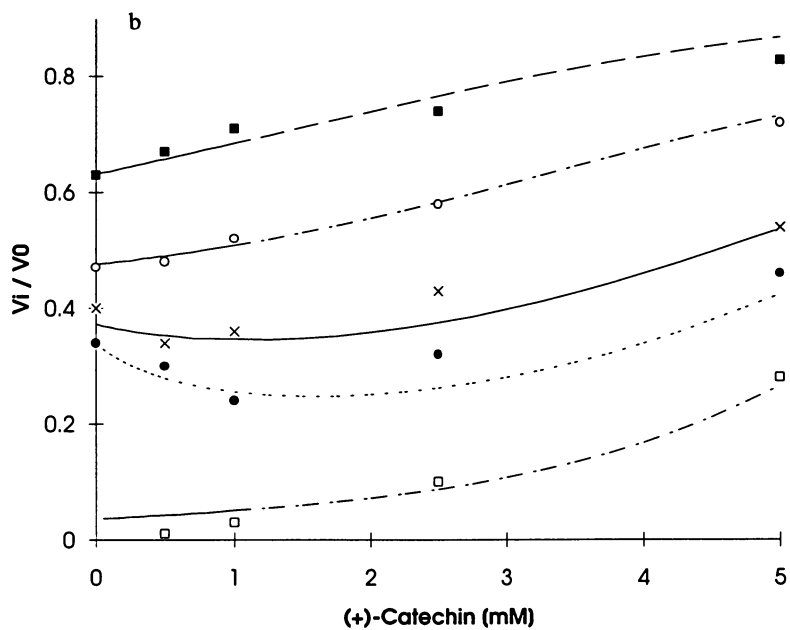
Figure 2. Continued.

of free caffeic acid results in a greater activation than the inhibition due to the decrease of free 4-methylcatechol leading to an apparent activation of PPO.

Effect of β-CD on Color Formation during PPO Oxidation in Binary Mixture of Phenols. Following the nature of the phenols involved in the oxidation, the resulting pigments varied widely in color intensity and hue (*5*). Thus, it has been established that the relative proportion among the different classes of phenolic substrate was probably the most important factor that contributed to the extent of enzymatic browning in apple varieties (*40*). Since β-CD binds the different phenols according to their K_D values, this compound is able to modify the balance among the different free phenolic substrates available for the PPO and therefore the resulting color of the oxidized solution. In this respect, the color variations of two phenolic compound mixtures have been compared during PPO oxidation with or without β-CD. The color variations were followed by tristimulus colorimetry for the 3 mixtures described in the preceeding paragraph. The amounts of free substrates in the presence of β-CD have also been calculated and the measurement of color variation of the resulting solution (equivalent solution) has been included in this study. The obtained results are given in Figure 3 for one example of each binary mixture. An excellent correlation was observed between the L*, a* and b* variations of the solutions with β-CD and the equivalent solutions. Moreover, the two latter solutions gave results which differed from the solution without β-CD.

Concerning the chlorogenic acid / catechin mixtures (Figures 3a and 3b), the L* values decreased rapidly in the first 3 minutes, then increased until 10 minutes and slowly decreased between 10 and 30 minutes. During the first part of the reaction, the dissolved oxygen was consumed (data not shown) and the enzymatic reaction probably stopped owing to the lack of oxygen. With β-CD, the inhibition (the residual activity was close to 0.64 in both cases) slowed down the enzymatic reaction which stopped later (after 5 minutes). Thus, darker solutions were obtained after 5 minutes (and later on) although the initial darkening rates were lower. Moreover for the chlorogenic acid / (+)-catechin mixture (Figure 3b), the hue was modified by the presence of β-CD since the a* values were higher and the b* values were lower corresponding to solutions less yellow and more red. The catechins mainly gave yellow pigments whereas chlorogenic acid gave pink to red pigments (*8*). Since β-CD preferentially bound (+)-catechin (the ratio chlorogenic acid / (+)-catechin was equal to 7 without β-CD and more than 40 with β-CD), this explains that the color turned from yellow to red. However, a preferential binding of the catechin *o*-quinones cannot be ruled out since β-CD induced also a similar change,although less marked, in hue for the (-)-epicatechin / chlorogenic acid mixture.

Concerning the 4-methylcatechol / caffeic acid mixture, addition of β-CD gave darker solutions after 15 minutes of oxidation with higher a* values and almost no effect on the b* values. The chosen conditions (3 mM in 4-methylcatechol and 1 mM in caffeic acid) corresponded to a slight activation (residual activity was equal to 1.06) of oxygen uptake which could explain the darkening of the solution.

In conclusion of this study, CD were always inhibitors of PPO activity in a single phenolic solution. These compounds acted by complexation of the substrate. However, in multiphenolic solutions, the effects were variable. Thus, following the

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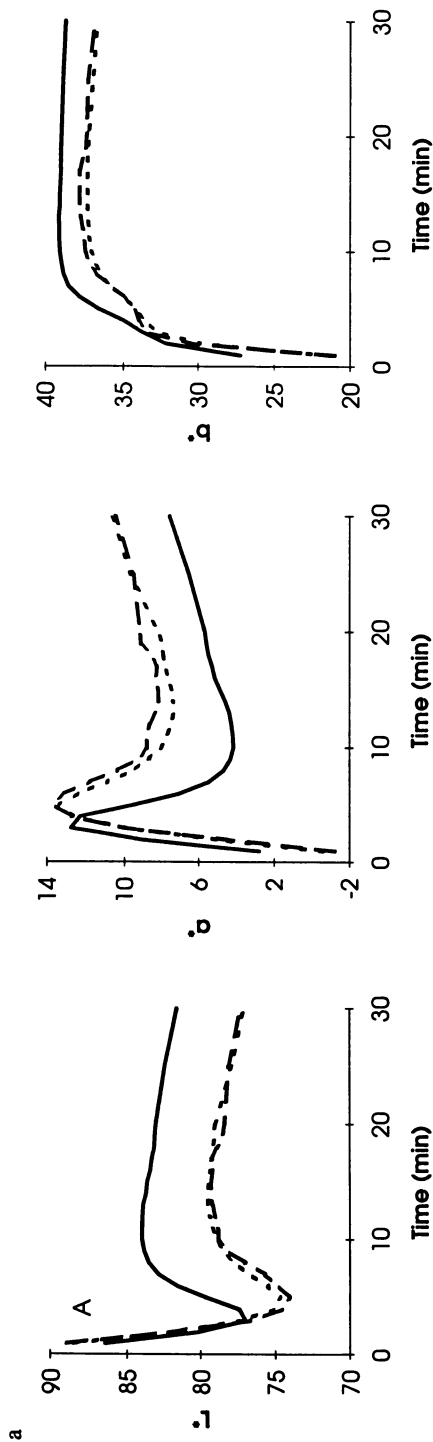
Figure 3. Effect of β-CD on L*, a* and b* variations of a two phenolic compound mixture during the oxidation catalyzed by purified apple PPO.
3a. Chlorogenic acid (2 mM) / (-)-Epicatechin (2 mM) with β-CD = 4 mM
3b. Chlorogenic acid (3.5 mM) / (+)-Catechin (0.5 mM) with β-CD = 4 mM
3c. 4-Methylcatechol (3 mM) / Caffeic acid (1 mM) with β-CD = 4 mM

Full lines were control experiments without β-CD. Dotted lines were experiments with β-CD = 4 mM. Dashed lines were experiments with equivalent solutions in which the concentrations of the phenolic compounds were: chlorogenic acid 1.02 mM and (-)-epicatechin 1.12 mM (3a); chlorogenic acid 1.87 mM and (+)-catechin 0.045 mM (3b); 4-methylcatechol 2.53 mM and caffeic acid 0.70 mM (3c) (equivalent solutions were without β-CD).
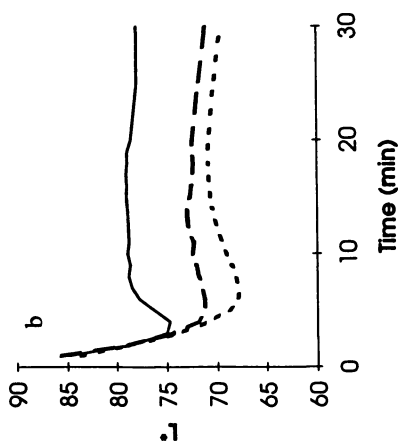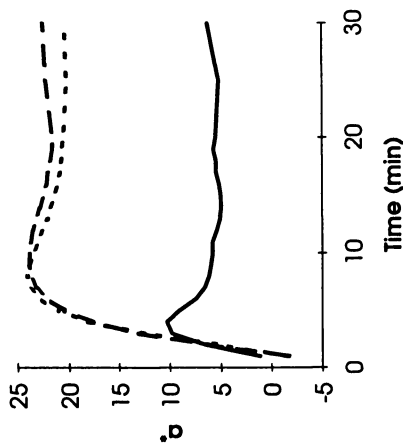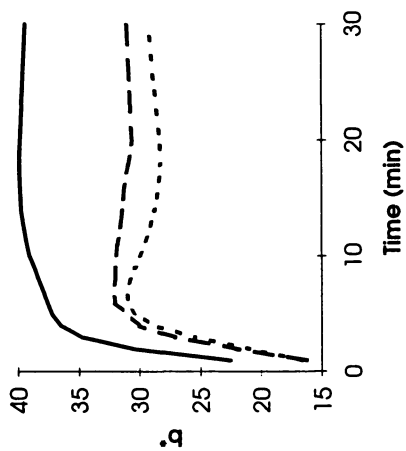
Figure 3.   Continued. (*Also continued on next page.*)
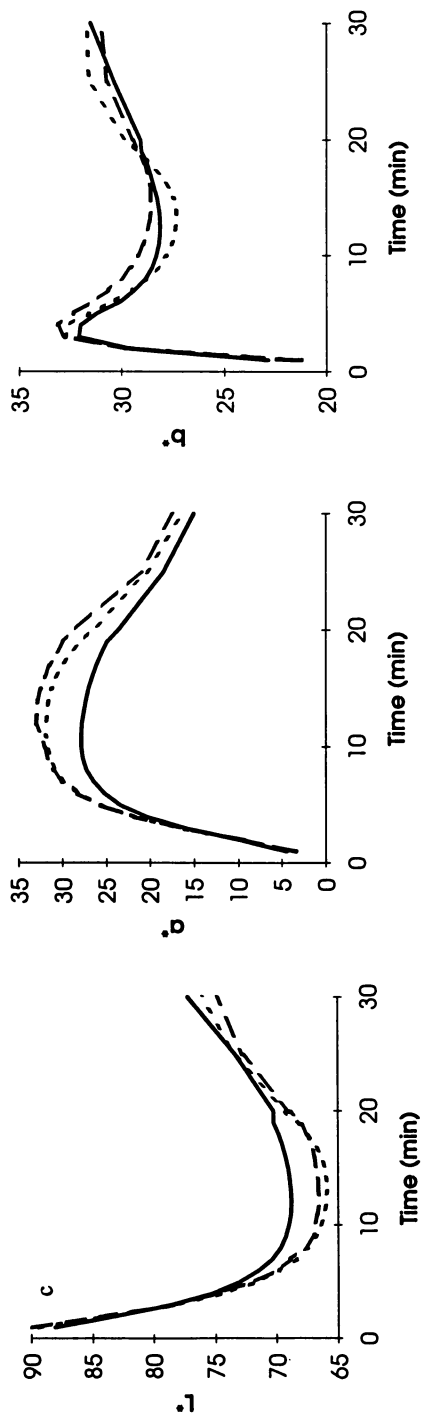
Figure 3. Continued.

phenolic compounds present in the solution, addition of β-CD can result in a large or a small decrease or a slight activation of the oxygen uptake catalyzed by PPO. Moreover, since the balance among the available phenolic substrates can be highly modified, CD can induce great changes in the color of the oxidized solutions. This could probably explain the effects observed by Crouzet et al. (*26*) who indicated that the inhibition of enzymatic browning by CD in apple juice was variety dependent.

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Chapter 24

Sulfite Substitutes for the Prevention of Enzymatic Browning in Foods

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Sulfites have been used extensively in the food industry to control enzymatic browning. However, within the last ten years, there has been an effort to avoid the use of sulfiting agents in foods due to safety, regulatory, and labeling issues. Safe alternatives to sulfites have been developed. The family of erythorbates, erythorbic acid and sodium erythorbate, are stereoisomers of the ascorbates and function in a similar manner as antioxidants. These compounds are reducing agents and are preferentially oxidized in foods, thus preventing or minimizing oxidative flavor and color deterioration. Erythorbates can prevent enzymatic browning in many products such as fruits, vegetables, and beverages. Enzymatic browning also occurs in crustaceans and results in the formation of dark pigments, a defect called blackspot. EverFresh (4-hexylresorcinol), unlike sulfites, inhibits polyphenoloxidase present in and under the shell of shrimp, thus preventing the formation of blackspot.

Browning reactions during processing and storage of foods are responsible for the majority of product and profit losses among food processors. There are four major types of browning in foods: Maillard, carmelization, ascorbic acid oxidation, and phenolase or enzymatic browning (*1*). Enzymatic browning results in objectionable surface discoloration which decreases the commercial value and consumer acceptance of foods. Many food products are susceptible to enzymatic browning including fruits and vegetables, beverages, and crustaceans. Scientists in the food industry are currently investigating innovative ways to control or inhibit enzymatic browning in foods.

Enzymatic Browning

Enzymatic browning is caused by the action of a family of enzymes generally known

as polyphenoloxidases (PPOs) (Figure 1). Most PPOs are mixed function oxidases that catalyze the hydroxylation of colorless monophenols to colorless diphenols (cresolase activity) and their further oxidation to colored and highly reactive orthoquinones (catecholase activity) (2). The orthoquinones polymerize and/or react with endogenous amino acids and proteins to form complex brown pigments. In intact tissue the endogenous phenolic substances are separated from PPO and browning does not occur. However, when the tissue is cut or damaged and exposed to air, which is typical in food processing operations, browning rapidly occurs (1,3).

Phenolase enzymes have been isolated from food sources and are characterized as being oligomers and containing one copper prosthetic group per subunit (1,4). The optimum pH for PPO activity is between pH 5 and 7, and the enzyme is relatively heat labile (5).

Preventing or inhibiting enzymatic browning is currently attempted in many ways. The methodology is to eliminate from the reaction one or more of its essential components: oxygen, enzyme, copper, or substrate (1). Oxygen can be excluded from the reaction site by immersion in water, syrup, brine, or by vacuum or modified atmospheric packaging (3). However, when the package is opened and oxygen is reintroduced, browning will occur rapidly.

The PPO enzyme can be inhibited by the use of heat treatments or the addition of antibrowning agents. Heat treatments can denature the enzyme complex. Steam blanching which is used primarily for frozen and canned foods effectively inhibits PPO (2). However, blanching can adversely effect the flavor and texture of fresh produce (3).

Antibrowning agents such as acidifiers, chelators, reducing agents, and other chemical compounds can inhibit or control enzymatic browning. Acidifiers such as citric, malic, and phosphoric acids will lower the pH of the system to below 3 where PPO is inactive (1). However, it is not always possible to lower the pH of fresh foods due to the significant acid flavor that prevails. Chelators such as EDTA, phosphates, and citric acid can chelate the copper prosthetic group at the enzyme-active site or reduce the level of copper available for incorporation into the enzyme (2). Chelating agents slow down the enzymatic reaction but do not completely inhibit it. PPO can be inhibited by certain chemical compounds which sterically hinder the enzyme or bind to its active sight and render it incapable of catalyzing the enzymatic reaction. Additionally, phenolic substrates can be removed from the reaction by complexing with such substances as cyclodextrins (2) or by chemical modification (2). However, cost and regulatory issues currently preclude the commercialization of these two procedures. The addition of reducing agents, sulfites and ascorbates, is the standard method today for controlling enzymatic browning.

Sulfites

The most common method for controlling browning in foods is the use of sulfiting agents. As food ingredients, the generic term sulfites includes sulfur dioxide (SO_2), sodium metabisulfite ($Na_2S_2O_5$), sodium bisulfite ($NaHSO_3$), sodium sulfite (Na_2SO_3), potassium metabisulfite ($K_2S_2O_5$), and potassium bisulfite ($KHSO_3$) (5,6). Sulfites are unique compounds because they can perform many functions in foods. Sulfites can control both enzymatic and nonenzymatic browning; function as antimicrobial agents

and control the growth of microorganisms in foods; and act as bleaching agents, antioxidants, and reducing agents (*6*).

Sulfites may control enzymatic browning in foods in several ways. First, there is an indication that sulfites react with PPO itself. Sulfites may irreversibly inhibit PPO by modification of the protein structure (*7*). Secondly, sulfites may interact with the intermediates in the reaction and thus, prevent the formation of the brown pigments (*6*). There is evidence of the formation of quinone-sulfite complexes which indicates that sulfites may complex with diphenols or quinones, therefore removing them from the reaction (*7*). Lastly, sulfites are best known as reducing agents due to their ability to reduce the colored orthoquinones back to the colorless and less reactive diphenols (*2*) (Figure 2). In some cases high concentrations of sulfite are used to bleach colored pigments that have already formed (*2*). Since sulfites, for the most part, do not irreversibly inhibit enzymatic browning, they are consumed in the reaction and thus required concentrations are dependent on the length of time that the reaction must be inhibited (*6*).

Although sulfites have found widespread use in controlling enzymatic browning in foods, there are disadvantages to their use. Within the last ten years, the safety of sulfite use has been questioned. There have been documented cases of allergic reactions in individuals who consumed sulfited products. Sulfites have been implicated in inducing asthmatic episodes and causing other hypersensitivity reactions, such as anaphylactoid reactions and contact sensitivity (*6*). Several sensitive individuals, such as steroid-dependent asthmatics, have died due to consumption of sulfited foods (*2*). Due to these health issues, the FDA in 1986 prohibited the use of sulfites on fruits and vegetables served or sold raw to consumers (*8*). Additionally, in 1988 new rules were proposed which set specific labelling criteria for sulfite-treated foods and set allowable sulfite residuals (*9*). Lastly, the FDA tried unsuccessfully in 1990 to ban sulfites in fresh potatoes (*10*).

There has also been concern over handling of sulfites. In enclosed areas and in the presence of moisture such as in holds of fishing ships, sulfur dioxide vapors can collect and cause asphyxiation. There have been several deaths among fisherman in the United States attributed to suffocation by sulfur dioxide vapors (*11*).

Finally, sulfite use and abuse can give a negative impact on the flavor, odor, and texture of certain treated foods. A more in-depth review of the use of sulfiting agents in foods and associated hazards is given by Taylor et al. (*6*).

Sulfite Alternatives

With the intense regulatory and consumer pressure to remove sulfites from foods, the food industry has made great efforts to develop alternatives to sulfites. Two such alternatives are the family of erythorbates, erythorbic acid and sodium erythorbate, and EverFresh (4-hexylresorcinol).

Erythorbates. Erythorbic acid (isoascorbic acid) and sodium erythorbate (sodium isoascorbate) are stereoisomers of the ascorbates. The difference between the two families of compounds is the configuration of the -OH and -H groups on the fifth carbon. The erythorbates and ascorbates have similar antioxidant activity but dissimilar vitamin C activity. Erythorbates are generally recognized as having only

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|
HCOH

Ascorbic
Acid

CH$_2$OH
|
HOCH
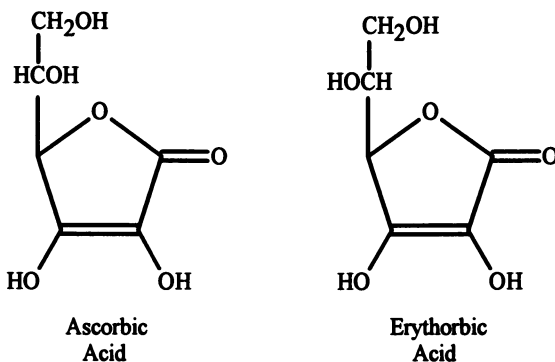
Erythorbic
Acid

5 to 7% of the antiscorbutic potency of ascorbates due to their poor absorption and retention by tissues (*12*). Erythorbates are more economical than ascorbates because they are inherently less expensive to manufacture.   Thus, in applications where vitamin C activity is not desired but antioxidant activity is needed, erythorbates represent the product of choice.   Using erythorbates as antioxidants in place of ascorbates can give savings of as much as 35 to 40%.

Erythorbates have three functions in controlling enzymatic browning in foods. Erythorbates can act as free radical scavengers and chelators, alter the redox potential of the system, and act as reducing agents (*2*).   Like sulfites, erythorbates are best known as strong reducing agents because they reduce the reactive orthoquinones back to diphenols (refer to Figure 2).

Erythorbates have been successfully used as sulfite and ascorbic acid substitutes in many foods.   Product applications include fresh and processed fruits and vegetables, prepared salads, fruit fillings and toppings, and carbonated and still beverages as well as beer and wine.   Erythorbic acid has been effectively used to control browning in cut fruits (*13*), frozen peaches (*14*), salad vegetables (*15*), apples and apple juice (*16*), potatoes (Pfizer Inc, unpublished data), and fruit juice (*17*). Combinations of erythorbic acid and citric acid are commonly used as antibrowning treatments.   The addition of citric acid aids in lowering the pH of the system and chelating copper, both of which lead to inhibition of PPO.   The erythorbic acid or erythorbic acid - citric acid combination can be utilized in foods in several ways: direct addition to the packing media (syrup, water, etc.); as a dip solution; direct addition to the food matrix; and in combination with a vacuum treatment to improve infiltration of the treatment into the food product (*18*).

Experimental.   Erythorbic acid and ascorbic acid were compared on an equivalent weight basis for controlling enzymatic browning in fresh cut pineapple. Fresh whole pineapple was peeled, cored, and sliced.   Slices were dipped for 2 minutes into one of three treatment solutions: 1) water (control); 2) 0.3% erythorbic acid; and 3) 0.3% ascorbic acid.   The treated slices were placed on foam plates and covered with plastic wrap.   The pineapple slices were stored at 32 °F (0 °C) for up to three weeks.   At two day intervals the pineapple samples were visually evaluated for the formation of brown pigments.
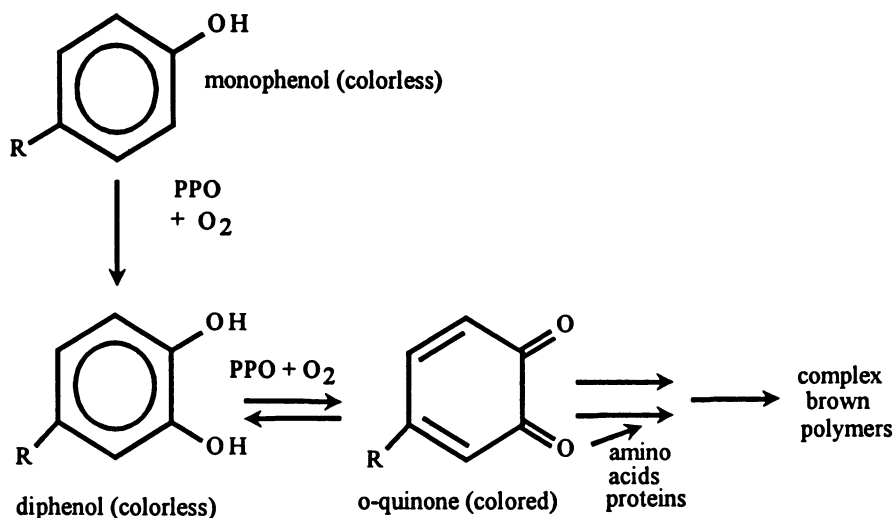
Figure 1. Action of polyphenoloxidase in enzymatic browning. (Adapted from ref. 2).
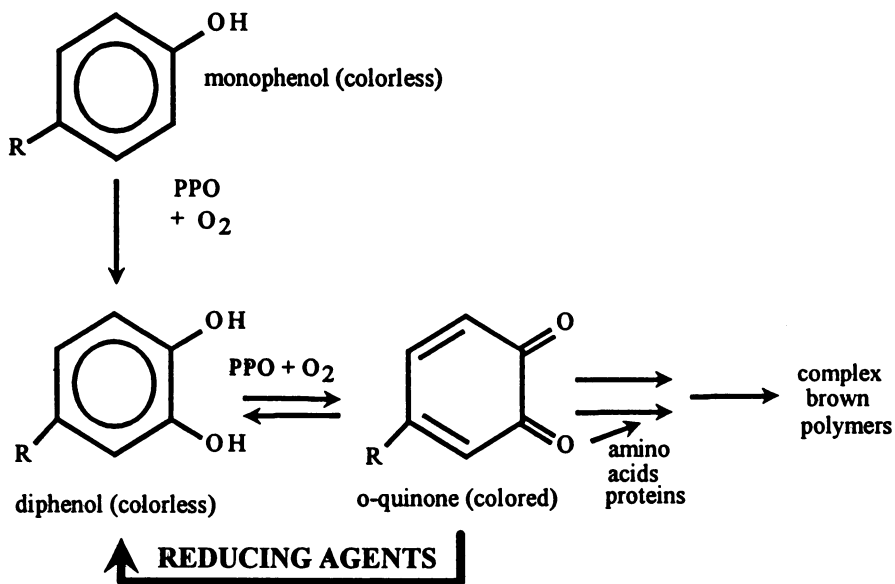
Figure 2. Effect of reducing agents on controlling enzymatic browning. (Adapted from ref. 2).

Results. As can be seen in Figure 3, erythorbic acid and ascorbic acid were equally effective in preventing browning in the pineapple slices for the entire 21 day storage period. There were no visual differences between the erythorbic acid-treated pineapple and the ascorbic acid-treated pineapple. Pineapple treated with water showed browning in as little as 8 days.

Further experiments showed equivalent antioxidant activity between erythorbates and ascorbates in the following foods: aloe vera gel, avocado puree, frozen peaches, frozen bananas, and refrigerated and dehydrated apples (*19*).

Safety and Regulatory Status. Erythorbates are generally recognized as safe (GRAS) as chemical preservatives in the United States (*20*). They are approved in at least 23 other countries including Canada, Mexico, China, Japan, and Brazil. Currently, erythorbic acid and sodium erythorbate are included for review in the European Union's Miscellaneous Additives Directive Annex III D. They have been used as antioxidants and curing accelerators in foods for over 50 years.

EverFresh. EverFresh is a patented product (patent #5,049,438) composed of 4-hexylresorcinol (4-HR) as the active ingredient and sodium chloride as the carrier agent.

OH

OH

CH_2 $(CH_2)_4CH_3$
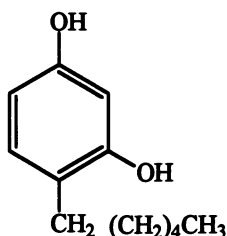
4-Hexylresorcinol

EverFresh is used for controlling enzymatic browning, or blackspot, in crustaceans. EverFresh is premeasured and packaged for easy use by shrimp farmers, boaters, and processors, and it represents an effective, safe, and economical alternative to sulfites.

In crustaceans, polyphenoloxidase serves in the synthesis of compounds necessary for hardening of the cuticle after molting. However, PPO is also responsible for the formation of black pigments on the head, fins, legs, and shell of commercial products (*21*). These blackspots decrease the acceptance and commercial value of crustaceans (*22*). Blackspotting occurs when crustaceans are exposed to oxygen during refrigeration, ice storage, and post-freeze thawing (*22*). Current methods to control blackspot involve the use of sulfites. Regulations allow shrimp and other crustaceans to be dipped for one minute in a 1.25% sodium bisulfite treatment before further processing or storage. This treatment allows for a maximum of 100 ppm of residual sulfite in the consumed product (*9,23-24*).

As mentioned previously, sulfites are used to control enzymatic browning, and thus blackspot, due to their ability to reduce orthoquinones back to diphenols. In contrast, 4-hexylresorcinol interacts with PPO and renders it incapable of catalyzing

the enzymatic reaction (2) (Figure 4). 4-Hexylresorcinol acts as an enzyme inhibitor and stops the reaction in its earliest phase, whereas sulfite treats reaction products that have already formed. 4-Hexylresorcinol has several advantages over using sulfites in foods including its specific mode of inhibitory action, its lower use level required for effectiveness, its inability to bleach preformed pigments, and its chemical stability (2). Several laboratory and field studies have shown the effectiveness of 4-hexylresorcinol in controlling enzymatic browning in shrimp. McEvily et al. (25) reported that a one minute dip in a 50 ppm 4-hexylresorcinol solution was more effective than the typical 1.25% sodium metabisulfite dip for controlling blackspot in pink (*Penaeus duorarum*) and brown (*Penaeus aztecus*) shrimp stored on ice for 14 days. Benner et al. (26) confirmed these findings. Additional studies showed 4-hexylresorcinol to be more effective than sulfites in rock (*Sicyonia brevirostris*), white (*Penaeus vannamei*), nylon (*Heterocarpus laevigatus*), and kuruma (*Penaeus japonicus*) shrimp (22,25,27).

Experimental. Two experiments were run in the field using 4-hexylresorcinol currently packaged as EverFresh. The first experiment involved treating raw brown (*Penaeus aztecus*) shrimp freshly harvested from the Gulf of Mexico. The shrimp were deheaded and dipped for 1 minute in one of three solutions: 1) fresh water (control); 2) 1.25% sodium metabisulfite; and 3) EverFresh. The shrimp were allowed to drain for 15 seconds and were packaged in perforated Ziplock storage bags. The bags were stored at 2 °C for up to 14 days. Every two days, the shrimp were visually evaluated for the percent of blackspot (calculated as the number of shrimp with blackspot divided by the total number of shrimp in the sample X 100).

The second experiment involved black tiger (*Penaeus monodon*) shrimp harvested in Thailand. These shrimp were deheaded and peeled until the last segment and tail remained, dipped in a treatment solution, cooked, and stored. The raw shrimp were dipped for 1 minute into one of the following solutions: 1) fresh water (control); 2) 0.6% BL-7P (0.8% L-tryptophan, 67.2% sodium pyrosulfite, 11.3% sodium erythorbate, 4.7% sodium polyphosphate, 4.6% disodium dihydrogen pyrophosphate, 3.3% trisodium citrate, 3.2% disodium L-tartrate, 2.5% monosodium L-glutamate and 2.4% sodium metaphosphate); and 3) EverFresh. The shrimp were drained, cooked by steaming for 3 to 4 minutes, packaged in polyethylene bags, and stored at -20 °C for up to 30 days. The shrimp were evaluated for the percent of blackspot at various intervals during the storage period.

Results. EverFresh controlled blackspot for a longer period of time than water or the current acceptable treatment of sulfite. After 7 days of storage at 2 °C, brown raw head-off shrimp treated with water showed 54% blackspot and the sulfite-treated shrimp had 11% blackspot, whereas the EverFresh-treated shrimp had only 3.6% blackspot (Figure 5). After 14 days of storage, the amount of blackspot had increased in both the control and sulfite-treated shrimp to 75% and 25%, respectively. However, the EverFresh-treated shrimp had remained at only 3.6% blackspot.

Results of the second experiment are shown in Figure 6. Cooked tail-on black tiger shrimp treated with EverFresh had only 6.4% blackspot after 7 days at -20 °C, whereas shrimp treated with sulfite or with water had 23% and 44% blackspot, respectively. As expected, after increased storage EverFresh outperformed sulfite for
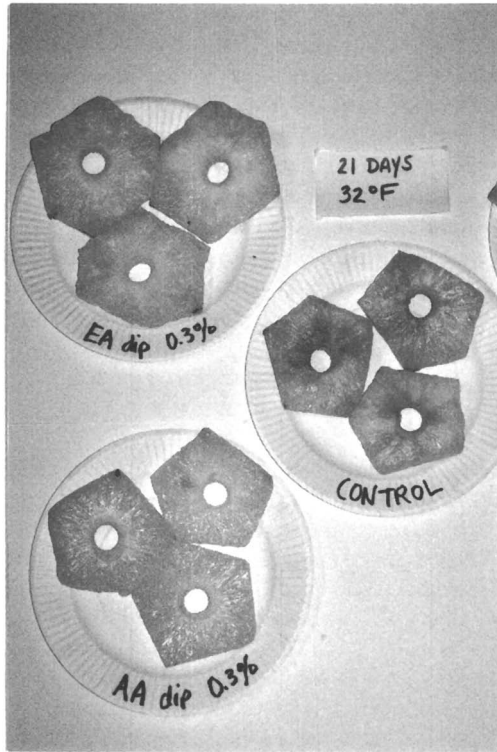
Figure 3. Fresh pineapple slices stored for 21 days at 32 °F (0 °C). EA = erythorbic acid; AA = ascorbic acid.
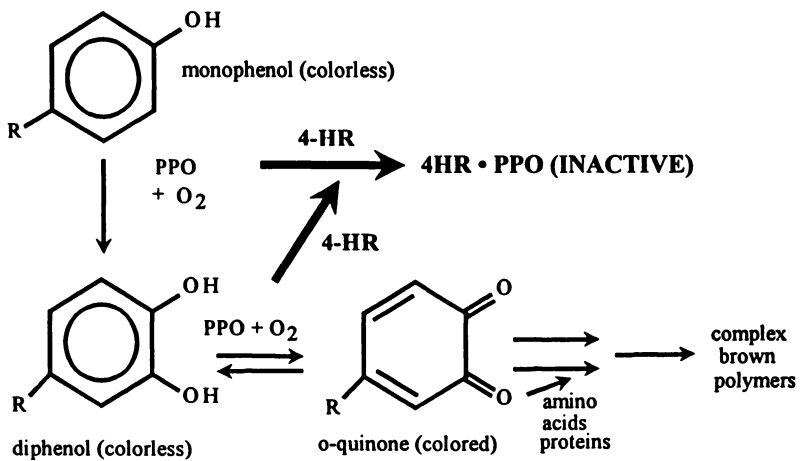
Figure 4. Effect of 4-hexylresorcinol on inhibiting polyphenoloxidase and enzymatic browning.
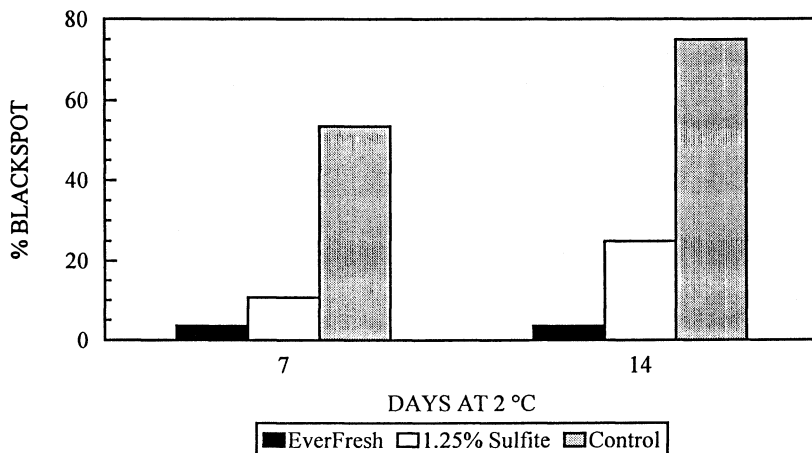
Figure 5. % Blackspot in raw head-off brown shrimp stored at 2 °C.
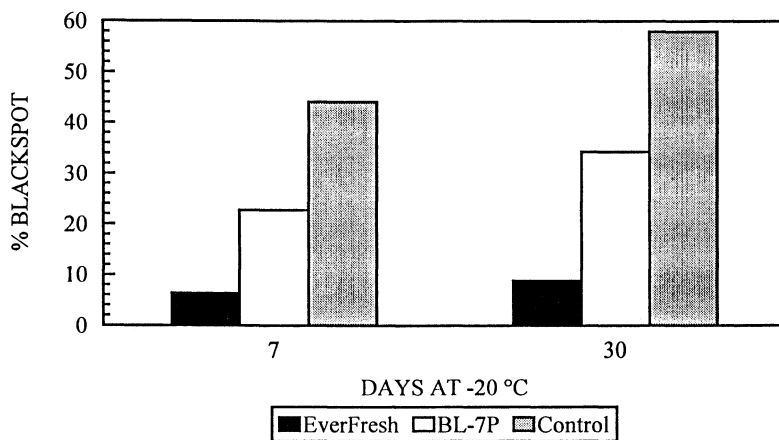
Figure 6. % Blackspot in cooked tail-on black tiger shrimp stored at -20 °C.

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controlling the development of blackspot. After 30 days at -20 °C, the water-treated shrimp had 58% blackspot, the sulfite-treated shrimp had 34% blackspot, and the EverFresh-treated shrimp had only 9% blackspot.

Safety and Regulatory Status. The active ingredient in EverFresh (4-hexylresorcinol) has an extensive history of safe use by consumers. 4-Hexylresorcinol has been used for over 50 years in over-the-counter consumer goods such as throat lozenges. A recent review of toxicological, mutagenic, carcinogenic, and allergenic studies determined that 4-hexylresorcinol presents no risk at the levels used to treat shrimp (28). This determination is further supported by the low residual 4-hexylresorcinol level on consumed EverFresh-treated shrimp. When using the recommended EverFresh treatment protocol, shrimp contains on average less than 1 ppm of 4-hexylresorcinol in the final consumed product (29,30).

EverFresh is permitted in many countries as a blackspot inhibitor for shrimp and other crustaceans. Currently in the United States, 4-hexylresorcinol was judged to be GRAS (generally recognized as safe) for use on shrimp by a prestigous LSRO panel of FASEB (28). A GRAS petition for use on shrimp was accepted for filing by the USFDA on March 10, 1992 (31). The acceptance of this petition initiated the introduction of EverFresh to the US market. Several major US shrimp processors are currently using EverFresh for blackspot control. In addition, EverFresh is considered a processing aid under United States regulations (32). EverFresh is also approved in Ecuador, China, India, Indonesia, Thailand, and Malaysia. Other country approvals are pending.

Conclusion

Erythorbates and EverFresh have many advantages for their use as sulfite substitutes to control enzymatic browning in foods. First, these products are effective and can be easily and directly substituted into the existing process without further modification. Secondly, these antibrowning agents are safer for industry personnel to use and the subsequent treated food products are safer for individuals to consume. Thirdly, the use of these ingredients in place of ascorbates and sulfites, respectively, can save food processors a significant amount of money by decreasing their level of throwaway product due to discoloration. Finally, the use of erythorbates and EverFresh allows processors the ability to market high quality, sulfite-free foods.

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