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ENZYME REACTIONS WITH PHENOLIC COMPOUNDS*: EFFECTS OF O-METHYLTRANSFERASE AND HIGH pH ON THE POLYPHENOL OXIDASE SUBSTRATES IN APPLE†

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Abstract—An anaerobic methylating treatment with a catechol O-methyltransferase system at pH 8 permanently prevented oxidative darkening of apple juice and fruit sections by modifying their polyphenol oxidase (PPO) substrates. Control non-methylating treatments of juice resulted in normal PPO darkening of endogenous substrates. Similar control treatments applied to fruit sections resulted in less darkening than occurred in untreated sections, and the observed effect resembled that of O-methyltransferase treatment in that the oxidative substrates in the tissue appeared to be permanently modified. This effect on the substrates was found to be entirely a result of the mildly alkaline pH of the system. The possibility that the effect was due to alkaline extraction of PPO substrates from the apple surfaces was shown to be unlikely, suggesting that substrate modification may occur as a result of *in situ* O-methyltransferase, when it is brought to its active pH range.

INTRODUCTION

ORTHO-DIPHENOLIC compounds have been reported to be the major substrates for polyphenol oxidase (PPO) (*o*-diphenol:O_2 oxidoreductase) in fruits[1–4] and also key intermediates in the enzymatic oxidation of monophenols.[5] Previous experiments with chlorogenic and caffeic acids—taken as model *o*-diphenol substrates of PPO—indicated that enzymatic O-methylation of these *o*-diphenols can be achieved *in vitro*, and this methylation prevents their oxidative darkening with PPO.[6] If *o*-diphenols are the sole, or controlling, natural substrates of PPO in fruit tissues, then O-methylation of them ought to prevent oxidative darkening of the fruit. This paper reports such an effect on apple sections and juice treated with catechol O-methylating enzyme system. In addition, a direct effect of an alkaline pH alone in diminishing the PPO substrates is noted.

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RESULTS

Two types of experiments were run: treatments of apple juice and of cut apple fruit surfaces.

Apple Juice

After anaerobic incubation for 3 hr with reagents of the methylating system (2·0 ml juice in 0·01 % ascorbic acid, 1·0 ml of O-methyltransferase, 0·6 ml 0·08 M S-adenosylmethionine iodide (AMe), 0·2 ml 0·2 M $MgCl_2$, and 0·2 ml 0·5 M pH 9·0 *tris*-(hydroxymethyl)-aminomethane-HCl (TRIS), final pH 8·2), the apple juices remained light in color, as on first mixing. When juice with the "complete" methylation mixture added and a control (minus enzyme and minus S-adenosylmethionine) were brought to pH 6 and shaken in air for 2 hr (or overnight), the juice with the methylation mixture added remained a light cream color whereas the unmethylated control became a deep brown. Other experiments omitting methylating enzyme or S-adenosylmethionine (AMe) did not give the light color of the "complete" system. It is of interest that a similar experiment in which AMe was replaced by methionine plus adenosine triphosphate gave comparable results.

Incubation with the methylating system had the effect of permanently modifying the substrates present in the juice so that they were no longer susceptible to oxidation with PPO. This was established by the addition of a PPO preparation[3] or additional substrate (1 μmol chlorogenic acid) to 0·2-ml portions of the treated, reacidified juice. The PPO of treated juice, or supplementary PPO added to the juice, produced no darkening; but on the addition of PPO substrate to the methyltransferase-treated juice, rapid darkening ensued, demonstrating that endogenous substrates of PPO had been modified by the transmethylation treatment.

Cut Apples

Incubation with the methyltransferase system on cut apple surfaces also prevented their surface darkening on exposure to air. This effect is recorded in Table 1, Experiment A. After

TABLE 1. EFFECTS OF TREATMENTS ON THE OXIDATIVE BROWNING OF THE SURFACES OF CUT APPLES

Experiment	Treatment	Terminal pH	Effect
A	Water	4·3	Brown
	Methyltransferase system	7·0	White
	Boiled-enzyme transferase system	7·2	Slight brown
B	Untreated		Brown
	Buffer only, 0·1 M KH_2PO_4	3·9	Brown
	" " " phosphates	6·2	Light brown
	" " " K_2HPO_4	7·8	White
	" " " K_2HPO_4, plus KOH	8·9	White

Experiment A was a "well experiment" with K_2HPO_4 as buffer, Experiment B was a "dip experiment", as described in the Experimental section. Anaerobic incubations: 2 hr.

the described treatments, rinsings, and exposure to air (Experimental section) rapid darkening of the water control took place but no darkening of the enzyme-treated surface. Treatment with the O-methyltransferase system had the effect of permanently retaining the surface color of a freshly cut apple. Another result occurred that was unexpected in light of the earlier

juice experiments: after the control treatment with boiled methyltransferase, again little darkening appeared, necessitating a further exploration of the experimental factors involved in the induced loss of oxidative darkening by cut fruit.

It was found that a pH change alone, which duplicated the pH of the described boiled enzyme sample, had the same effect. The methyltransferase experiments were run in mildly alkaline solutions close to the pH 7·5–8·2 optimum of the enzyme.[7] Well experiments using only buffers indicated that an anaerobic incubation with any of several slightly alkaline buffers (phosphate, bicarbonate, or *tris*-(hydroxymethyl)–aminomethane–HCl) would stabilize the color of the apple surface when it was subsequently reacidified and exposed to air. Sodium or potassium buffers, in the range 0·1–0·4 N cation concentration, were tested and found effective. The effect of pH on the inhibition of darkening of apple sections ("dip experiment") is seen in Table 1, Experiment B. Each apple section in the experiment was immersed only half-way, so that the upper portion acted as an untreated control in each case.

Incubation in the pH range 8–9 (in 0·1 or 0·2 M K_2HPO_4) gave maximum stabilization of the surface constituents against oxidation on re-exposure to air. Immersion in buffer below pH 5 (e.g. 0·2 M KH_2PO_4) gave no stabilization effect, while immersion at pH below 3 or above 10, or in distilled water, gave rise to necrotic darkening. The prevention of oxidative darkening by slightly alkaline buffer was a permanent effect, surviving air-drying of the segments for several days. This effect, just as with the methylation experiments, was upon the substrates of PPO: when an external substrate, such as dihydroxyphenylalanine, was added to the treated sections rapid darkening occurred, indicating the PPO enzyme to be still vigorously active at the apple surfaces where the slightly alkaline treatment had prevented darkening of the *in situ* constituents. The buffer effect described is different from the well-known effect of NaCl (Cl-ion) which prevents oxidation by inhibiting PPO itself.[8] The latter conclusion was confirmed by experiments in which apple sections were immersed into 0·1 and 0·4 M chloride salts, after which substrate was added.

In order to test whether the alkaline buffer treatment had caused a differential leaching of PPO substrates from the fruit surface, buffers from alkaline and acidic incubations (both well and dip experiments) were removed by pipet, brought to pH 5, and shaken in air with and without addition of supplementary PPO preparation. No difference in darkening of constituents leached from the apple was observed. Absorption spectra of incubation solutions did reveal an unstable compound with a high absorption peak (264 mμ) present in the slightly alkaline (K_2HPO_4) extracts and little absorption in parallel acidic (KH_2PO_4) extracts. The alkaline peak did not represent differential extraction, however. The peak disappeared when the solution was acidified and appeared again, reversibly, when the pH was again made alkaline, if the extracts were kept cold and manipulated rapidly or under nitrogen. Hence, the different absorption curves represent only different ultraviolet absorbing forms of the same extracted unidentified compound, and do not signify a differential extraction of PPO substrate from the apple cells.

DISCUSSION

Chlorogenic acid[1,2] and (−)-epicatechin[1,3] are reported to be the major substrates of PPO in apple fruit. But, *whatever* might be the particular PPO substrate compounds active in the fruit, or the sequence in which their oxidation normally occurs, the results reported

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here indicate that reaction of the constituents with the catechol-O-methyltransferase system prevents their participation in the reactions of oxidative polymer formation known as "oxidative browning". These findings, considering the *o*-diphenol specificity of the enzyme,[7] lend generalized support to the empirical results of the previously cited publications in confirming that *o*-diphenols (catechols) do indeed constitute the important substrates concerned in the oxidative browning of fruit.

The results with apple juice are clearly interpretable: incubation with a specific transmethylating system permanently modified the constituent PPO substrates, presumably by their methylation. But with apple fruit surface treatments, while the results are clear, interpretation is not. Incubation with the animal methyltransferase system at its optimum pH of about 8 permanently eliminated the surface substrates of PPO. A pH 8 buffer alone, however, had almost the same effect. Either the natural surface substrates were permanently modified to inactive compounds, or, they were differentially leached out at slightly alkaline pH (in contrast to acidic pH). Our preliminary observations favor the former occurrence—substrate modification—although this would be difficult to establish conclusively with the small amounts of substrate compounds present in the cut surface.

The pH conditions which permanently diminished the oxidative darkening of PPO substrates in the cut fruit were in the same range as the pH optimum of the animal O-methyltransferase, hence the *in situ* presence and operation of a plant O-methyltransferase system is suggested. A search for O-methyltransferase in plant tissues, in order to determine its properties, has been successfully made, and it is, indeed, optimally active at slightly alkaline pH.[9] We are now conducting tracer experiments as highly sensitive criteria for determining whether *in situ* O-methylation in the low-substrate fruit tissues does indeed take place in response to the described pH change, and possibly also as a means of identifying methylated derivatives of the normally present PPO substrates.

The findings described suggest that exposure of plant tissues to O-methyltransferase or to slightly alkaline buffer is a mild, non-toxic treatment which can be used to avoid the oxidative effects of PPO. The incubation period may be reduced considerably (even to 5 min) and still be effective. This aspect of our observations has obvious implications with regard to the undesirable darkening of marketable plant products.

EXPERIMENTAL

Methylation System

The reagents and conditions for reaction were similar to those reported.[6] S-Adenosylmethionine iodide (AMe) was obtained from the California Corporation for Biochemical Research.* In juice experiments, the following quantities of reagents were used per ml reaction mixture: 4 mg (as protein[10]) rat liver catechol-O-methyltransferase enzyme, 12 μmoles AMe, 10 μmoles $MgCl_2$, and 100 μmoles pH 9·0 *tris*-(hydroxymethyl)-aminomethane-HCl (TRIS) buffer (final pH of mixture, 8·2). Controls on the enzyme component contained either boiled enzyme (15 min at 100°) or dilute buffer corresponding to the concentration found in the enzyme solution without added enzyme. All incubations were carried out in an anaerobic environment to avoid premature PPO darkening.

* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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Apple Juice Experiments

The juice from green-mature Pippin apples was expressed cold and filtered through a Whatman No. 1 paper (Acme centrifugal juicer), then stored frozen in the presence of 0·01% ascorbic acid. Methylation treatments (0·5 ml juice per ml reaction mixture) were carried out in Thunberg tubes. Buffer and apple juice were each held in compartments separated from the other reagents. The tubes were flushed with nitrogen and evacuated at 0°, then tipped and brought to 38°. After an incubation period the tubes were opened, acidified to pH 6 with 0·15 ml 1 M KH_2PO_4, and shaken in air at room temperature.

Apple Surface Experiments

Green-mature Gravenstein apples were cut in half (in "well experiments") or radially sectioned (in "dip experiments"). In the well experiments, plastic rings were inserted into the cut surfaces to form a well into which reagents could be added. Reagents were added in the proportions described under "methylation system" (about 0·3 ml total volume), but including one drop 0·1% ascorbic acid added initially to each well to reduce traces of oxygen present. TRIS or K_2HPO_4 was used as buffer. All manipulations were carried out rapidly in a cold room; each half-apple was placed into a small bell-jar under nitrogen flow, and the bell-jar was then sealed and brought to room temperature for incubation. After the incubation period (½–3 hr), the contents of the wells were removed by pipet, the wells were rinsed with water and 0·2 M KH_2PO_4, and the apples were allowed to stand in air.

Another procedure ("dip experiments") was to section Pippin apples under 0·1% ascorbic acid and place them into reagents in a small beaker which was then placed in a bell-jar under nitrogen and brought to room temperature for incubation, as described. After incubation for 2 hr, the sections were dipped for 2 min into water and two changes of KH_2PO_4, then blotted and exposed to air for 1·5 hr. 0·1 ml of 0·02 M dihydroxyphenylalanine was added to well inserts during the air exposure period.

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