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EVIDENCE ABOUT THE CATECHOLOXIDASE ACTIVITY OF THE ENZYME ASCORBATE OXIDASE EXTRACTED FROM *CUCURBITA PEPO MEDULLOSA*

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Summary

Pure ascorbate oxidase (L-ascorbate:oxygen oxidoreductase, EC 1.10.3.3) isolated from *Cucurbita pepo medullosa*, which is known to be specific for ascorbic acid, shows a secondary catecholoxidase activity at approx. pH 6.7.

This activity was tested against natural and synthetic compounds possessing a catechol-like structure.

Among natural compounds (+)-catechin furnishes the same complex oxidation mixture obtained with other oxidases.

Among synthetic compounds, 3,5-di-*t*-butylcatechol and 4-*t*-butylcatechol give the corresponding *o*-quinones.

The significance of this secondary activity in the darkening process of fruits and vegetables which contain ascorbate oxidase is also discussed.

Introduction

A previous communication from this laboratory reported the isolation of an ascorbic acid oxidase (L-ascorbate:oxygen oxidoreductase, EC 1.10.3.3) from *Cucurbita pepo medullosa* [1]. This enzyme contains copper [2—3] and catalyses the oxidation of ascorbic acid, maximally around pH 5.6.

During the course of the isolation and purification of this enzyme, we observed that the crude enzyme extract must be rapidly and completely separated from low molecular weight cell components in order to avoid progressive darkening, which lowers the yield of the purified enzyme.

This observation has let to some questions regarding the possibility that the enzyme would be capable of oxidising various natural compounds present in

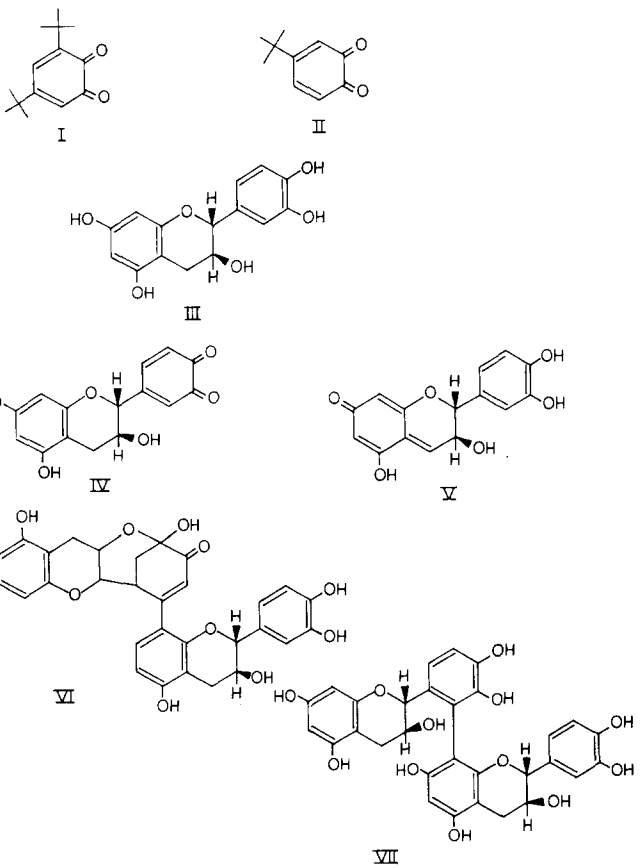
vegetable cells. The enzyme is known to be strictly specific with regard to the aerobic oxidation of L-ascorbic acid, which leads to dehydroascorbic acid and water.

Since nothing is known [4] about the oxidation of ascorbic oxidase with regard to compounds other than ascorbic acid, it has been considered important to study the possibility of such reactions.

The substrates, which were subjected to the action of ascorbate oxidase in our study, have been chosen from those compounds normally present in vegetable cells, which are known to give rise to high molecular weight compounds, like tannins and lignins, that are presumably responsible for the characteristic browning of vegetables which occurs in their senescent stage or as result of tissue lesion formation incurred by vegetables during storage [5].

Thus, some hydroxycinnamic acids, catechols and flavanoids have been tested, together with other compounds such as 3,5-di-*t*-butylcatechol (I), 4-*t*-butylcatechol (II) and L-DOPA which, although completely synthetic and not present in vegetable organs, have structural analogies with the natural products mentioned above.

Particular attention has been focussed on the flavan (+)-catechin(III) because its oxidation products could be isolated, although with some difficulty, from the reaction mixture and because it is widely found in the vegetable kingdom, mainly localised in the peripheral tissues of vegetable organs in areas where the enzyme ascorbate oxidase is also present [6—7].



The results of the present research revealed that the enzyme ascorbic acid oxidase might play an important role in the enzymatic oxidative darkening processes of vegetable extracts and has led to the formulation of a new hypothesis on the biological function of this cuproprotein.

Material and Methods

The chemical reagents used in this work were of analytical grade and were not further purified.

The compounds (+)-catechin, quercetin, chlorogenic acid, caffeic acid, ferulic acid and *p*-cumaric acid were all products purchased from Fluka A.G. Buchs.

The 3,5-di-*t*-butylcatechol, 4-*t*-butylcatechol, hydroquinone, and 3,4-dihydroxyphenylalanine (DOPA) were synthetic compounds of analytical grade from Fluka and C. Erba, Milan. Phenolase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.15.1 (formerly 1.10.3.1)) was purchased from Worthington Biochemical Corporation Freehold.

Peroxidase (donor:hydrogen peroxide oxidoreductase, EC 1.11.1.7) from *Cucurbita pepo medullosa* was purified and characterized by one of us.

Isolation and purification were accomplished using ammonium sulphate precipitation acetone, chromatography on DEAE-cellulose and preparative gel electrophoresis. The cathodic protein had a Rein Zahl of 1.2.

Catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6) analytical grade was purchased from Boehringer Mannheim.

The enzyme ascorbic oxidase purified and characterized by us, was found to be free from impurities detectable by means of electrophoretic technique and ultracentrifugation (Figs. 1, 2 and 3). Analysis by neutronic activation of the metals in the purified protein used in the oxidation tests, has revealed the presence of 8 copper atoms per mole of enzyme. A molecular weight of 140 000 was determined for the native protein by sedimentation equilibrium. The same analysis has shown that the purified enzyme has a content of other metals equal to or less than 0.1 μg/mol of enzyme [8].

The resin Chelex 100 from Bio Rad, Richmond, successively washed with 0.1 M NaOH, 0.1 M HCl, and deionized water prior to use to remove all extra-

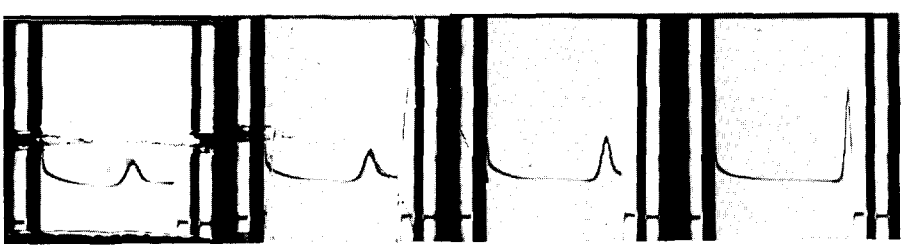


Fig. 1. Purity test for the ascorbic acid oxidase used in the study. Schlieren pattern of ascorbic acid oxidase, 1.5 mg/ml in 0.1 phosphate buffer, pH 7.00, at 23°C. Photographs show the sedimentation pattern, the exposure was made 15, 30, 45 and 60 min after reaching maximum speed (56 000 rev/min). Bar angle 60°. The $S^0_{20,w}$ value of the sedimenting peak is 7.05 S and a molecular weight of 140 000 has been estimated.

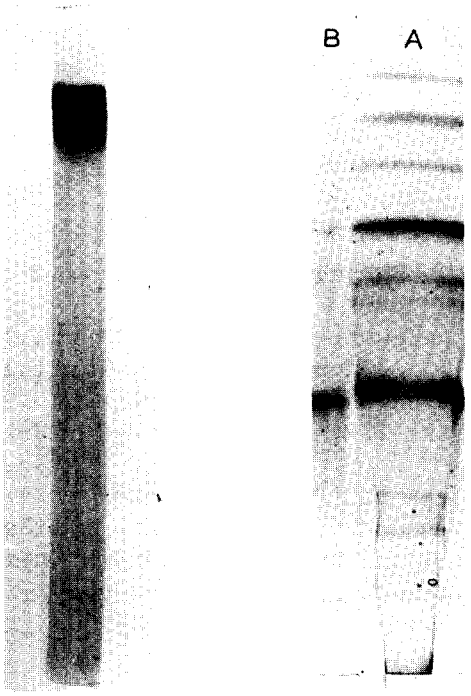


Fig. 2. Analytical polyacrilamide gel electrophoresis of ascorbic acid oxidase in Tris/glycine buffer, pH 8.6. The electrophoresis was carried out on 8% standard polyacrilamide gel at pH 8.6 for 6 h at 4°C. The gel columns were 0.6 × 7 cm and a current of 2 mA per tube was applied.

Fig. 3. The SDS electrophoresis was performed in gel state of 10% acrylamide (Bis being 25:1 of acrylamide) in 0.125 M Tris/borate, pH 8.9, 0.1% SDS. The stacking gel was 6% acrylamide in 0.115 M Tris · Cl, pH 6.8, 0.1% SDS. Electrode buffer: 0.125 M Tris/glycine, pH 8.9 and 0.1% SDS. Samples were dissolved in 0.05 M Tris/Cl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 6 M urea. The run was done at 4°C at 50 mA. Markers from the bottom to the top: bovine serum albumin (M_r = 68 000), ovalbumin (44 000), concanavalin (25 800, 18 000, 13 000, 10 000 [14]). A, markers; B, ascorbate oxidase.

neous free ionic copper from ascorbate oxidase. The pH of the suspension after this treatment was roughly neutral. The resin was stored at 4°C until required for use. A column 5 cm in length, diameter 0.5 cm, was filled with the prepared resin and washed with 10 mM phosphate buffer, pH 7. The pH of the eluate after passage of 100 ml of buffer solution was about pH 7.

1 ml of ascorbate oxidase solution was added to the column (5 mg/ml of the enzyme) 20 ml of phosphate buffer, pH 7, were added subsequently to elute the enzyme completely from the column.

The eluate was then concentrated by means of ultrafiltration with colloidal bags.

Protein determination was by the Lowry method [9].

Enzymatic reaction

10 mg of substrate were transferred to a 20 ml test-tube, 0.5 ml of methanol was added followed by 0.1 M phosphate buffer, pH 6.7 up to a final volume of 10 ml. The enzyme (2 μg protein), was added and the mixture left to stand for 15 h.

In the case of (+)-catechin the unreacted substrate was extracted with ether, the reaction products with ethyl acetate and high molecular weight compounds with *n*-butanol. In the case of 3,5-di-*t*-butylcatechol the precipitated reaction product is collected by filtration.

In the other cases, the unreacted product is extracted with ethyl acetate while the reaction products are not or are only partially extracted.

Thin-layer chromatography

Merck F 254 silica gel plates measuring 20 × 20 cm, 0.25 mm thick, and with fluorescence indicator were used.

The eluent mixture consisted of butyl acetate/methanol/water/formic acid (8:1:1:1, v/v). After evaporation of the solvent with a rotatory evaporator, the reaction products were dissolved in methanol and deposited on the plates.

Chromatography was carried out for 90 min at 20°C and at the end of this period the solvent front had run about 17 cm.

The plate was examined under ultraviolet light. The silica layers corresponding to ultraviolet-visible spots were then scratched from the plate and the reaction products extracted with ethyl acetate and then treated overnight with pyridine and acetic anhydride in order to obtain the acetylated derivatives.

Dilution with methanol and evaporation gave crude acetylderivatives.

Determination of the optical and mass spectra

Optical spectra were determined with a Perkin Elmer 402 spectrophotometer. After acetylation of products, the NMR spectra were taken with a Varian XL-100 instrument in a solution of C^2HCl_3; chemical shifts are in ppm from TMS. Mass spectra were obtained with a LKB 9000 mass spectrometer.

Stoichioimetry of the oxidation reaction catalysed by ascorbic oxidase

The substrate (22.2 mg) 3,5-di-*t*-butylcatechol in methanol was dissolved in 0.1 M phosphate buffer, pH 6.7. The solution was placed in a glass cell (total volume 1.5 ml) connected to a Clark electrode. Ascorbic acid oxidase was added to the cell and the oxygen uptake was recorded. The system had been previously calibrated with ascorbic acid and ascorbic acid oxidase.

It is known that the oxygen uptake is stoichiometrically proportional to the quantity of ascorbic acid present in the medium [10] as expressed by the equation which shows that for every mole of ascorbic acid oxidised, 1/2 mol of O_2 is used:

$$\text{Ascorbic acid} + \tfrac{1}{2}O_2 \xrightarrow{\text{ascorbate oxidase}} \text{dehydroascorbic acid} + H_2O$$

Thus is was possible to arrive at the stoichiometry of the enzymatic reaction from the cell oxygen up-take, by comparison of the oxygen up-take from 1 mol of ascorbic acid to that consumed by 1 mol of 3,5-di-*t*-butylcatechol.

The reaction was effected with an enzyme:substrate ratio of 1:1. Under these conditions the substrates were completely oxidised after a period of 5 min using 0.1 mM substrate and 0.1 mM enzyme of 140 000 molecular weight [9—11].

Results

(a) Characteristics of the oxidation reaction of the substrates used

The activity of ascorbate oxidase was tested against a series of substrates, as given under Methods, the concentration of the substrate being greater than that of the enzyme (10^{-4} M substrate compared with 10^{-6} M enzyme).

All the substrates give rise to the formation of coloured and highly polar products in the presence of ascorbate oxidase. That the reaction had taken place was demonstrated by thin-layer chromatographic analysis of the reaction mixture in the case of catechin and from the quantity of original substrate recovered by extraction with a suitable solvent as described in Methods.

In particular, among the various substrates, 3,5-di-*t*-butylcatechol and 4-*t*-butylcatechol gave a single reaction product, the corresponding quinone. Under the analytical conditions of our procedures the flavan (+)-catechin showed, instead, more polar reaction products.

In the case of the other substances, the complexity of the reaction mixture composed of highly polar materials prevented characterization of the products.

Fig. 4 gives the thin layer chromatographic pattern of the reaction between (+)-catechin and enzyme: two reaction products are seen alongside the unreacted product (C), one of which (A) is highly polar while the polarity of the second is similar to that of the catechin (B).

Isolation and the determination of their structure are described below. In additon polymeric products * (P) which do not move in thin-layer chromatography are present.

The activity of the cuproprotein towards the compound (+)-catechin has been tested at several pH values. Fig. 5 gives the quantity, in mg, of the reaction products isolated from the chromatographic plate, as a function of the pH.

Fig. 6 shows the enzymatic activity measured at various concentrations of catechin. The results are plotted according to the Lineweaver-Burk method.

Fig. 7 illustrates a series of comparative chromatograms regarding the reaction products obtained with the substrate (+)-catechin ($3.4 \cdot 10^{-4}$ M) in 0.1 M phosphate buffer, pH 6.7, with different oxidase enzymes, fungal phenolase, peroxidase, with ascorbic acid oxidase treated or not with Chelex 100 resin and with catalase.

Furthermore, the oxidation reaction was carried out in the presence of copper sulphate and hydrogen peroxide separately.

The enzymatic reactions of catalase added to the (+)-catechin plus ascorbic acid oxidase show no differences compared with the previous chromatograms; nor does Chelex 100 show any effect. The chromatographic plates obtained with copper sulphate $1 \cdot 10^{-5}$ M and hydrogen peroxide $1 \cdot 10^{-5}$ M do not reveal reaction products similar to those of the chromatograms obtained with the (+)-catechin plus enzymes.

In the test where ascorbic acid was present in the proportion of 1:1 with the

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* By "polymeric products" we mean highly polar products which have no mobility in thin-layer chromatography in the eluent system used. We therefore do not intend to offer any indication of structure of the number of monomer units and certainly not of the presence of only one or more products.

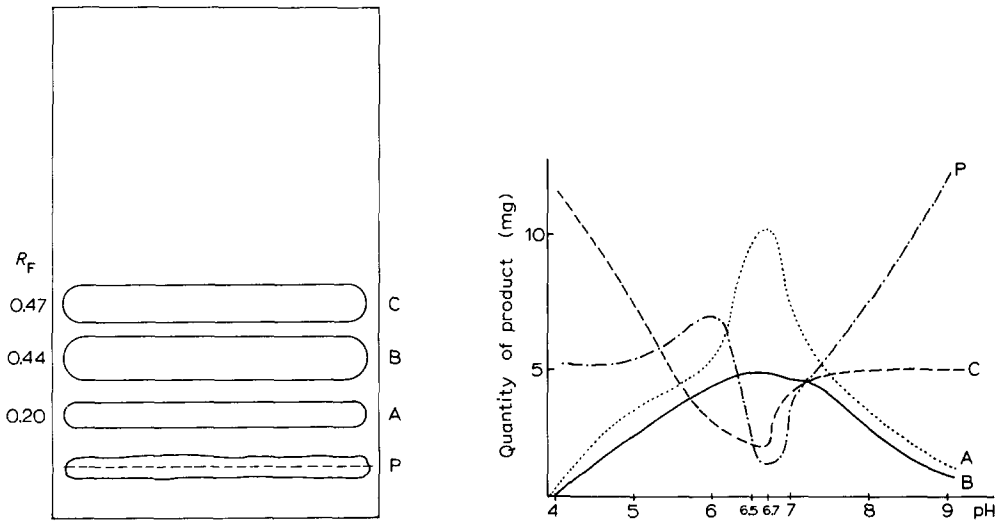


Fig. 4. Thin-layer chromatogram obtained with Merck 2.5 mm silicagel plate with fluorescent indicator, revealed with fumes of iodine. The spots indicated by the letters C, B, A and P indicate: C, (+)-catechin; B, dimer of catechin (dehydrodicatechin A); A, 6,8-dicatechin; P = polymers of catechin. The eluent was the upper part of the mixture butyl acetate/methanol/HCOOH/HCl, 8:1:1:1.

Fig. 5. Activity of the enzyme ascorbic oxidase on (+)-catechin as function of the pH. The graph illustrates the weights (mg) of products A, B, C and P. The reaction products were obtained with various enzymatic tests at different pH values and successively isolated by thin-layer chromatography as in Fig. 4. Letters as in Fig. 4.

catechin substrate, there was a delay of several hours before the reaction products appeared. The chromatogram was then found be the same as the others obtained with various enzymes.

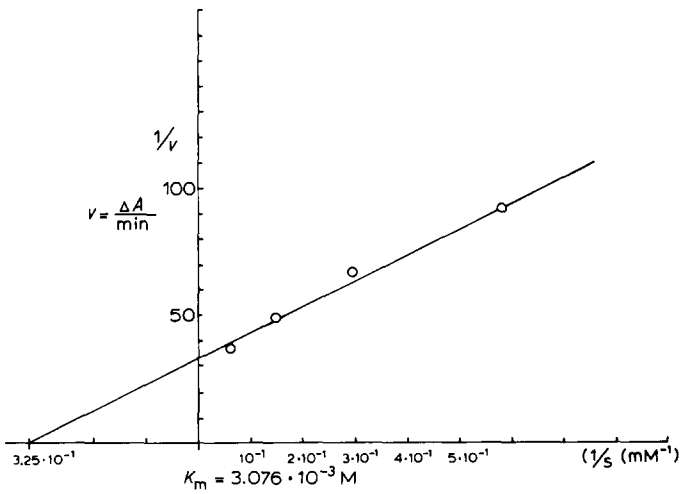


Fig. 6. Determination of the K_m of the enzyme ascorbic acid oxidase, with the compound (+)-catechin, in 0.1 M phosphate buffer, pH 6.7. The quantity of enzyme used was $5 \cdot 10^{-4}$ M. The catechin substrate was used in concentrations from $3.4 \cdot 10^{-4}$ M to $1.3 \cdot 10^{-2}$ M. The absorbance of the reaction products was read at 385 nm, at 30 s intervals.

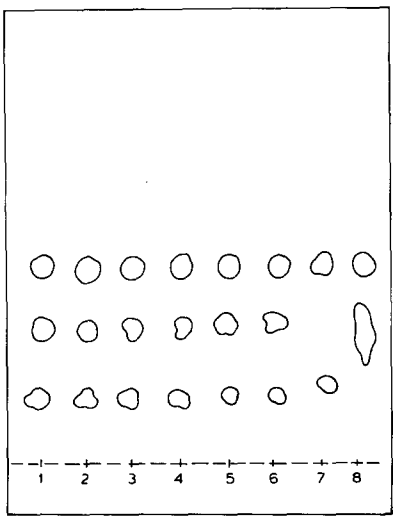


Fig. 7. Thin-layer chromatography on silicagel of the (+)-catechin reaction products (1 mg/ml; total volume of the reaction mixture, 10 ml) at room temperature in 0.1 M phosphate buffer, pH 6.7. The enzymatic reaction was obtained with 4 different enzymes: ascorbic acid oxidase treated with Chelex 100 resin or untreated, pure peroxidase extracted from squash (R_F = 1.2), phenolase (Worthington), and catalase (Boehringer). The enzyme catalase was used in mixture with ascorbic acid oxidase. The quantity of enzymes used for each test was 2 μg of protein for each enzyme. The catechin oxidation reaction was also obtained with hydrogen peroxide ($1 \cdot 10^{-5}$ M) and copper sulphate ($1 \cdot 10^{-5}$ M). 1, ascorbate oxidase; 2, fungal oxidase; 3, peroxidases from green squashes; 4, ascorbate oxidase plus ascorbic acid; 5, ascorbic oxidase treated with Chelex 100; 7, copper sulphate; 8, hydrogen peroxidase.

(b) Structures of the oxidation products of the enzymatic reaction of catechin, 3,5-di-t-butylcatechol and 4-t-butylcatechol

At the end of the enzymatic reaction the unreacted catechin is extracted with ether. Successively the reaction products indicated by A and B in Fig. 4, are extracted with ethyl acetate and separated by means of preparative thin-layer chromatography. Product B is a yellow solid which crystallizes from acetone/water and melts at 280° C, $[\alpha]_D^{20} = 400°$ (c = 1, acetone). Its ultraviolet spectrum in MeOH shows absorption maxima at 385, 276, 255 nm (log ϵ 4.23, 3.70 and 3.83). The highest peak in *m/e* units in the mass spectrum is seen at 288 atomic mass units, apparently indicating a dehydrocatechin structure.

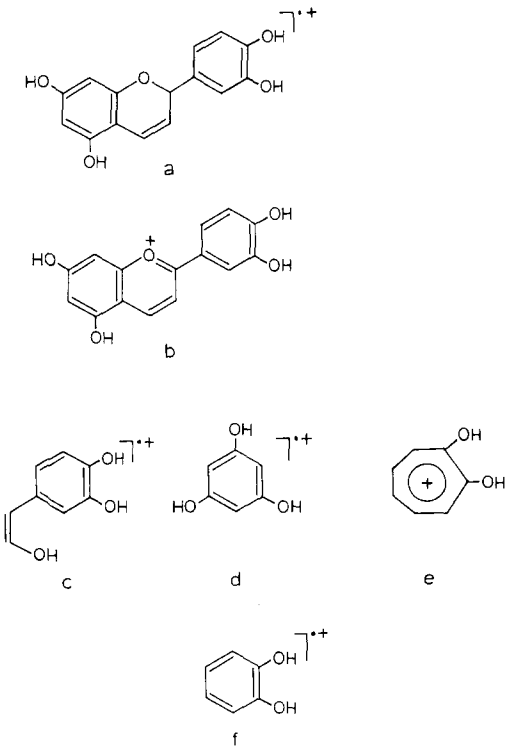
Other peaks present in the spectrum appear at *m/e* 256, 232, 180, 152, 126, 123 and 110. This spectrum was compared with that of catechin which, apart from the molecular ion at *m/e* 290, shows peaks at *m/e* 272, 271, 152, 136, 123 and 110, respectively attributable to ions a, b, c, d, e and f (Scheme I). On the basis of the presumed fragmentations, neither an *o*-quinone structure (IV) nor a quinonmetide structure (V) could explain such a spectrum.

In fact, apart from peaks at *m/e* 123 and 110, structure IV explains the peak at *m/e* 150 but not that at *m/e* 152. The opposite is true for structure V. In particular it is to be noted that there is no loss of water from the molecular ion.

To throw some light on the structure of compound B, it was acetylated under usual conditions.

A colourless amorphous acetate was obtained, which was homogeneous on

Scheme I. Fragmentation ions of (+)-catechin.



thin-layer chromatography (eluents: CH_3-CH_2-CH_2-CH_2O-CO-CH_3/CH_3OH/HCOOH/H_2O (8:1:1:1), CH_2Cl_2/CH_3OH/H_2O (80:19:1). Its NMR spectrum in C^2HCl_3 showed, among other acetoxyl groups at 1.98, 2.16, 2.18, 2.24, 2.27 (2 groups) and 2.30 and a complex signal for a H-C-OAc group at 5.30.

These data lead to the assumption that compound B has a structure derived from the condensation of two catechin molecules. Catechin dimerization products are widely reported in the literature [12—13] and research along these lines has shown that the enzymatic oxidation of catechin with fungal phenolase, laccase, horseradish peroxidase, gives a yellow product of structure VI known as dehydrodicatechin A [12].

As reported in the Methods, comparison of the reaction of (+)-catechin with ascorbate and fungal oxidase phenolase indicate the identity of our compound B with the yellow dehydrodicatechin A (VI).

The identity of two products has been proved after isolation by direct comparison (superimposed infrared, thin layer chromatography). Furthermore, the same yellow product is obtained on chemically oxidising (+)-catechin with metallic reagents such as cerium ammonium nitrate, potassium iodate and silver nitrate.

Comparison between the enzymatic reaction of catechin with ascorbate oxidase and with the above mentioned enzymes has established the nature of the other oxidation product A; in fact this product appears also in the oxidation of (+)-catechin with peroxidase, etc., and the identity has been established

by comparing the chromatographic behaviour with different solvents.

Byung-Zun Ahn has demonstrated that the product in question has structure (VII) of 6′:8-dicatechin [13]. The mass spectrum obtained using the silyl derivative showes peaks at 930, 648, 368 and 282 *m/e* in agreement with the proposed structure.

The oxidation products of 3,5-di-*t*-butylcatechol (I) and 4-*t*-butylcatechol have been identified as the corresponding *o*-quinones through direct comparison (thin-layer chromatography, infra-red, ultraviolet) with authentic samples.

Discussion

The aerobic oxidation experiments performed with the pure enzyme, ascorbic oxidase, characterised chemically and physically (ultracentrifugation, electrophoresis and neutronic activation analysis of the metals) shows that ascorbate oxidase possesses a secondary activity towards such compounds.

The authors therefore suggest that the enzyme should not be considered strictly specific towards L-ascorbic acid since it is capable of catalysing the aerobic oxidation of various compounds which contain a functional *o*-diphenolic group.

The presence of ascorbic acid prevents the enzymatic oxidation of (+)-catechin since reduction of the oxygen occurs at a much higher rate ($K_m = 2.4 \cdot 10^{-4}$ M) with ascorbic acid, compared to (+)-catechin ($K_m = 3.076 \cdot 10^{-3}$ M). Successive oxidation of the (+)-catechin occurs after several hours, following complete diffusion of oxygen in the medium and/or enzymatic inactivation which takes place as a result of the catalysis of the first reaction with ascorbic acid.

The oxidation of *o*-diphenols in the presence of ascorbic acid oxidase is attributable to the enzyme's catalytic action because treatment of the enzyme with Chelex 100 and the addition of catalase to the enzyme system does not alter the oxidative activity. Furthermore, Cu^{2+} and hydrogen peroxide are capable of oxidising the catechin substate but the reaction products are not the same as those obtained with the enzyme. On oxidation of the substrate 3,5-di-*t*-butylcatechol the reaction products are the corresponding quinone and water. In the case (+)-catechin substrate, the reaction does not go to completion and the reaction products affect the rate of reaction reducing it until the oxidation reaction is blocked.

The in vitro reactivity revealed by our experiments suggests that the enzyme in vegetable cells can oxidise *o*-diphenolic metabolites.

For this to occur, the enzyme and the *o*-diphenol compounds must come into contact through the desctruction of the normal cellular structure. This can happen after the tissue has been damaged, for instance on cutting. In this case, ascorbic acid oxidised and then the *o*-diphenolic metabolites undergo the enzymatic attack, affording the characteristic browning substances.

A similar situation occurs during the senescence stage of vegetables. In fact, when the organism ages, a number of factors (enzymatic catabolism, dehydration, etc.) contribute to mix the cell content, thus reducing the ascorbic acid endowment and allowing the successive enzymatic attack on *o*-diphenolic metabolites. Among these compounds, flavanol (+)-catechin is one of the most abundant substrates.

Consequently our findings on the secondary activity of ascorbate oxidase clarify the origin of the well known process of darkening at least in the fruits and vegetables which contain ascorbate oxidase and *o*-diphenolic compounds.

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