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Comparative Study of the Products of the Peroxidase-Catalyzed and the Polyphenoloxidase-Catalyzed (+)-Catechin Oxidation. Their Possible Implications in Strawberry (*Fragaria* × *ananassa*) Browning Reactions

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The peroxidase- and polyphenoloxidase-catalyzed oxidations of (+)-catechin yield several products showing different degrees of polymerization, which are apparently responsible for the pigment decay and the associated browning reaction that occurs in processed strawberry fruits and their derived foods. In this work, we have purified both peroxidase and polyphenoloxidase from Oso Grande cv. strawberry fruits, and comparatively analyzed the products of their enzyme-mediated (+)-catechin oxidation. The joint analysis by reversed-phase and size-exclusion HPLC of the (+)-catechin oxidation products obtained with both enzymes indicate that they were qualitatively the same: dehydrodicatechin B4, a (+)-catechin quinone methide, dehydrodicatechin A, a (+)-catechin trimer, and a (+)-catechin oligomer with polymerization degree equal to or greater than 5. The main quantitative differences between the oxidative reactions were the great amount of oligomer formed in the case of the polyphenoloxidase-mediated reaction and the low amount of (+)-catechin reacted in the case of the peroxidase-mediated reaction. One of the possible reasons for such low levels of (+)-catechin consumption in the case of the peroxidase-mediated reaction was the possible inhibition by products of the enzyme-catalyzed oxidation. In fact, the peroxidase-mediated (+)-catechin oxidation was differentially inhibited by dehydrodicatechin A, showing a competitive type inhibition and a k_I of 6.4 μM. In light of these observations, these results suggest that brown polymer formation, estimated as oligomeric compounds resulting from (+)-catechin oxidation, in strawberries is mainly due to polyphenoloxidase, and although peroxidase also plays an important role, it is apparently autoregulated by product (dehydrodicatechin A) inhibition.

KEYWORDS: (+)-Catechin, (+)-catechin oligomers; (+)-catechin oxidation; dehydrodicatechin A; peroxidase; polyphenoloxidase; product inhibition

INTRODUCTION

Strawberries (*Fragaria* × *ananassa*) are very susceptible to undesirable alterations in texture, flavor, and color as a consequence of injury during processing (*1*). One of the most important causes of color deterioration in processed strawberry fruits is enzymatic browning, a phenomenon caused by the oxidation of polyphenolic compounds, which leads to the formation of dark brown polymers of a quinoidal nature (*2*). This process is accompanied by changes in the native color of the jam, which may lead to substantial economic loss due to unacceptability by consumers.

Pigment instability in canned syrup strawberries has been associated in part with oxidative phenomena caused by a basic peroxidase isoenzyme which remains active after appertization (treatment at 100 °C in a boiling water bath followed by fast cooling) (*3*), and which is capable of efficiently oxidizing (+)-catechin at μM H_2O_2 concentrations (*4*). Peroxidases, which are located in the concentric array of the vascular bundles and in the vascular connections with the seeds (*5*, *6*), together with polyphenol oxidases, which are located almost exclusively in the cortex and to a lesser extent in the pith (*6*), are considered to be the main enzymes responsible for pigment decay in processed strawberries and their derived foods (*1*, *7*−*9*). In this way, strawberry peroxidases (*4*) and polyphenoloxidases (*7*) are capable of oxidizing (+)-catechin, one of the major phenolic compounds in strawberries (*9*) and now regarded as both an antioxidant and a pro-oxidant compound (*10*−*13*).

Results in support of this observation have been obtained from several lines of evidence. Thus, when phenolic stability in processing-ripe strawberries was studied in response to aging under mildly oxidizing conditions (*9*), it was found that phenolic metabolism may be either oxidative (H_2O_2 independent) or peroxidative (H_2O_2 dependent), and that treating strawberry slices with H_2O_2 stimulates oxidative phenomena that take place in the absence of H_2O_2, such as (+)-catechin degradation and

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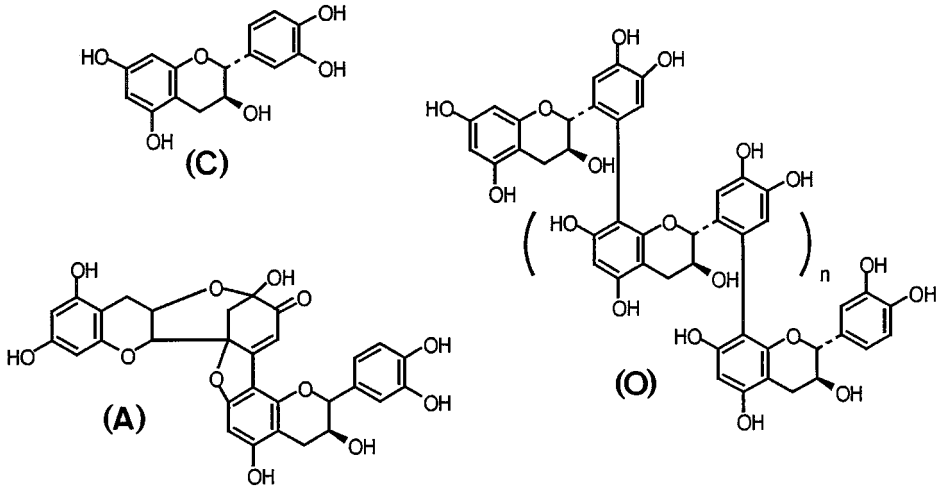
Chart 1. Structure of (+)-catechin (C) and of the Products of the Oxidative Coupling of (+)-catechin, Dehydrodicatechin A (A), and (+)-catechin Oligomers (O).



brown polymer formation. These results suggested a synergisic role for both peroxidase and polyphenol oxidase in (+)-catechin degradation and brown polymer formation during the aging of strawberry fruits under mildly oxidizing conditions.

The joint participation of peroxidase and polyphenoloxidase in the formation of both desirable and undesirable color modifications also seems to occur in strawberry fruits during maturation, ripening, and aging. In fact, when we studied the anthocyanin degradation index, color density, polymeric color, and the percentage of contribution of tannins as indices of desirable (or undesirable) color modifications in strawberries during fruit development, it was found that the anthocyanin degradation index, polymeric color, and tannins showed an exponential decay from the immature green stage to the processing-ripe stage (*14*), which correlated well with the levels of peroxidase activity measured during fruit development (*6*). Color density was more related to the levels of polyphenol oxidase (*6*). There seems to be, then, a strong relationship between polyphenol oxidase and peroxidase levels with both desirable (color density) and undesirable (anthocyanin degradation index, polymeric color, and tannins) color modifications during strawberry fruit development.

However, the levels of peroxidase and polyphenoloxidase themselves are not apparently the only factors that determine strawberry susceptibility to color alterations and enzymatic browning (*15*). Thus, it is known that the color stability of processed strawberry fruits differs according to cultivars (*13*, *14*). For example, Oso Grande and Chandler, two widely used strawberry cultivars in the food industry, show distinctive features, and the color (pigment) stability of processed Chandler is greater than that of Oso Grande (*14*), with the former being more suitable for processing. Differences in anthocyanin contents themselves cannot explain the color stability of Chandler against Oso Grande (*6*).

When levels of peroxidase and polyphenol oxidase, and levels of (+)-catechin, were studied in fruits of these two strawberry cultivars, it was found that the color stability of Chandler was apparently determined by moderately lower endogenous levels of peroxidase and polyphenol oxidase in the processing-ripe stage, accompanied by a very low (+)-catechin content (*6*). All these results together clearly indicate that endogenous peroxidase, endogenous polyphenoloxidase, and endogenous levels of catechin are the main factors responsible for determining the extent of browning reactions in strawberry fruits and their derived foods. In this work, we present a comparative study of the nature of the products of the peroxidase-catalyzed and polyphenoloxidase-catalyzed (+)-catechin oxidation.

MATERIALS AND METHODS

Plant Material. Strawberries (*Fragaria* × *ananassa* var. Oso Grande) were grown in-field at Huelva (Spain) and sampled at the processing-ripe (dark red color, slightly soft) stage. Fruits were frozen at −20 °C until use.

Chemicals. (+)-Catechin was obtained from Sigma Chemical Co (Madrid, Spain). Dehydrodicatechin A and (+)-catechin oligomers (**Chart 1**) were obtained by oxidation of (+)-catechin with horseradish peroxidase type IX (Sigma) according to López-Serrano and Ros Barceló (*16*). Dehydrodicatechin A ($C_{30}H_{24}O_{12}$) was purified with a yield of 90%, and analytical data (^{1}H NMR, ^{13}C NMR, and FAB) were in accordance with those reported by Weinges et al. (*17*) and Guyot et al. (*18*).

(+)-Catechin oligomers were purified with a yield of 81%. The δ values in ^{13}C NMR analysis of this oligomeric fraction closely resembled those obtained for (+)-catechin, and it was deduced that the oligomers were composed of repetitive units of (+)-catechin, presumably B-type dehydro-oligocatechins (**Chart 1**), linked by C−C interflavan bonds (*16*, *19*). When the minimal M_r for this oligomeric fraction was estimated by size-exclusion HPLC as described below, it was about 1600, indicating that these oligomers were composed of at least 5 units of (+)-catechin (M_r = 290).

Preparation of the Crude Enzymatic Extract. A 5-kg portion of strawberries was homogenized with a mechanical blade (Waring commercial heavy duty blender) in 5 L of acetone at −20 °C, and the residue was filtered through filter paper under vacuum. The protein precipitate was washed with acetone at −20 °C until all the pigments were removed, and then dried under a N_2 stream. The acetone powder was resuspended in 50 mM Tris−HCl (pH 7.5), containing 1 M KCl and 0.1 M $CaCl_2$, and gently stirred overnight. After incubation with this extraction buffer, abundant pectin precipitates appeared, which were removed by filtration through gauze. These pectin precipitates, which contained peroxidase and polyphenoloxidase activities, were incubated with the same buffer without $CaCl_2$, and filtered again. The filtrates were mixed, dialyzed overnight against 50 mM Tris−HCl (pH 7.5), and concentrated first with Aquacide I (Calbiochem, Calbiochem-Novabiochem Corp., La Jolla, CA), and then by centrifugation at 3000g_{max} using Centriprep-10 (Amicon, Amicon Inc., Beverly, MA). The concentrated protein fraction obtained after these preliminary steps constituted the crude enzymatic extract.

Peroxidase and Polyphenoloxidase Purification. All chromatographies described were performed using the Bio-Rad's Econo System (Bio-Rad Laboratories, Richmond, CA) at 5 °C.

Hydrophobic Chromatography on Phenyl-Sepharose CL-4B. The crude enzymatic extract was salted out with $(NH_4)_2$ SO_4 up to 1.5 M,

chromatographed on a Phenyl-Sepharose CL-4B (Sigma Chemical Co.) 40 × 1.5 cm gel bed column at a flow rate of 2.0 mL min^{-1}, and fractions of 7.0 mL were recovered. The eluent chromatography program was as follows: 100% A and 0% B from 0 to 125 min , 0% to 100% B from 125 to 235 min, and 100% B from 235 to 315 min, where buffer A was composed of 50 mM Tris−HCl (pH 7.5) containing 1.5 M $(NH_4)_2$ SO_4, and buffer B was composed exclusively of 50 mM Tris−HCl (pH 7.5).

Affinity Chromatography on Concanavalin A−Sepharose 4B. The unbound peroxidase fraction and the bound polyphenoloxidase fraction obtained from the hydrophobic chromatography were dialyzed against 50 mM Tris−HCl (pH 7.5), loaded on a concanavalin A−Sepharose 4B (Sigma Chemical Co.) 26 × 1 cm bed gel column and chromatographed at a flow rate of 1.0 mL min^{-1}. Fractions of 4.0 mL were recovered. The eluent chromatography program was as follows: 100% A and 0% B from 0 to 80 min, 0 to 100% B from 80 to 160 min, and 100 % B from 160 to 240 min, where buffer A was composed of 50 mM Tris−HCl (pH 7.5) containing 0.5 M NaCl, and buffer B was composed of 50 mM Tris−HCl (pH 7.5), containing 2.5 M NaCl and 0.5 M methyl-α-D-mannopyranoside

Ion-Exchange Chromatography on Econo-Pack CM Cartridges. The peroxidase and polyphenoloxidase fractions obtained from the concanavalin-A chromatography were dialyzed against 50 mM Mes−Na (pH 6.0) and loaded on three coupled 5.0-mL-capacity CM cartridges (Bio-Rad) at a flow rate of 1.0 mL min^{-1}. Fractions of 2.0 mL were recovered. The eluent chromatography program was as follows: 100% A and 0% B from 0 to 45 min, 0 to 100% B from 45 to 90 min, and 100 % B from 90 to 140 min, where buffer A was composed of 50 mM Mes−Na (pH 6.0), and buffer B was composed of 50 mM Mes−Na (pH 6.0) containing 1.0 M KCl.

Size-Exclusion Chromatography on Sephacryl S-200 HR. The polyphenol oxidase fractions obtained from the CM chromatography contained a certain amount of residual peroxidase activity. To purify this polyphenoloxidase activity from peroxidase, this enzyme fraction was dialyzed against 50 mM Tris−HCl (pH 7.5), and chromatographed on a Sephacryl S-200 HR (Pharmacia Biotech, Uppsala, Sweden) 47 × 1.5 cm gel bed column equilibrated with 50 mM Tris−HCl (pH 7.5) at a flow rate of 0.7 mL min^{-1}. Fractions of 1.0 mL were recovered.

Assessment of Purity of Purified Peroxidase and Polyphenoloxidase Fractions. The purity of peroxidase (absence of contamination by polyphenoloxidase) and polyphenoloxidase (absence of contamination by peroxidase) was checked by isoelectric focusing (IEF) in 3.5−10.5 pH gradients according to López-Serrano and Ros Barceló (*4*). IEF allows the simultaneous resolution of peroxidase (isoelectric point about 10.5) from polyphenoloxidase (isoelectric point 7.1−7.3) on the same running gel (*4*).

Molecular Mass Determination of Peroxidase and Polyphenoloxidase by Size-Exclusion Chromatography. The molecular mass determination of both peroxidase and polyphenoloxidase was performed by size-exclusion chromatography on Sephacryl S-200 HR as described above. The system was calibrated with the following molecular mass markers (Sigma Chemical Co.): cytochrome *c* (12.4 kDa), carbonic anhydrase (29.0 kDa), bovine albumin (66.0 kDa), alcohol dehydrogenase (150.0 kDa), and β-amylase (200.0 kDa).

Determination of Enzymatic Activities and Protein. The spectrophotometric determinations of enzymatic activities were carried out at 30 °C in 0.1 M Tris−acetate (pH 5.0) using a Uvikon 940 spectrophotometer (Kontron Instruments, Madrid, Spain) (*4*). Peroxidase activity was determined during the purification steps in a reaction medium containing 1.0 mM 4-methoxy-α-naphthol and 0.33 mM H_2O_2, by monitoring the increases in absorbance at 593 nm (ϵ_{593} = 21000 M^{-1} cm^{-1}). Peroxidase activity was also determined using 1.0 mM (+)-catechin by monitoring the increases in absorbance at 390 nm (ϵ_{390} = 4680 M^{-1} cm^{-1}) in a reaction medium containing 0.5 mM H_2O_2. Polyphenol oxidase activity was determined in a reaction medium similar to that used for peroxidase, but omitting H_2O_2. Activities were expressed in nkat (nmoles of substrate oxidized s^{-1}). The protein content in each fraction was estimated with the Bio-Rad Protein Assay (based on Bradford reagent).

Inhibition Studies with Dehydrodicatechin A. To study the nature of the inhibition of both the peroxidase-catalyzed and the polyphenoloxidase-catalyzed oxidation of (+)-catechin by dehydrodicatechin A, Michaelis−Menten kinetic curves were analyzed for several (+)-catechin concentrations at five dehydrodicatechin A concentrations (5, 10, 15, 20, and 30 μM). Inhibition type, inhibition constants (k_I), and inhibition degrees were deduced and calculated, respectively, from the $1/v$ vs [dehydrodicatechin A] Dixon's plots. I_{50} values were defined as the dehydrodicatechin A concentration which provokes 50% inhibition for both the peroxidase-catalyzed and the polyphenoloxidase-catalyzed (+)-catechin oxidations.

Oxidation of (+)-Catechin by Peroxidase and Polyphenoloxidase. The enzymatic oxidation of (+)-catechin by peroxidase and polyphenoloxidase from strawberries was performed in a way similar to that described by López-Serrano and Ros Barceló (*16*) for the oxidation of (+)-catechin by horseradish peroxidase. For this, two reaction media of 1.0 mL containing 4.0 mM (+)-catechin in 0.1 M Tris−acetate buffer (pH 5.0) were prepared. To one, purified strawberry polyphenoloxidase (free of peroxidase) was added. To the other, purified strawberry peroxidase (free of polyphenoloxidase) and 1.0 mM H_2O_2 were added. The activities were adjusted to supply 5 nkat of each enzyme to the reaction medium. To overcome possible denaturation of the enzymes during the incubation period, two successive additions of peroxidase and polyphenoloxidase were realized. In the case of the peroxidase-containing reaction medium, five further additions of H_2O_2 up to 1.0 mM were periodically performed. The reactions were stopped after 24 h by the addition of 1 mL of methanol. The samples were centrifuged at 14000*g* for 5 min to remove proteins, filtered through a 0.45-μm filter, and analyzed directly by HPLC.

HPLC Analyses of (+)-Catechin Oxidation Products. HPLC analysis was carried out in a Waters system (Millipore Corp., Waters Chromatography, Milford, MA) comprising a model 600 controller, a model 600 pump, a Rheodyne 7725i manual injector, and a Waters 996 photodiode array detector. The data were processed with the Waters Millenium 2010 LC version 2.10 software.

Reversed-phase (RP) HPLC was carried out at 25 °C on a 25 cm × 4.6 mm i.d. Waters Spherisorb S5-ODS2 column using a flow rate of 1 mL/min. Solvent A was 2.5% acetic acid in water and solvent B was acetonitrile. A linear gradient from 0 to 10% B in 5 min, from 10 to 30% B in 20 min, and from 30 to 50% B in 20 min was used.

Size-exclusion (SE) HPLC was carried out at 25 °C on a 30 cm × 7.8 mm i.d. TSK-Gel G2500HR column (TosoHaas, TOSOH Corp., Montgomeryville, PA) using dimethylformamide as eluent at a flow rate of 1 mL/min.

Samples of 20 μL each were injected and chromatographed under the above conditions. Because of the different λ_{max} of the (+)-catechin oxidation products (*16*), chromatographic profiles were obtained as Maxplot (λ_{max} chromatograms), defined in the Waters Millenium software as the chromatographic channel where each data point is the absorbance maximum of the spectrum acquired at a given point in time. Quantification of (+)-catechin oxidation products was performed at 280 nm using (+)-catechin as standard. The minimum detectable quantity of (+)-catechin in SE HPLC was found to be approximately 0.03 μg with a signal-to-noise ratio of 2 at 0.0005 AUFS. The precision of the analysis was assessed by replicate analyses of (+)-catechin, and then the variations were calculated. The response for a series of five injections of (+)-catechin resulted in relative standard deviations of 0.5% for the retention time (t_R) and 3.6% for the peak area.

RESULTS AND DISCUSSION

Purification of Peroxidase and Polyphenoloxidase from Strawberry Fruits. Peroxidase and polyphenoloxidase were purified from Oso Grande strawberry fruits in a three-step purification protocol, involving hydrophobic chromatography on phenyl Sepharose, lectin-based chromatography on concanavaline A, and cationic ion-exchange chromatography on carboxymethyl-cellulose. Although at 2.0 M $(NH_4)_2$ SO_4, both strawberry peroxidase and polyphenoloxidase are firmly bound to phenyl Sepharose, salting with 1.5 M $(NH_4)_2$ SO_4 allows the effective separation of peroxidase and polyphenoloxidase during chromatography. Under these conditions, a peroxidase fraction,

Table 1. Purification Chart for Peroxidase from Oso Grande Strawberries

	peroxidase activity			
purification step	total (nkat)	specific (nkat/mg protein)	purification factor	yield (%)
crude extract	807.5	15	1	100.0
phenyl sepharose	209.9	74	5	26.0
concanavaline A	60.9	441	29	7.5
carboxymethylcellulose	57.0	57011	3707	7.0

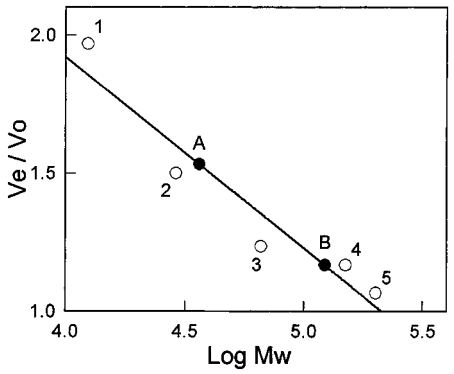


Figure 1. Ve/Vo vs log Mw plot for the determination of the molecular mass of Oso Grande strawberry peroxidase (A) and polyphenoloxidase (B) by size-exclusion chromatography on Sephacryl S-200 HR. The system was calibrated with the following molecular mass markers: cytochrome *c* (12.4 kDa, 1), carbonic anhydrase (29.0 kDa, 2), bovine albumin (66.0 kDa, 3), alcohol dehydrogenase (150.0 kDa, 4), and β-amylase (200.0 kDa, 5).

which is not retained by the column, may be purified free of polyphenoloxidase, which is almost totally retained by the column together with another bound peroxidase fraction. The unbound peroxidase fraction was further purified by chromatography on concanavaline A and carboxymethyl cellulose, to yield a pure peroxidase fraction, totally free of polyphenoloxidase, with a specific activity of 57.01 μkat mg^{-1} protein and a purification factor of 3707 (**Table 1**).

Oso Grande strawberry peroxidase showed a molecular mass of 36.0 kDa, as estimated by size-exclusion chromatography on Sephacryl S-200 (**Figure 1**), somewhat lower than that reported for two Selva strawberry peroxidases, which showed molecular masses of 58.1 kDa and 65.5 kDa (*8*) in this same chromatographic system. The differences between Oso Grande and Selva strawberry peroxidases may be due to the lower glycosylation of the former, as was confirmed from its low affinity for (binding to) concanavaline A-sepharose during chromatography.

The low molecular mass of Oso Grande strawberry peroxidase was borne in mind when purifying Oso Grande strawberry polyphenoloxidase free of peroxidase. In fact, the molecular masses of strawberry polyphenoloxidases have been reported as 111.0 kDa for Tioga (*20*) and of 135.0 kDa for Selva (*21*) cultivars. With this in mind, Oso Grande strawberry polyphenoloxidase was further purified by size-exclusion chromatography (**Table 2**). The purification steps yielded a pure polyphenoloxidase fraction, totally free of peroxidase, with a specific activity of 21.46 μkat mg^{-1} protein and a purification factor of 165 (**Table 2**). Oso Grande strawberry polyphenoloxidase showed a molecular mass of 122.0 kDa, as estimated by size-exclusion chromatography on Sephacryl S-200 (**Figure 1**), that is in the same range as that shown by other strawberry polyphenoloxidases (*20*, *21*).

Table 2. Purification Chart for Polyphenoloxidase from Oso Grande Strawberries

	polyphenol oxidase			
purification step	total (nkat)	specific (nkat/mg protein)	purification factor	yield (%)
crude extract	6831	130	1	100
phenyl sepharose	3905	635	5	57
concanavaline A	384	8346	64	5.6
carboxymethyl cellulose	314	5712	44	4.6
sephacryl S200	150	21464	165	2.2

These pure (36000 M_r)-peroxidase (free of polyphenoloxidase) and (122000 M_r)-polyphenoloxidase (free of peroxidase) fractions were used to determine the products of (+)-catechin oxidation by these metallo-enzymes.

Products of the Peroxidase- and Polyphenoloxidase-Mediated (+)-Catechin Oxidation. The peroxidase-catalyzed oxidation of (+)-catechin (RH) involves one-electron oxidation (*4*), and yields unstable mono-radical species, $2\ RH + H_2O_2 \rightarrow 2\ R^\bullet + 2\ H_2O$, which couple to generate dimers, $2\ R^\bullet \rightarrow R_2$. These dimers can be further oxidized by peroxidase, $2\ R_2H + H_2O_2 \rightarrow 2\ R_2^\bullet + 2\ H_2O$, yielding heterogeneous product mixtures of different degrees of polymerization, $R_2^\bullet + R^\bullet \rightarrow R_3$, etc. These oligomeric products result, in the last instance, from the repeated condensation reactions between the A ring of one unit and the B ring of another, through a mechanism which is known as "head to tail" polymerization. A similar heterogeneity for the (+)-catechin oxidation products is found when polyphenoloxidase is used as oxidizing agent (*18*, *22*, *23*). Some of these oligomeric compounds show biological activity and are capable of inhibiting plant β-glucosidases (*24*) and bacterial glucosyltransferases (*25*). Recently, we developed (*16*) a rapid method for the detection and quantification of these oligomeric compounds by HPLC, based on the joint use of both reversed-phase (RP) and size-exclusion (SE) chromatography. This method allowed the analysis of the complete set of dimers, trimers, tetramers, and oligomers resulting from the horseradish peroxidase-mediated (+)-catechin oxidation (*16*).

In fact, HPLC analyses using C_{18} reversed-phase (RP) of both the peroxidase-mediated (**Figure 2A** and **B**), and the polyphenoloxidase-mediated (**Figure 2C** and **D**), (+)-catechin oxidation products resolve these complex mixtures in a product series (B = dehydrodicatechin B4, Q = (+)-catechin quinone methide, A = dehydrodicatechin A, and T = (+)-catechin trimer). However, RP-HPLC also yields a hunchback profile, which hinders both qualitative and quantitative analysis of (+)-catechin oligomers by HPLC, especially in the case of reaction media to which polyphenoloxidase was added (**Figure 2C** and **D**).

This hunchback profile was avoided when these complex reaction media were analyzed by size-exclusion (SE) HPLC on a TSK-Gel G2500HR column using dimethylformamide as mobile phase (**Figure 3B** and **D**). SE HPLC is useful not only for the analysis of (+)-oligomers (peak O, **Figure 3B** and **3D**), but also for the simultaneous resolution of other (+)-catechin oxidation products of a lower polymerization degree. Thus, in this system, (+)-catechin oligomers (peak O, retention time, t_R = 5.36 min) can clearly be separated from (+)-catechin dimers (peak D, t_R = 7.51 min), and from (+)-catechin itself (peak C, t_R = 8.54 min). However, SE HPLC is unable to resolve (+)-catechin dimers, such as dehydrodicatechin A (peak A) and

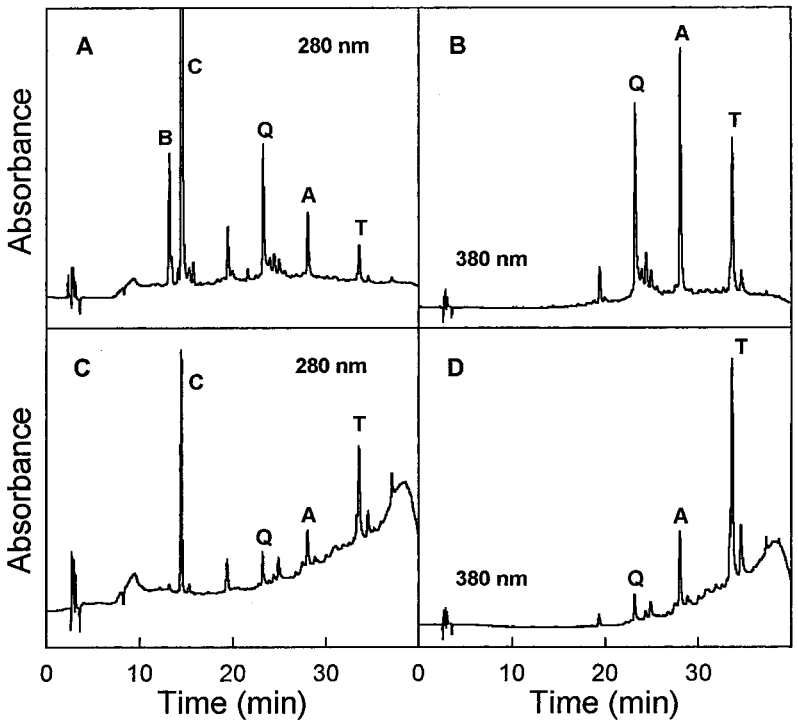


Figure 2. HPLC chromatogram at 280 nm (panels A and C) and at 380 nm (panels B and D) on a Spherisorb S5-ODS2 peroxidase-catalyzed (panels A and B) and strawberry polyphenoloxidase-catalyzed (panels C and D) oxidation of (+)-catechin. HPLC conditions are described in the Materials and Methods section. Peak A, dehydrodicatechin A; peak B, dehydrodicatechin B4; peak C, (+)-catechin; peak Q, (+)-catechin quinone methide; and peak T, (+)-catechin trimer.

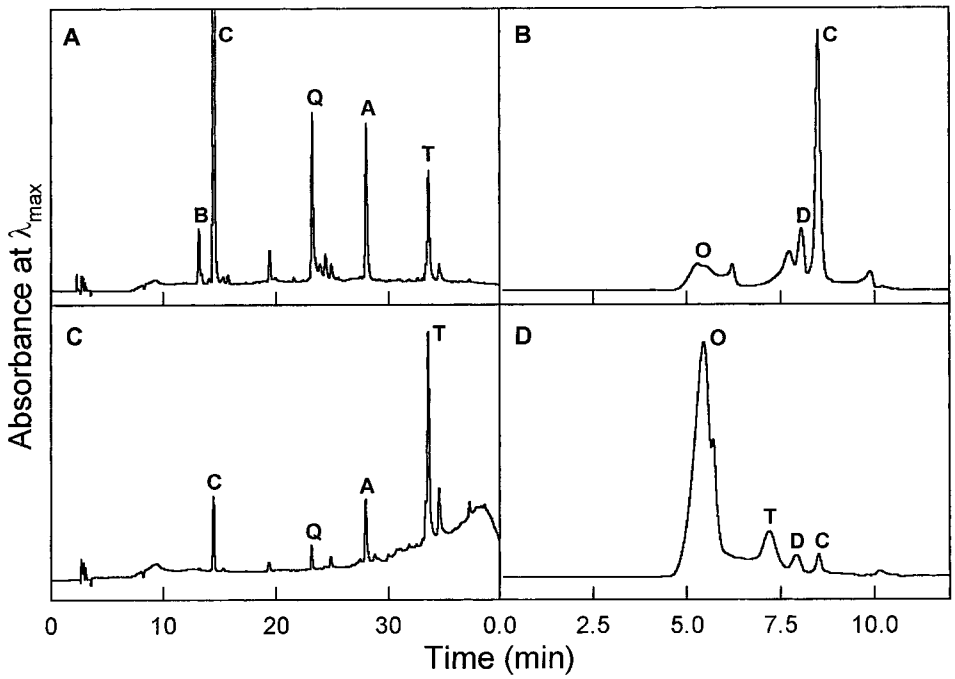


Figure 3. HPLC chromatogram at λ_{max} on a Spherisorb S5-ODS2 column (panels A and C) and on a TSK-Gel G2500HR column (panels B and D) of the products of the strawberry peroxidase-catalyzed (panels A and B) and strawberry polyphenoloxidase-catalyzed (panels C and D) oxidation of (+)-catechin. HPLC conditions are described in the Materials and Methods section. Peak A, dehydrodicatechin A; peak B, dehydrodicatechin B4; peak C, (+)-catechin; peak D, (+)-catechin dimers; peak O, (+)-catechin oligomer; peak Q, (+)-catechin quinone methide; and peak T, (+)-catechin trimer.

dehydrodicatechin B4 (peak B), because of their similar M_r values. For this reason, both RP (on a Spherisorb S5-ODS2 column) and SE (on a TSK-Gel G2500HR column) HPLC methods were now used together to analyze and to dissect the complex product mixtures which result from both strawberry peroxidase and strawberry polyphenoloxidase-catalyzed (+)-catechin oxidation. Besides, and due to the different λ_{max} of the (+)-catechin oxidation products (*16*), which can be clearly seen when comparing **Figure 2A** and **2C** with **Figure 2B** and **2D**, respectively, chromatographic profiles were obtained as Maxplot (λ_{max} chromatograms), defined in the Waters Millenium software as the chromatographic channel where each data point is the absorbance maximum of the spectrum acquired at a given point in time.

Table 3. Levels of (+)-catechin and of the Identified (+)-catechin Oxidation Products during the Peroxidase-Mediated and Polyphenoloxidase-Mediated (+)-Catechin Oxidation after 24 h of Reaction[a]

		peroxidase		polyphenoloxidase	
	compound	μg	%	μg	%
B	dehydrodicatechin B4	37.8	3.26	0.6	0.05
C	(+)-catechin	755.5	65.13	33.1	2.85
Q	(+)-catechin quinone methide	34.9	3.01	4.8	0.41
A	dehydrodicatechin A	18.3	1.58	5.4	0.47
T	(+)-catechin trimer	8.3	0.71	17.1	1.47
O	oligomers	270.4	23.31	1088.9	93.87
	total	1125.2	97.00	1149.9	99.12

[a] The initial (+)-catechin amount in reaction media was 1160 μg.

The joint analysis by RP (**Figure 3A**) and SE (**Figure 3B**) HPLC of the (+)-catechin oxidation products obtained by oxidation with strawberry peroxidase, indicates (**Table 3**) that they were mainly dehydrodicatechin B4 (3.26%), (+)-catechin quinone methide (3.01%), dehydrodicatechin A (1.58%), (+)-catechin trimer (0.71%), and (+)-catechin oligomers (23.31%) with a polymerization degree equal to or greater than 5. Surprisingly, in this peroxidase-mediated oxidative medium, a great amount of (+)-catechin (65.13%) remained unreacted after 24 h of reaction, and after two and five additions of enzyme and H_2O_2, respectively.

The joint analysis by RP (**Figure 3C**) and SE (**Figure 3D**) HPLC of the (+)-catechin oxidation products obtained by oxidation with strawberry polyphenoloxidase indicates (**Table 3**) that they were mainly dehydrodicatechin B4 (0.05%), (+)-catechin quinone methide (0.41%), dehydrodicatechin A (0.47%), (+)-catechin trimer (1.47%), and (+)-catechin oligomers (93.87%) with a polymerization degree equal to or greater than 5. In this last case, only a low amount of (+)-catechin (2.85%) was unreacted.

Thus, the main difference between the nature of the peroxidase-mediated and the polyphenoloxidase-mediated (+)-catechin oxidation products was the great amount of oligomers formed in the case of the polyphenoloxidase-mediated reaction (93.87% compared with the 23.31% obtained in the case of peroxidase). Of note is the low amount of (+)-catechin unreacted in the case of the peroxidase-mediated reaction (65.13% compared with the 2.85% obtained in the case of polyphenoloxidase). The last observation was surprising as the capability of plant peroxidases to oxidize phenolic compounds to highly polymeric compounds is well-known (*2*).

One of the possible reasons for such low levels of substrate consumption in the case of the peroxidase-mediated reaction was the possible inhibition by products of the enzyme-catalyzed oxidation. To test this hypothesis, individual products of the peroxidase-mediated reaction were checked as possible inhibitors of the enzyme after isolation by preparative chromatography on Sephadex LH-20 (*16*). Of them all, only dehydrodicatechin A inhibits strawberry peroxidase to any great extent at the concentration found in the reaction medium after 24 h of reaction, which was calculated to be 37.5 μM. In fact, the peroxidase-mediated oxidation of (+)-catechin was clearly inhibited by dehydrodicatechin A at 20 μM (**Figure 4**). The strawberry polyphenoloxidase-mediated oxidation of (+)-catechin was also inhibited by dehydrodicatechin A (**Figure 4**), but in this case the inhibition was appreciably less, as may be clearly seen from the curves of inhibitor (dehydrodicatechin A) titration (**Figure 4**). In the case of both the peroxidase-mediated

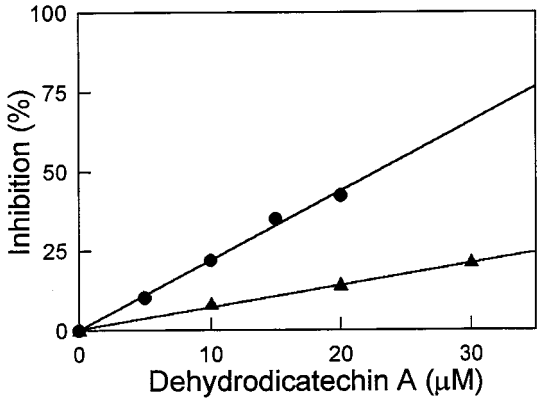


Figure 4. Titration plots for the inhibition by dehydrodicatechin A of the strawberry peroxidase-catalyzed (●) and strawberry polyphenoloxidase-catalyzed (▲) oxidation of (+)-catechin.

Table 4. Values of Unreacted (+)-Catechin, (+)-Catechin Oligomers, Dehydrodicatechin A, and Theoretical Inhibition of the Enzyme-Mediated (+)-Catechin Oxidation on the Basis of the Accumulated Dehydrodicatechin A in the Reaction Medium after 24 h of Reaction

values after 24 h of oxidation	peroxidase	polyphenoloxidase
unreacted (+)-catechin (%)	65.1	2.8
(+)-catechin oligomers (%)	23.3	93.8
dehydrodicatechin A (μM)	37.5	11.3
theoretical inhibition (%)	82.2	8.0

(+)-catechin oxidation and the polyphenoloxidase-mediated (+)-catechin oxidation, dehydrodicatechin A provokes a reduction in the (+)-catechin steady-state oxidation rate of the reaction.

All these results suggest that the low substrate [(+)-catechin] consumption in the case of the peroxidase-mediated (+)-catechin oxidation could be due to the formation of dehydrodicatechin A, which acts as a strong strawberry peroxidase inhibitor at the concentration present in the reaction medium. In the case of the strawberry polyphenoloxidase-mediated (+)-catechin oxidation, dehydrodicatechin A seems to be only a weak inhibitor, and levels of dehydrodicatechin A in the reaction medium never exceeded 12 μM. These two items probably act as nonrestrictive factors for substrate [(+)-catechin] consumption during the polyphenoloxidase-catalyzed reaction. All these results are summarized in **Table 4** for a direct comparison.

Characterization of the Inhibition of Peroxidase by Dehydrodicatechin A. To study the nature of the inhibition of the strawberry peroxidase-catalyzed oxidation of (+)-catechin by dehydrodicatechin A, Michaelis−Menten kinetic curves were analyzed for several (+)-catechin concentrations at three dehydrodicatechin A concentrations (5.0, 10, and 15 μM). Inhibition type and inhibition constants (k_I) were deduced and calculated, respectively, from the 1/v vs [dehydrodicatechin A] Dixon's plots. The type of inhibition for strawberry peroxidase by dehydrodicatechin A was competitive with a k_I value of 6.4 μM. It was not possible to calculate inhibition type and inhibition constants of dehydrodicatechin A for the strawberry polyphenoloxidase because of the unresolved nature of the Dixon's plots. I_{50} values, defined as the dehydrodicatechin A concentration which provokes 50% inhibition for both the peroxidase-catalyzed and polyphenoloxidase-catalyzed (+)-catechin oxidations, were 22.8 μM and 71.6 μM, respectively, confirming the differential effect of dehydrodicatechin A on the peroxidase vs. the polyphenoloxidase catalyzed (+)-catechin oxidation.

In such a scenario, these results strongly suggest that brown polymer formation, estimated as oligomeric compounds resulting

from catechin oxidation, in strawberries is mainly due to polyphenoloxidase. Although peroxidase also plays an important role, probably in cooperation with polyphenoloxidase due to the ability of this copper-containing enzyme to generate H_2O_2 during catechin oxidation (*10*, *11*), the participation of peroxidase in (+)-catechin oxidation is apparently auto-regulated by product (dehydrodicatechin A) inhibition. Further studies are in progress to determine the levels of dehydrodicatechin A in brown strawberries in order to ascertain the in situ extent of such inhibition.

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