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Enzymatic Oxidative Reaction of Catechin and Chlorogenic Acid in a Model System

Jan Oszmianski[‡] and Chang Y. Lee*

Department of Food Science and Technology, Cornell University, Geneva, New York 14456

Enzymatic oxidation of catechin and chlorogenic acid and their mixture was studied at 20 °C and at pH 3.5 and 6.5. The reaction products were monitored by HPLC with diode array detection. The rate of reaction at pH 6.5 was higher than at pH 3.5. The reaction products of catechin oxidation were dimers and other polymers of catechin with low polarity; chlorogenic acid produced mainly polymers. However, the enzymatic oxidation products of the catechin–chlorogenic acid mixture were mainly copolymers which had higher polarity than catechin or chlorogenic acid and were less brown in color than the oxidation products of catechin or chlorogenic acid alone.

Catechin and chlorogenic acid are the major phenolic compounds found in many fruits such as apples, pears, peaches, plums, cherries, and apricots (Risch and Herrmann, 1988). These two polyphenols are well-known substrates of polyphenol oxidase (PPO). PPO catalyzes the oxidation of diphenols to *o*-quinones, which upon further reaction leads to brown pigments. The enzymatically oxidized phenolic acid *o*-quinones were reported to oxidize other polyphenols, such as flavans, by a coupled oxidation reaction (Cheynier et al., 1988). The *o*-quinone formed by enzymatic or coupled oxidation can also react with a hydroquinone to yield a condensation product (Singleton, 1987). Dimers or oligomers are reported to be generated by condensation of hydroquinone with quinone or by condensation of quinones of phenolic acid and catechin (Cheynier et al., 1988). Oszmianski et al. (1985) reported a rapid depletion of grape seed polyphenols by PPO as compared to that by chemical oxidation. Cilliers and Singleton (1989) found more than a dozen oxidation products from a nonenzymatic oxidative browning reaction of caffeic acid.

The purpose of the present work was to study the PPO reaction products of individual chlorogenic acid and catechin and the mixture of the two at pH 3.5 and 6.5 in relation to browning in model systems.

MATERIALS AND METHODS

Materials. Standard catechin, chlorogenic acid, and tyrosinase (monophenol monooxygenase, polyphenol oxidase; EC 1.14.18.1) were obtained from Sigma Chemical Co. Catechin and chlorogenic acid (2 mM) were dissolved individually or together in 0.02 M acetic acid buffer solutions at pH 3.5 and 6.5 and filtered through 0.45-μm membrane filters. Tyrosinase (0.5 mg/mL) was dissolved in the same buffer solutions.

Sampling. Phenolic solutions (19.5 mL) and the enzyme solution (0.5 mL, 565 units) were thoroughly mixed and incubated at 20 °C with constant agitation on a magnetic stirrer. The oxidation reaction was terminated by acidification of an ali-

[‡] On leave from the Agricultural University, 50-375 Wroclaw, Norwida 25, Poland.

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quot in a sample vial according to the method of Cilliers and Singleton (1989). This was done by adding 1 mL of reactant solution to a vial that contained 1 mL of 50% acetonitrile and 3% HCl. Acetonitrile was necessary to prevent precipitation and loss of the oxidation products. Sampling was at 0, 1, 3, 6, 15, 30, 60, and 120 min. To study the solubilization of the oxidation product, an additional sample, collected at 120 min, was first filtered through a 0.45-μm membrane and then added to the acetonitrile–acid solution.

HPLC. All samples were analyzed immediately by using an HPLC (Hewlett-Packard 1090M with a diode array detector) equipped with a C18 NOVA column (Waters Associates) and connected to a Hewlett-Packard 300 series Chem-Station according to the method of Jaworski and Lee (1987). Four wavelengths, 280, 320, 380, and 420 nm, were monitored simultaneously, and scanning was carried out at 230 and 500 nm. The elution gradient was a linear system run from 5% to 100% of mobile phase A (40% acetonitrile) with mobile phase B (5% acetic acid) for 30 min. A calibration curve was obtained by injecting 20 μL each of seven standards containing different concentrations of chlorogenic acid and catechin from 0.02 to 10 mM. Injection was tested by injecting eight times 1 mM chlorogenic acid.

Browning. Color change was measured at 420 nm with a Varian Cary 219 spectrophotometer using a 1.0-cm path length. All analyses were repeated three times.

RESULTS AND DISCUSSION

The disappearance of chlorogenic acid and catechin and the formation of enzymatic oxidation products were monitored by HPLC. For the quantitation of chlorogenic acid, linear calibration curves at 320 nm of $y = 13197x + 1095$ ($r = 0.999$) and at 280 nm of $y = 6863x + 488$ ($r = 0.999$) were obtained. For catechin a linear calibration curve at 280 nm $y = 2995x + 165$ ($r = 0.999$) was obtained. Typical chromatographic profiles of the oxidation products of chlorogenic acid, catechin, and the mixture of chlorogenic acid–catechin detected at 280 nm are shown in Figure 1. Profiles of the oxidation products are relatively similar at both pH values, although proportionate amounts and ratios varied. All six oxidation products from chlorogenic acid have chromatographic elution times longer than that of chlorogenic acid (Figure 1A). This would indicate that these oxidation products have higher molecular weights and/or they are less polar than chlorogenic acid. Peaks A and B in Figure 1A were the major oxidation products of chlorogenic acid. However, all of the chlorogenic acid oxidation products showed a maximum absorbance at or near 320 nm, a value that is significantly different from 420 nm, which is the maximum absorbance for brown color.

The reaction products of catechin were monitored at 280, 380, and 420 nm according to their maximum absorbance wavelengths. Figure 1B is a typical chromatogram of catechin oxidation at pH 6.5 when monitored at 280 nm. Enzymatic oxidation of catechin also produced several compounds that had longer elution times than that of catechin. In addition, a couple of reaction products (peak A and minor peaks) that had shorter elution times than catechin were observed. One of them, peak A in Figure 1B, appeared to be procyanidin B3 (dimer of catechin), since it showed the same retention time and the same spectrum as the standard procyanidin B3 (Lee and Jaworski, 1987). On the basis of the oxidation reaction sequence proposed by Singleton (1987) and Cheynier et al. (1988), enzymatic oxidation of catechin may be able to produce the dimer, procyanidin B3. Compounds B and C showed a maximum absorbance at 420 nm, and compounds D–F showed a maximum absorbance at 380 nm. All of these reaction products from catechin appeared to contribute to the brown color. An

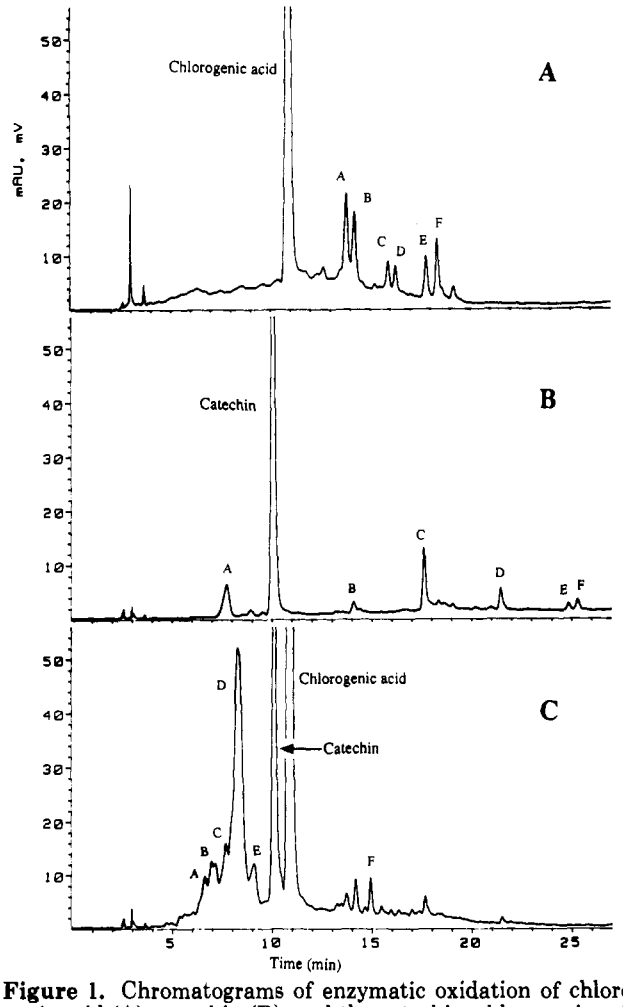

Figure 1. Chromatograms of enzymatic oxidation of chlorogenic acid (A), catechin (B), and the catechin–chlorogenic acid mixture (C) at pH 6.5 after a 2-h reaction. Peaks A–F are the oxidation products.

interesting observation was that all of the reaction products from chlorogenic acid dissolved well in the buffer solutions at pH 3.5 and 6.5 but the solubility of the oxidation products of catechin, such as peaks E and F, was very poor.

During enzymatic oxidation of the catechin–chlorogenic acid mixture, six new peaks were observed in the chromatogram (Figure 1C), five of which had shorter elution times than those of chlorogenic acid and catechin. All six compounds showed spectra and maximum absorptions that differed from those of the reaction products of chlorogenic acid or catechin alone. All showed maximum absorptions at near 280 and 320 nm (Figure 2C). These new compounds may correspond to the copolymers of chlorogenic acid–catechin as proposed by Cheynier et al. (1988) for catechin and caffeoyl tartaric mixtures. The total area of the new peaks (A–E, copolymers) from the catechin–chlorogenic acid mixture was 5–6 times larger than that of polymers produced by individual polyphenols at both pH values. This confirms the recent report of Cheynier et al. (1989), who found that caftaric acid–catechin copolymers were formed more readily than single caftaric acid oligomers in the oxidation reaction of the caftaric acid–catechin mixture. The participation of catechin *o*-quinones in copolymerization with chlorogenic acid in our experiment appeared to slow down the formation of catechin polymers.

Table I gives the first-order reaction rate of each phenolic and their mixture at a temperature of 20 °C and at pH 3.5 and 6.5. The catechin oxidation rate was more

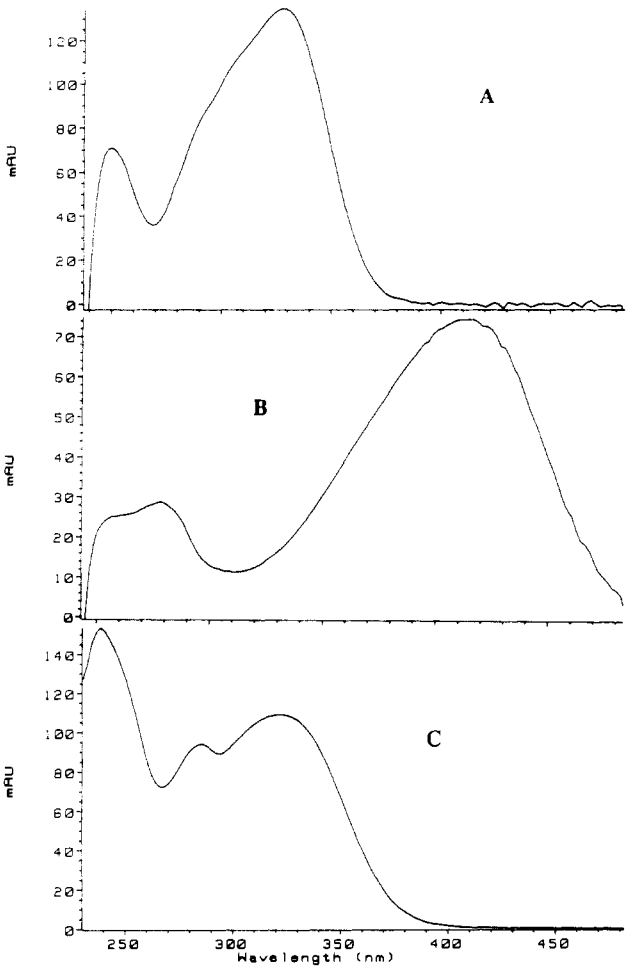

Figure 2. Typical spectra of the enzymatic reaction products. (A) Peak A from chlorogenic acid in Figure 1A; (B) peak C from catechin in Figure 1B; (C) peak D from the catechin–chlorogenic acid mixture in Figure 1C.

Table I. Rate Constants[a] ($\times 10^{-4}$ min^{-1}) for the First-Order Enzymatic Oxidation Reactions of Catechin and Chlorogenic Acid and Their Mixtures at pH 3.5 and 6.5, at 20 °C

			mixture	
pH	catechin (r)	chlorogenic acid (r)	catechin (r)	chlorogenic acid (r)
3.5	24.2(0.93)	87.6(0.94)	33.5(0.96)	31.4(0.95)
6.5	82.4(0.88)	150.0(0.94)	100.3(0.95)	67.6(0.93)

[a] The number of data points used in calculation for each value was seven.

rapid at pH 6.5 (reaction rate 82.4 $\times$ 10^{-4} min^{-1}) than at pH 3.5 (24.2 $\times$ 10^{-4} min^{-1}). Chlorogenic acid showed the same pattern of oxidation. This increased reaction rate at high pH is very similar to the nonenzymatic oxidation rate of caffeic acid at different pH values (Cilliers and Singleton, 1989), even though the conditions were not entirely the same.

In the catechin–chlorogenic acid mixture, the rate of catechin oxidation was faster than that of catechin alone, while the rate of chlorogenic acid oxidation was slower than that of chlorogenic acid alone. This may be due to the fact that chlorogenic acid quinones oxidize catechin by a coupled reaction mechanism (Cheynier et al., 1988), so that chlorogenic acid quinone was converted back to chlorogenic acid. Consequently, the degree of browning after a 2-h reaction of the catechin–chlorogenic acid mixture at pH 6.5 was less than that of catechin alone (Figure 3). The intensity of brown color formed by catechin oxidation is higher than that from chlorogenic acid. This is due mainly to the different maximum absorbance of the reaction products as described above. The catechin reaction products are more brownish in color and have absorption maxima at near 400 nm, while the reaction products of chlorogenic acid and that of the catechin–chlorogenic acid mixture are yellowish and have absorption maxima at 320 nm.

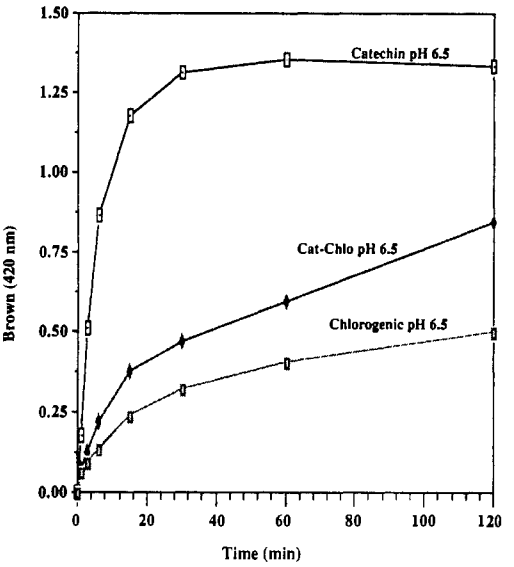

Figure 3. Browning rate of catechin, chlorogenic acid, and their mixture at pH 6.5.

It is concluded from this experiment that the rate of enzymatic oxidation of polyphenols that leads to browning of fruit and vegetable products depends not only upon the concentration of polyphenols and PPO activity but also on the nature of the polyphenols that are copresent in a product.

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