Enzymatic Oxidation of Phloretin Glucoside in Model System

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Enzymatic oxidation of phloretin glucoside and its effect on chlorogenic acid and catechin in relation to the browning reactions were studied in model systems. Phloretin glucoside alone oxidized slowly and formed the light yellowish reaction products. However, the addition of chlorogenic acid and catechin to phloretin accelerated the reaction of phloretin oxidation by decreasing the lag period of the enzymatic browning reaction significantly. The degree of browning (absorbance at 420 nm) in the mixture was approximately 2 times higher than that of phloretin alone. This synergistic effect of phloretin glucoside with chlorogenic acid or catechin must be taken into account in the browning reactions of apple products.

INTRODUCTION

Enzymatic oxidation of phenolic compounds is of great concern in the determination of the quality of fruit and vegetable products. The brown color development is determined by the polyphenol oxidase activity and the amount of the polyphenol substrates. In apples the most prevalent substrates for this enzymatic reaction are chlorogenic acid and catechins (Macheix, 1970; Walker, 1962). Apples are also known to contain other polyphenols in a significant concentration, such as dihydrochalcone glycosides (Oleszek et al., 1988). Durkee and Poapst (1965) reported that phloretin glucoside produced yellow color but was not a good browning substrate. Challice and Williams (1970) demonstrated that phloretin glycosides were very effective in vitro substrates for catechol oxidase. Recently, Oleszek et al. (1989) reported that phloretin glucoside oxidized very slowly at the early stage of enzymatic oxidation, but the mixture of phloretin glucoside and epicatechin showed a synergistic effect by increasing the reaction rate significantly. The degree of browning was severalfold higher than those of other apple polyphenols. Lea (1984) also reported that the procyanidins themselves are not a substrate of polyphenol oxidase but they are easily oxidized in the presence of chlorogenic acid. The purpose of this research was to study enzymatic oxidation of phloretin glucoside and the effect of catechin and chlorogenic acid in a model system.

MATERIALS AND METHOD

Materials. Standards catechin, chlorogenic acid, phloretin glucoside, and tyrosinase (polyphenol oxidase) were obtained from Sigma Chemical Co.

Model System. Phloretin glucoside (1 mM) was dissolved with 0.01, 0.05, 0.1, and 1.0 mM catechin or chlorogenic acid in 0.02 M acetic buffer solution at pH 5.5 and filtered through the 0.45-μm membrane filter. Phenolic solution (19.5 mL) and enzyme solution (0.5 mL, 565 units) were thoroughly mixed and incubated at 23 °C for 0, 3, 6, 10, 30, 60, 90, and 120 min with constant agitation on a magnetic stirrer (air saturation). After each incubation, 1 mL of reactant was transferred to a vial that contained 1 mL of 50% acetonitrile and 2.5% sulfuric acid to terminate the reaction (Cilliers and Singleton, 1989).

HPLC Analysis. Concentration of polyphenols in each reactant was analyzed immediately by HPLC (Hewlett-Packard 1090M with diode array detector) equipped with a C_{18} Nova column (Waters Associates) according to the method of Jaworski and Lee (1987). The elution was carried out by a linear gradient system run from 5% to 100% mobile phase A (40% acetonitrile) with phase B (5% acetic acid) for 30 min. Three wavelengths, 280, 320, and 420 nm, were monitored simultaneously, and scanning was carried out from 230 to 500 nm.

Browning. Color of the reactant was measured at 420 nm with a Varian Cary 219 spectrophotometer.

RESULTS AND DISCUSSION

Oxidation of phloretin glucoside in the presence of catechin and chlorogenic acid was monitored by HPLC. Chromatographic profiles of oxidation products of phloretin glucoside alone and the mixture of phloretin glucoside and chlorogenic acid detected at 280 nm are shown in Figure 1. The initial reaction products of phloretin glucoside alone were mainly two peaks, labeled a and b (Figure 1A). Their elution times were shorter (higher polarity) than that of phloretin glucoside (retention time, 11.3 min). However, the mixture of phloretin glucoside and chlorogenic acid produced several reaction products (Figure 1B). They were probably the copolymerization products that were similar to those oxidation products of the other polyphenols mixture (Oszmianski and Lee, 1990). When the same chromatograms (Figure 1) were monitored at 420 nm to detect the colored (yellow) products, two major peaks, c and d, were observed as shown in Figure 2. The sizes of these two peaks from phloretin glucoside alone (Figure 2A) were small compared to that of the mixture of phloretin glucoside and chlorogenic acid (Figure 2B). Additional reaction products were seen in the chromatograms. A similar observation was made in the mixture of phloretin glucoside and catechin. This indicated that chlorogenic acid and catechin have a synergistic effect by accelerating oxidation of phloretin glucoside in the presence of polyphenol oxidase and produced highly colored products. Distinctive spectra differences between these two reaction products are shown in Figure 3: peaks a and b showed maximum absorption near 280 nm (Figure 3A) and peaks c and d were near 420 nm (Figure 3B).

When the effect of chlorogenic acid on the formation of oxidation products of phloretin glucoside is monitored by HPLC, it was found that phloretin glucoside alone started to show peaks a and b after 30 min of reaction time, but the mixture of phloretin glucoside and chlorogenic acid produced peaks a and b within 3 min (Figure 4). Chlorogenic acid not only accelerated the reaction by producing colorless compounds a and b but also promoted further oxidation of these compounds to the colored compounds c and d (Figure 5). Increasing the amount of chlorogenic

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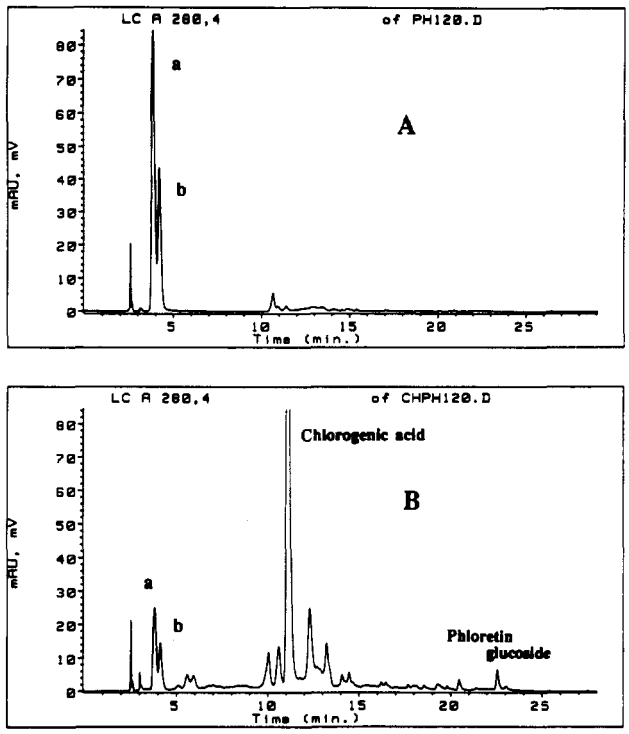

Figure 1. Chromatograms (HPLC) monitored at 280 nm: (A) oxidation products of phloretin glucoside and (B) oxidation products of phloretin glucoside and chlorogenic acid mixture, at pH 5.5 after 2 h of reaction. Peaks a and b are the initial oxidation products of phloretin glucoside.

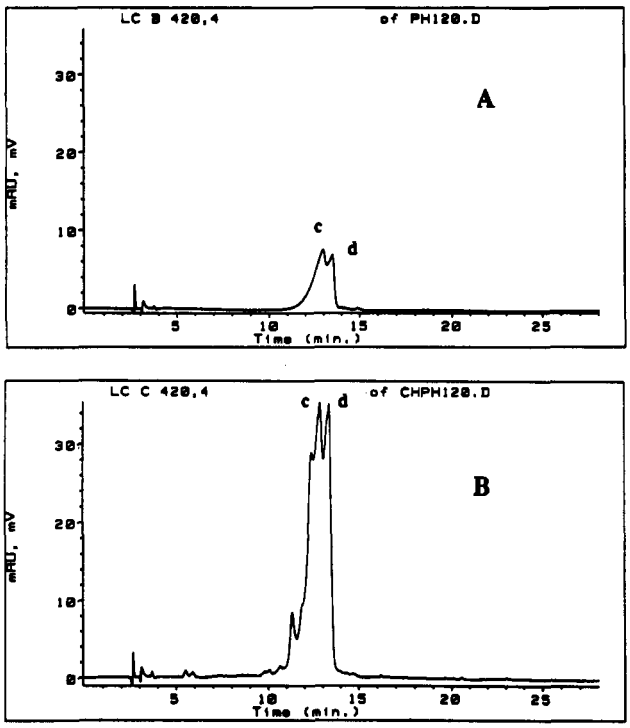

Figure 2. Chromatograms (HPLC) monitored at 420 nm: (A) oxidation products of phloretin glucoside and (B) oxidation products of phloretin glucoside and chlorogenic acid mixture, at pH 5.5 after 2 h of reaction. Peaks c and d are the oxidation products of phloretin glucoside.

acid added accelerated the reactions; the concentrations of c and d were proportional to the concentration of chlorogenic acid added to phloretin glucoside. The compounds a and b are probably the hydroxylation products of phloretin glucoside, and the compounds c and d are the oxidation products of hydroxylated phloretin glucoside.

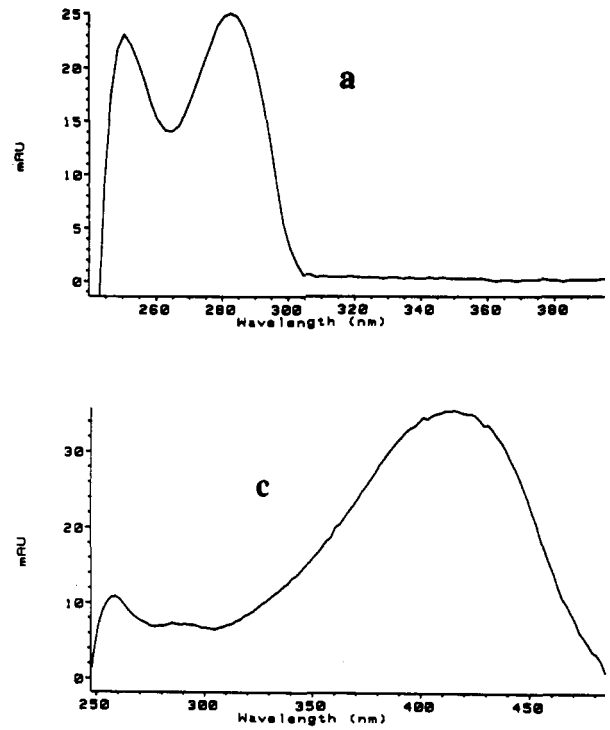

Figure 3. Spectra of the oxidation products of phloretin glucoside a from Figure 1 and c Figure 2.

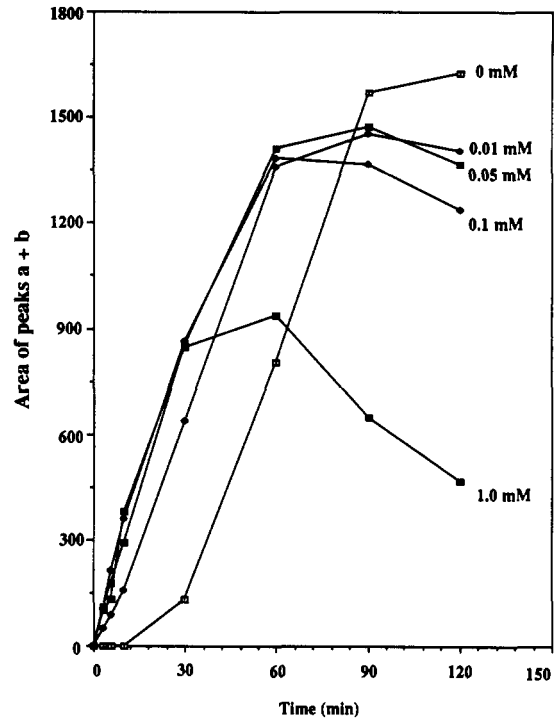

Figure 4. Effect of chlorogenic acid concentration added to phloretin glucoside on the reaction products (peaks a and b in Figure 1) monitored at 280 nm.

The hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones are commonly found in most polyphenol oxidase preparations from potatoes, apples, or bananas (Vamos-Vigyazo, 1981). This hydroxylation can be explained by the lag period of phloretin glucoside oxidation in this experiment. Table I shows the effects of catechin and chlorogenic acid on oxidation of phloretin glucoside expressed in the lag period observed at 420 nm. The lag period observed in phloretin glucoside alone was 54 min. Addition of chlorogenic acid or catechin to phloretin glucoside decreased the lag period rapidly

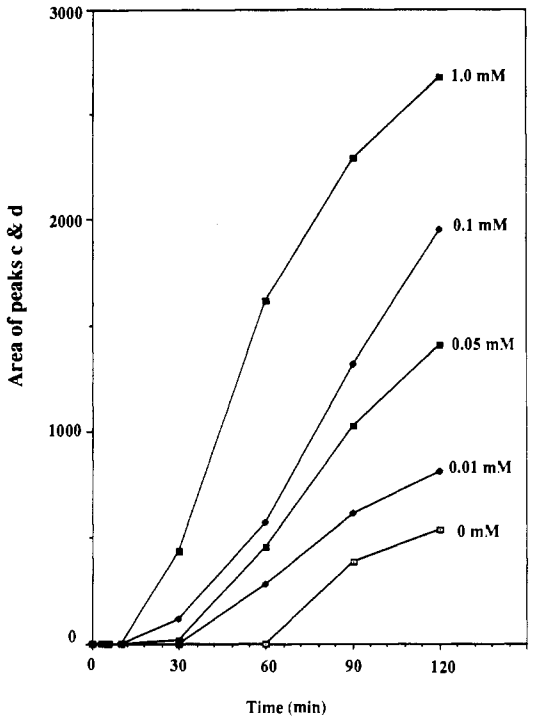

Figure 5. Effect of chlorogenic acid concentration added to phloretin glucoside on the reaction products (peaks c and d in Figure 2) monitored at 420 nm.

Table I. Effect of Catechin or Chlorogenic Acid Added to Phloretin Glucoside on the Lag Period and Color during Enzymatic Browning[a]

phloretin glucoside, mM	added catechin or chlorogenic acid, mM	catechin		chlorogenic acid	
		lag, min	A_{420nm} (after 2 h)	lag, min	A_{420nm} (after 2 h)
1	0	54	0.727	54	0.727
1	0.01	23	1.389	27	1.020
1	0.05	21	1.538	22	1.410
1	0.10	19	1.555	20	1.514
1	1.00	2	1.574	6	1.568

[a] Each value is the average of three replications.

from 54 to 6 and 2 min, respectively. After 2 h of oxidation, phloretin glucoside showed absorbance (A_{420nm}) of 0.727, catechin, 0.833, and chlorogenic acid, 0.400. However, the mixture of phloretin glucoside and catechin (1:1 mM) showed absorbance 1.574, and the mixture of phloretin glucoside and chlorogenic acid (1:1 mM) was 1.568. Whitaker (1972) explained that when a monophenol is added alone as a substrate of polyphenol oxidase, there is a delay in the reaction, and this lag period is due to the need for an *o*-diphenolic compound. However, this lag period can be eliminated by addition of a small amount of an *o*-diphenol at the beginning of the reaction, and in the absence of added *o*-diphenol there is a lag period until the enzyme can build up a sufficient amount of the *o*-diphenol to permit the reaction to proceed. This reaction mechanism explains the rapid change in the lag period of phloretin glucoside in the presence of *o*-diphenols. This synergistic effect between phloretin glucoside and chlorogenic acid or catechin confirms the previous finding of Oleszek et al. (1989), who observed a strong browning reaction in phloretin glycosides in the presence of chlorogenic acid or flavans.

The oxidation rate of phloretin glucoside was higher (207.6×10^{-4} mM/min) than that of catechin (36.5×10^{-4} mM/min) and chlorogenic acid (188.5×10^{-4} mM/min). However, the mixture of phloretin glucoside and catechin or chlorogenic acid increased the oxidation rate proportionally to the increased concentration of *o*-diphenols (Table II). Catechin showed a slightly higher effect than chlorogenic acid. The exception was the sample with 1 mM catechin at which the concentration was too high and the reaction products probably inhibited the enzyme to slow down the reaction. This result of the oxidation rate of phloretin glucoside confirms the result of Challice and Williams (1970) that dihydrochalcone glycosides of the phloridzin type appeared to be the most effective in vitro substrates for phenolase.

Table II. Rate Constant k ($\times 10^{-4}$ mM/min) for the First-Order Enzymatic Oxidation Reaction of 1 mM Phloretin Glucoside with Catechin and Chlorogenic Acid[a]

phloretin glucoside, mM	added catechin or chlorogenic acid, mM	catechin k (r)	chlorogenic acid k (r)
1	0	207 (0.998)	207 (0.998)
1	0.01	325 (0.998)	216 (0.972)
1	0.05	354 (0.994)	266 (0.983)
1	0.10	359 (0.990)	268 (0.998)
1	1.00	214 (0.998)	345 (0.995)

[a] Each value is the average of three replications.

Since the combination of phloretin glucoside with catechin or chlorogenic acid accelerated the enzymatic oxidation and produced strongly colored oxidation products in a model system, this synergistic effect must be considered carefully in the processing of apples because these phenolic compounds are present in significant amounts in many apple cultivars (Oleszek et a., 1988; Burda et al., 1990).

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Registry No. Phloretin glucoside, 60-81-1; catechin, 120-80-9; chlorogenic acid, 327-97-9; tyrosinase, 9002-10-2.