Inhibition of Polyphenol Oxidase Activity and Browning by Honey

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The inhibitory effect of honey on polyphenol oxidase (PPO) and on browning reactions was studied in apple slices, grape juice, and model systems. Browning was prevented by immersing apple slices in a honey solution. Grape juice preparations with added honey exhibited less browning and retained a higher concentration of polyphenols than juices made with no added honey. In model solutions of caffeoyl tartrate and epicatechin, the browning rate was inversely proportional to the added honey concentration. A kinetic study showed that honey is noncompetitive with epicatechin. Preincubation of PPO with honey progressively decreased the PPO activity with time. The compound responsible for this inhibitory effect of honey appeared to be a small peptide with an approximate molecular weight of 600.

Polyphenol oxidase (PPO) is a copper enzyme that is responsible for the enzymatic browning reactions of fruit and vegetable products. Browning is undesirable not only because of the discoloration of the products but also because the reaction produces off-flavors. A great number of studies have been conducted to prevent browning, including treatment with reducing agents, acidulants, chelating agents, other chemicals, and heat (Vamos-Vigyazo, 1981). Use of sulfur dioxide has been quite successful, but it has been limited recently due to the reactions that it engenders in a low percentage of the population. Since consumer demand for natural additives has been increasing, it is desirable to use naturally occurring compounds to inhibit browning. Avigad and Markus (1965) reported that cultures of *Dactylium dendroides* contain a small peptide that inhibits the activity of the copper enzyme galactose oxidase. Harel et al. (1967) showed that this peptide was also a good inhibitor of apple catechol oxidase and thus reduced the browning of apple slices. The presence of low molecular weight peptides (about 1000) that inhibit PPO activity was also reported in mushrooms (Madhosingh, 1975) and potatoes (Nilova et al., 1973). Kahn (1985) showed that some amino acids, such as lysine, glycine, histidine, and phenylalanine, were also effective inhibitors of PPO. Recently, we (Lee, 1984; Lee and Kime, 1984) reported that honey protein interacts with tannins and browning reaction products of apple juice and consequently produces clear juice. Only 1 of 10 bands of an electrophoregram of honey protein was responsible for this clarification activity (Lee et al., 1990).

In this paper we report the inhibitory effect of honey protein on PPO in apple slices, grape juice, and model systems. The effects of honey on the formation of *o*-quinone, rate of browning, inhibitory mode on PPO, and preincubation with PPO of the rate of browning were studied. A protein fraction that has a high inhibitory effect was separated from honey by using Bio-Gel P-2 gel and Sephadex G-15 columns.

MATERIALS AND METHODS

Materials. Standard epicatechin, *p*-coumaric acid, and tyrosinase [polyphenol oxidase (PPO) from mushroom] were obtained from Sigma Chemical Co.; *trans*-caffeoyl tartaric acid was isolated and purified in our laboratory according to the method of Singleton et al. (1978). Rhode Island Greening apples and Niagara grapes were obtained from the New York State Agricultural Experiment Station orchards during the 1989 season. Commercial honey (floral type; blend of clover and alfalfa) was obtained from a local producer.

Methods. *Effect of Honey on Browning of Apple Slices.* Peeled and sliced apples (300 g) were immersed immediately in 1 L of honey (10%) and sucrose solution (8%) for 30 min at room temperature. The treated slices were blotted with paper towels and then placed in a plastic container (7 cm in diameter). The browning rate was determined at 23 °C by measuring changes in L value (δL) over a 2-h period using a Hunter color difference meter (Model 25D-3). The control was apple slices immersed in distilled water for 30 min. All measurements were replicated three times.

Effect of Honey on Grape Juice Browning. Three sets of juice samples were prepared as follows: (1) Niagara grape berries (200 g); (2) Niagara grape berries (200 g) plus honey (5%); (3) Niagara grape berries (200 g) plus ascorbic acid (1%). Each set of samples was ground in a Waring Blendor at a slow speed for 1 min to homogenize the flesh and skins without crushing the seeds. The homogenate was filtered through a Kendall filter, and the juice was incubated at 20 °C for 60 min to enable browning to occur. The residual polyphenols were then extracted and analyzed by HPLC (Oszmianski and Lee, 1990).

Inhibitory Effect of Honey on Browning in Model Solutions. Various concentrations of honey, 0, 0.5, 1.0, 2.0, 4.0, and 10.0% in 2.5 g/L potassium bitartrate solution (pH 3.65), were added to 1 mL of 2 mM caffeoyl tartrate or epicatechin in potassium bitartrate (pH 3.65); to this was added 50 μL of PPO (0.5 mg/mL) solution. The rate of browning was measured at 420 nm.

Inhibitory Effect of Honey on Oxidation of Polyphenols in Model Solution. One milliliter of 2 mM caffeoyl tartrate or 2 mM epicatechin in the potassium bitartrate solution was incubated with 25 μL of PPO (0.5 mg/mL) for 0, 15, 30, 60, and 120 min, at 20 °C, with constant agitation. After each time period, 1 mL of 10% honey in potassium bitartrate solution was added. After an additional incubation of 0, 30, and 60 min, the solutions were analyzed for polyphenols by HPLC (Jaworski and Lee, 1987).

Analysis of Free Quinones. Ten milliliters of 2 mM caffeoyl tartrate or 2 mM epicatechin in potassium bitartrate (pH 3.65) solution was incubated at 20 °C with and without honey (5%) in the presence of 0.25 mL of PPO (0.5 mg/mL) for 0, 15, 30, 60, and 120 min, with constant agitation (air saturation). After each incubation, 1 mL of sample was mixed with 50 μL of benzenesulfinic acid solution (65.5 mg/mL) to trap the free quinones (Cheynier et al., 1989). After a few seconds, 1 mL of 1% sodium metabisulfite solution was added to stop the enzymatic reaction. Quinones were analyzed as stated above against standards of caffeoyl tartrate and epicatechin.

Kinetics of Honey Inhibition. To each 1 mL of 1.0, 1.4, 2.0,

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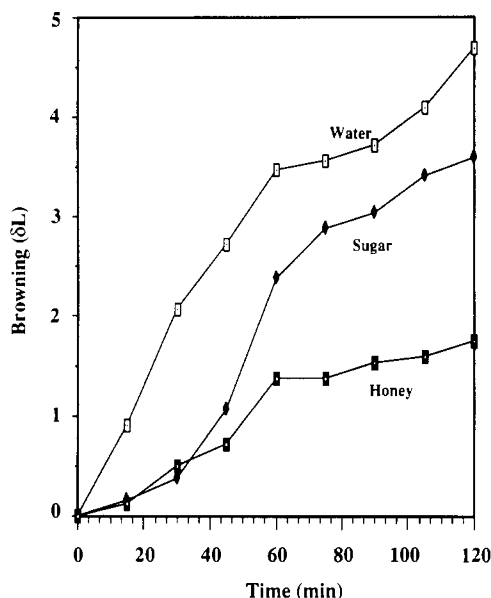

Figure 1. Enzymatic browning (δL value) of apple slices treated with honey, sucrose, and water.

3.0, and 4.0 mM epicatechin in potassium bitartrate solution was added 1 mL of 0, 1, 2, or 4% honey solution. The rate of browning was measured during 200 s after the addition of 50 μL of PPO.

Effect of Preincubation of PPO with Honey. PPO (50 μL) solution and 1 mL of 4% honey in 2.5 g/L potassium bitartrate solution (pH 3.65) were incubated at 20 °C for 0, 30, 60, 90, and 120 min; 1 mL of 4 mM epicatechin was added, and the rate of browning was measured at 420 nm by using a Hewlett-Packard 8452A spectrophotometer equipped with a diode array detector and a series 300 computer.

Column Chromatography Fractionation of Honey. Honey solution (10 mL of 60% solution) was passed through a 0.45 μM membrane filter and then applied to a gel column (Bio-Gel P-2, 1.5 × 30 cm) and eluted with 0.2 mM acetate buffer at pH 6.0 at the flow rate of 0.4 mL/min. The optical density of each fraction (4 mL) was measured at 280 nm, and the peptide content was analyzed by using ninhydrin reagent (Moore and Stein, 1948).

Molecular Weight of Peptide. Honey solution (0.5 mL of 80% solution) was applied to a gel column (Sephadex G-15, 1 × 26 cm) and eluted with ammonium acetate buffer, 0.1 M, pH 7.2, according to the method of Madhosingh (1975). The column was calibrated with standards of glutathione (M_r 307), flavin adenine dinucleotide (FAD, M_r 785), bacitracin (M_r 1411), and blue dextran obtained from Sigma Chemical Co. The proteins and FAD were monitored at 280 and 405 nm, respectively.

RESULTS AND DISCUSSION

Since sugar solutions inhibit discoloration by reducing the concentration of dissolved oxygen and the rate of diffusion of the oxygen of the air into fruit tissue (Joslyn and Ponting, 1951), the rates of browning of apple slices in 8% sucrose solution (the level of sugar in 10% honey solution) and in 10% honey were compared. The results showed that apple slices treated with honey exhibited the lowest browning rate followed by sucrose and water (Figure 1). The percent inhibition in apple slices after 2 h with honey and sucrose was 62 and 23%, respectively. This suggested that honey contains inhibitor(s) of PPO in addition to sugars.

To study the effect of honey on grape juice browning, honey was mixed with Niagara grapes prior to pressing into the juice. The residual polyphenol content after 60 min showed that a substantial amount of polyphenols had been oxidized in the plain juice compared to the juices with added ascorbic acid or honey (Table I). The concentration of caffeoyl tartrate and epicatechin in juice with honey was approximately 2 times higher than that in plain juice. The loss of polyphenols was directly correlated with the degree of browning: a high degree of browning was observed in the plain juice, while the least browning occurred in the juice supplemented with ascorbic acid. Honey was not as effective in preventing browning as was the ascorbic acid. When honey was added to the oxidized, plain juice for 30 min, a large decrease in the concentration of yellowish oxidation products (measured at 380 nm) occurred. This phenomenon may be due to a reaction of honey protein with the oxidation products, as was proposed by Wakayama and Lee (1987).

Table I. Effect of Honey on Polyphenols in Niagara Grape Juice after 60 min at 20 °C

	concn, mg/L		
polyphenols	plain juice	honey added juice	ascorbic acid added juice
cis-caffeoyl tartrate	4.73	6.51	10.60
trans-caffeoyl tartrate	97.52	196.03	487.55
cis-coumaroyl tartrate	33.42	39.78	41.55
trans-coumaroyl tartrate	32.16	57.42	63.59
catechin	11.48	13.93	29.05
epicatechin	12.48	27.09	40.97
gallocatechin gallate	45.30	54.28	56.29
absorbance at 420 nm	0.987	0.704	0.344

Table II. Effect of Different Concentrations of Honey on the Rate of Enzymatic Browning of Caffeoyl Tartrate and Epicatechin

honey concn, %	rate constant, AU/min × 10^{-3}	
	caffeoyl tartrate	epicatechin
0	43.75	28.55
0.25	36.65	13.53
0.5	31.46	11.37
1.0	23.38	7.13
2.0	14.95	3.94
5.0	5.43	1.19
10.0	1.39	0.19

In model solutions of caffeoyl tartrate or epicatechin with different honey concentrations, the rate of browning was inversely proportional to the level of honey (Table II). The oxidation rate constants for both polyphenols decreased as the concentration of honey was increased. Addition of 10% honey decreased the reaction rate 95% in caffeoyl tartrate and 99% in epicatechin.

The inhibitory effect of honey on polyphenol oxidation was monitored by analyzing individual compounds during oxidation. Figure 2 shows the concentration of caffeoyl tartrate during the course of browning in the presence of honey. Caffeoyl tartrate was oxidized rapidly by PPO, with more than 90% of the oxidation occurring within 30 min. However, addition of honey at time 0 retarded the oxidation significantly in that only 25% of caffeoyl tartrate was oxidized at 30 min. Addition of honey after the 15-min reaction time practically prevented the oxidation reaction completely, and there was a slight increase in the concentration of caffeoyl tartrate thereafter. Epicatechin was slowly oxidized by PPO when honey was added (data not presented), and a partial reverse reaction of *o*-quinone to epicatechin appeared to be taking place. This suggested that honey not only inhibits enzymatic oxidation of polyphenols but also converts a portion of the *o*-quinones that are formed to the original polyphenols. This type of reaction is known to occur with ascorbic acid, which rapidly reverses the *o*-quinones to polyphenols. Honey appeared to contain some kind of reduction mechanism that resembles ascorbic acid.

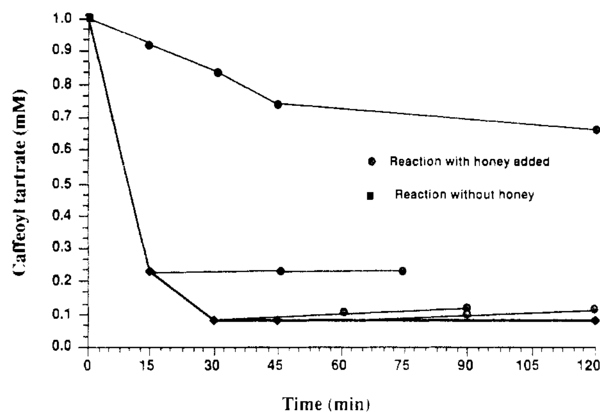

Figure 2. Inhibitory effect of honey on enzymatic reaction of caffeoyl tartrate at pH 6.0 and 20 °C. Concentration of caffeoyl tartrate was measured after honey was added to a reaction mixture of PPO–caffeoyl tartrate at 0, 15, 30, and 45 min.

Table III. Effect of Honey on the Evolution of *o*-Quinones in the PPO–Polyphenol System

	caffeoyl tartrate *o*-quinone, mM		epicatechin *o*-quinone, mM	
reaction time, min	without honey	with honey	without honey	with honey
0	0	0	0	0
15	0.485	0.035	0.123	0
30	0.531	0.052	0.021	0
60	0.418	0.064	0.007	0
120	0.226	0.023	0.004	0

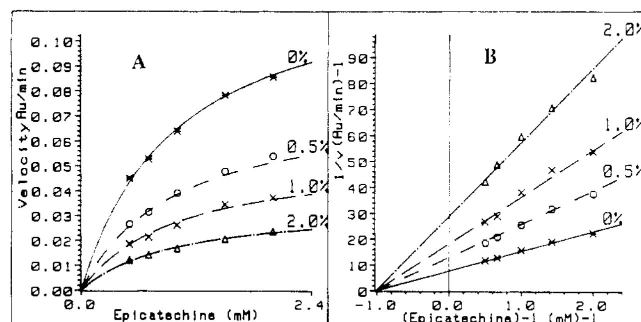

Figure 3. Kinetics of inhibitory effect of honey on browning. (A) Effect of honey concentration on the browning reaction rate of epicatechin. (B) Lineweaver–Burk plot of honey inhibition on the PPO–epicatechin reaction.

Enzymatic oxidation of caffeoyl tartrate and epicatechin produced corresponding *o*-quinones (Table III). The concentration of caffeoyl tartrate *o*-quinone increased up to the 30-min reaction time and then decreased. The concentration of epicatechin *o*-quinone that was formed was very low compared to that of caffeoyl tartrate. These results are similar to those of Cheynier et al. (1989). The addition of honey to the caffeoyl tartrate–PPO system decreased the concentration of *o*-quinone significantly, and no detectable epicatechin *o*-quinone was observed when honey was added to the epicatechin–PPO system. This result clearly indicated that honey contains certain compounds that inhibit the PPO activity and suppress the formation of *o*-quinones.

The kinetic study on the inhibitory effect of honey on PPO with respect to epicatechin is shown in Figure 3. The reaction rate of PPO–epicatechin decreased as the concentration of honey increased (Figure 3A). The double-reciprocal Lineweaver–Burk plot (Figure 3B) shows that honey is noncompetitive with epicatechin since it affects the apparent V_m but does not affect the apparent K_m, a kinetic pattern that is consistent with a sequential mechanism for the binding of honey and PPO before PPO reacts with epicatechin. The apparent K_i value was 0.731%.

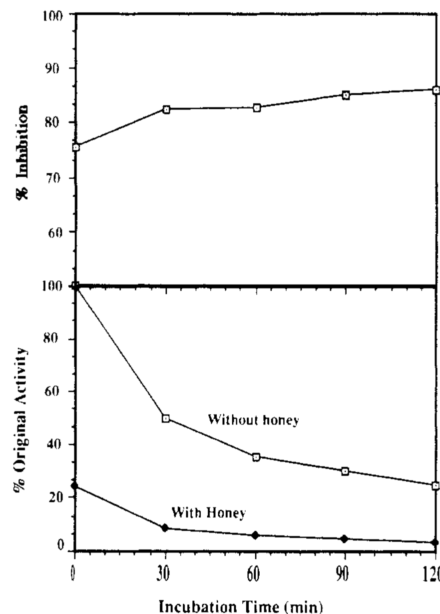

Figure 4. Inhibitory effect of honey on PPO during incubation.

This inhibitory effect of honey on PPO can be seen in another experiment in which PPO was incubated with honey and the activity was measured at different time intervals. When PPO was dissolved in potassium bitartrate solution at pH 3.65 and incubated at 20 °C for 120 min, the PPO activity decreased slowly with the time of incubation. When honey was added to PPO solutions, however, the activity decreased rapidly as the incubation time increased (Figure 4). This result is similar to that observed by Madhosingh (1975), who reported that two low molecular weight peptides (about 1000) from mushroom had strong inhibitory effects on dopa oxidation after incubation with enzyme. Harel et al. (1967) have also shown that preincubation of mushroom tyrosinase with a low molecular weight peptide inhibitor obtained from *D. dendroides* enhanced inhibition.

Honey solution was separated into 14 fractions by using Bio-Gel P-2, which has maximum 1800 molecular weight exclusion. The fractions that had a high inhibitory effect contained a higher peptide content and showed a high absorbance at 280 nm with a correlation coefficients of r = 0.838 and 0.957, respectively. These results suggest that honey contains some low molecular weight peptide components that have an inhibitory effect on PPO. The elution profile of this inhibitor on the Sephadex G-15 column along with the molecular standards indicated that the molecular weight of the peptide is about 600. Since proteins, peptides, and amino acids can affect PPO activity by chelating the essential copper at the active site of PPO and forming stable complexes with Cu^{2+} (Kahn, 1985), this may be the mechanism of action of the honey peptide. Its isolation and characterization are currently underway to obtain a better understanding of methods for the control of oxidation of polyphenols and for the development of practical uses for honey.

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Registry No. PPO, 9002-10-2; *trans*-caffeoyltartaric acid, 67879-58-7; *cis*-caffeoyltartaric acid, 84519-50-6; epicatechin, 490-46-0; *cis*-coumaroyltartaric acid, 67920-37-0; *trans*-coumaroyltartaric acid, 27174-07-8; catechin, 154-23-4; gallocatechin gallate, 5127-64-0.