Kinetic Study of Oxalic Acid Inhibition on Enzymatic Browning

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Oxalic acid has a strong antibrowning activity. The inhibitory pattern on catechol-PPO model system appeared to be competitive, with a K_i value of 2.0 mM. When the PPO was incubated with oxalic acid, the activity was not recovered via dialysis, but the inactivated enzyme partially recovered its activity when cupric ion was added. Comparing the relative antibrowning effectiveness of oxalic acid with other common antibrowning agents, oxalic acid with I_{50} value of 1.1 mM is as effective as kojic acid and more potent than cysteine and glutathione.

Keywords: *Oxalic acid; inhibition; enzymatic browning; kinetic study; polyphenol oxidase*

INTRODUCTION

Enzymatic browning of fresh fruits and vegetables is an unfavorable reaction caused by the action of polyphenol oxidase on the phenolic substrate in the presence of oxygen and leads to dark-colored pigments. This discoloration results in the loss of aesthetic and nutritional qualities of fresh fruits and vegetables. To prevent browning, sulfites have been successfully used as antibrowning agents. However, since the 1986 FDA ban on the use of sulfites on fresh fruits and vegetables, the food industry has sought a replacement for sulfites.

Considerable research has been conducted to find alternatives to sulfites as antibrowning agents, such as ascorbic acid and its derivatives (Duxbury, 1986; Langdon, 1987; Saper et al., 1989; Saper and Miller, 1992), citric acid (Lee-Kim and Hwang, 1997), honey (Oszmianski and Lee, 1990), amino acids and their derivatives (Kahn, 1985; Gurbuz and Lee, 1897; Molnar-Perl and Friedman, 1990a,b; Richard-Forget et al., 1992), kojic acid (Chen et al., 1991a,b; Kahn et al., 1995; Kahn and Zakin, 1995), 4-hexylresorcinol (Dawley and Flurkey, 1993; Monsalve-Gonzales et al., 1993, 1995), tropolone (Kahn and Andrawis, 1985), mimosine (Hashiguchi and Takahashi, 1977), and pyridinones (Hider and Lerch, 1989). Among these browning inhibitors, ascorbic acid based formulations, cysteine and 4-hexylresorcinol (4HR), have been used commercially with a limited success. The ascorbic acid based formulations require improvement to control color after the reducing activity has been depleted. Cysteine and 4-hexylresorcinol (4HR) are too expensive for commercial use. Additionally, cysteine may produce a sulfury odor at high concentration levels (Mathew and Parpia, 1971). 4HR is not approved by the FDA for fresh fruits and vegetables, except for controlling the discoloration of unpeeled shrimp.

In a comprehensive study utilizing more than three dozen chemical compounds in a search for effective antibrowning agents on apple slices, oxalic acid was found to be an excellent antibrowning agent (Son et al., 1999). Oxalic acid is a common acid found in many foods such as spinach (356−780 mg/100 g of fresh material), rhubarb (260−620 mg/100 g fresh material), and beet root (97−121 mg/100 g of fresh material) (Hodgkinson, 1977). Therefore, oxalic acid at controlled concentrations has a strong potential for practical application. There have been no reports on kinetic data and relative potency on the inhibitory activity of oxalic acid. Therefore, the objectives of this study were to determine the inhibitory modes of oxalic acid in the browning reactions and to compare its relative antibrowning effectiveness compared to commonly known inhibitors such as citric acid, tartaric acid, kojic acid, oxalacetic acid, cysteine, and glutathione.

MATERIALS AND METHODS

Materials. Catechol and mushroom PPO with an activity of 3400 units/mg were obtained from Sigma Chem. Co. (St. Louis, MO). Oxalic acid, oxalacetic acid, tartaric acid, kojic acid, citric acid, cysteine, and glutathione were from Acros Chemical Co. (Fair Lawn, NJ).

Methods. *Kinetic Study of Oxalic Acid Inhibition.* The inhibitory effect of oxalic acid (OA) on the catechol-PPO model system was measured by spectrophotometric and polarographic methods. All the experiments were performed in triplicate on duplicate samples.

Spectrometric Measurements. Various concentrations of catechol (0.3, 0.5, 1, 4, and 10 mM) and oxalic acid (0, 10, 30, 50, and 100 mM) were prepared in a citrate phosphate buffer (pH 5.0). Catechol solutions (2.4 mL) and oxalic acid solutions (0.3 mL) were mixed together in the cuvette. The reaction was initiated by the addition of 0.3 mL of PPO (0.1 mg/mL) into the catechol−oxalic acid mixture. Change in absorbance was monitored using a diode array spectrophotometer (Hewlett-Packard Inc., Palo Alto, CA) for 3 min at 420 nm. Maximal initial velocity (ΔOD/min) was determined from the initial linear portion of absorbance vs time graph by the software supplied by Hewlett-Packard Inc. (Palo Alto, CA). One unit of PPO activity (A) was defined as an increase in absorbance of 1.0/min/mg protein at 420 nm and 25 °C. The degree of inhibition on reaction was expressed as percentage inhibition ($I = 100(A^* - A)/A^*$, where A and A^* were enzyme activity with or without inhibitor, respectively). Kinetic parameters (K_m, apparent, V_{max}, and K_i) of oxalic acid inhibition were determined using double-reciprocal plots (Lineweaver−Burk) of enzyme activity vs catechol concentration.

Polarographic Measurements. PPO activity was also measured by oxygen uptake rate to find out whether oxalic acid

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acts on the reaction products or on enzyme, per se. Twenty milliliters of catechol (1 mM) and 2.5 mL of oxalic acid solutions (0, 20, and 100 mM) were mixed together into the test tube attached to the dissolved oxygen sensor (DO meter, model 50B, YSI Inc.). The mixture was saturated by air for 30 min. After adding 2.5 mL of PPO (0.1 mg/mL), change in percentage of the initial oxygen content was monitored for 30 min at room temperature. The rate of oxygen consumption (C) was determined from the plot of oxygen uptake vs time. Percentage inhibition (I) on the rate of oxygen consumption was defined as $I = 100(C^* - C)/C^*$, where C and C^* were the rate of oxygen consumption with or without inhibitor, respectively.

Dialysis and Addition of Cupric Ion. To know whether oxalic acid was reversibly combined with PPO, the residual enzyme activity of PPO−oxalic acid mixtures were measured after dialysis or adding cupric ion. The effect of dialysis on PPO−oxalic acid mixture was tested as follows. PPO (0.1 mg/mL) in citrate phosphate buffer (pH 5.0) was incubated with 0, 5, 10, and 50 mM oxalic acid for an hour at room temperature. Each sample was placed into the dialysis membrane (Spectrum Medical Indusria, Inc.) with a molecular weight cutoff of 6000−8000 and then dialyzed overnight against several changes of citrate phosphate buffer (pH 5.0) to remove the oxalic acid. Enzyme activity (A) before and after dialysis was measured using a spectrophotometer by adding 0.6 mL of the dialyzed solutions to the cuvette containing 2.4 mL of 1 mM catechol in of citrate phosphate buffer (pH 5.0). The effect of adding cupric ion on PPO−oxalic acid mixture was tested as follows. Thirty milliliters of PPO (0.1 mg/mL) was mixed with 30 mL of the various concentrations of oxalic acid (0, 10, and 50 mM) and incubated for an hour at room temperature. Ten milliliters of each PPO−oxalic acid mixture was placed into test tubes and then added with 0, 5, 10, 20, and 50 mM cupric chloride (5 mL) in citrate phosphate buffer (pH 5.0). Enzyme activity (A) was measured by using a spectrophotometer as previously described.

Relative Antibrowning Activity of Oxalic Acid. To compare the antibrowning activity of oxalic acid with commonly known inhibitors such as tartaric acid, citric acid, oxalacetic acid, glutathione, cysteine, and kojic acid, the relative inhibition activities were spectrophotometrically measured. The reaction mixture included 2.4 mL of 1 mM catechol in citrate phosphate buffer (pH 5.0), 0.3 mL of PPO (0.1 mg/mL), and 0.3 mL of various concentration of inhibitors. From the plot of each enzyme activity vs inhibitor concentration, I_{50} value of each inhibitor was obtained and compared, where I_{50} value is the mM concentration of each inhibitor needed to inhibit enzyme activity by 50%.

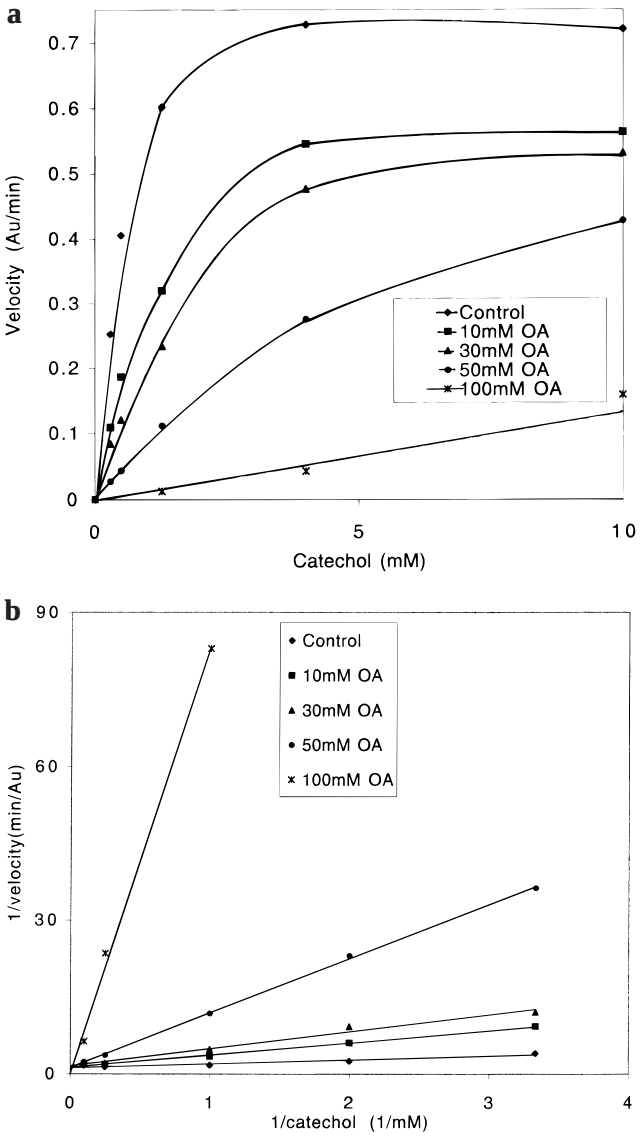

Figure 1. Inhibition kinetics of oxalic acid on browning. (a) Effect of oxalic acids (0, 10, 30, 50, and 100 mM) on the browning reaction rate of catechol. (b) Lineweaver−Burk plot of oxalic acid inhibition on the catechol-mushroom PPO system. (Standard deviations of all data were less than 5%.)

RESULTS AND DISCUSSION

The inhibition kinetics of oxalic acid on the catechol-PPO model system are shown in Figure 1. Enzymatic browning reaction rate decreased with increasing oxalic acid concentration in all substrate levels (Figure 1a). The K_m value for control was 0.64 mM, which is similar to that (0.69 mM) of Chen et al. (1991a) on a monophenol−mushroom PPO system. The inhibitory mode of oxalic acid was found to be of the competitive type since K_m values increased with oxalic acid concentration, while V_{max} value remained unchanged (Figure 1b). The inhibition constant (K_i) in our model system was 2.0 mM at pH 5.0. However, Sato (1980a) reported that the inhibition of ammonium oxalate on catechol-spinach PPO system was a noncompetitive type inhibitor, and its K_i value was 0.9 mM at pH 6.8. On the other hand, Ferra and Walker (1993) reported a mixed competitive type of oxalate inhibition in 4-methyl catechol-apple PPO system with inhibition constant (K_i) of 0.55 mM. On the basis of these reports and our results, it appears that the different source of PPO produces different types of inhibition mechanism.

To determine whether oxalic acid acts on the reaction product or on enzyme per se, the inhibition of oxalic acid on PPO was measured by both spectrophotometric and polarographic methods. The percent inhibition with respect to the inhibitor concentration is shown in Figure 2. Ascorbic acid was chosen as a reference since it acts on the reaction product and reduces *o*-benzoquinone to catechol. Ascorbic acid completely inhibits the rate of oxidation of catechol to colored products, but there was no inhibition in terms of the oxygen uptake during the reaction. However, in the inhibition of oxalic acid on catechol−PPO reaction, there was no significant difference in the percent inhibition measured by color change or oxygen uptake rate ($p < 0.05$). Oxalic acid seems to inhibit PPO per se, by chelating copper from the active site of the enzyme since oxalic acid has a high affinity to form metal complexes with copper ion (Furia, 1964).

To know whether oxalic acid reversibly combines with PPO, PPO was mixed with oxalic acid and then followed by dialysis or adding cupric ion. After dialysis of the mixtures, the residual enzyme activities were measured

Table 1. PPO Activity[a] Treated with Oxalic Acid and Kojic Acid before and after Dialysis

		oxalic acid			kojic acid
	control	5 mM	10 mM	50 mM	0.5 mM
PPO activity before dialysis	22.7 ± 1.2[b]	4.2 ± 0.5	0.2 ± 0.0	0.0 ± 0.0	18.1 ± 0.9
PPO activity after dialysis	18.1 ± 1.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	18.3 ± 0.6

[a] One unit of PPO activity (A) was defined as an increase in absorbance of 1.0/min/mg protein at 420 nm and 25 °C. [b] Data were presented as a mean ± SD.

Table 2. Effect of Various Concentrations of Cupric Chloride on Regenerated PPO Activity[a]

concd oxalic acid (mM)	concd cupric chloride				
	0 mM	5 mM	10 mM	20 mM	50 mM
0	21.2 ± 0.2[b]	20.7 ± 0.2	20.4 ± 0.6	18.3 ± 0.5	11.7 ± 1.7
10	1.8 ± 0.6 (8.5%)[c]	2.7 ± 0.5 (13%)	3.9 ± 0.3 (19%)	6.2 ± 0.3 (34%)	6.9 ± 0.5 (59%)
50	1.1 ± 0.3 (5.2%)	1.2 ± 0.2 (5.8%)	1.5 ± 0.3 (7.4%)	2.1 ± 0.5 (11%)	2.0 ± 0.5 (17%)

[a] One unit of PPO activity (A) was defined as an increase in absorbance of 1.0/min/mg protein at 420 nm and 25 °C. [b] Data were presented as a mean ± SD. [c] The percent recovery ($R = 100A/A^*$, where A and A^* were PPO activities in the presence or absence of oxalic acid, respectively) was indicated in the parentheses.

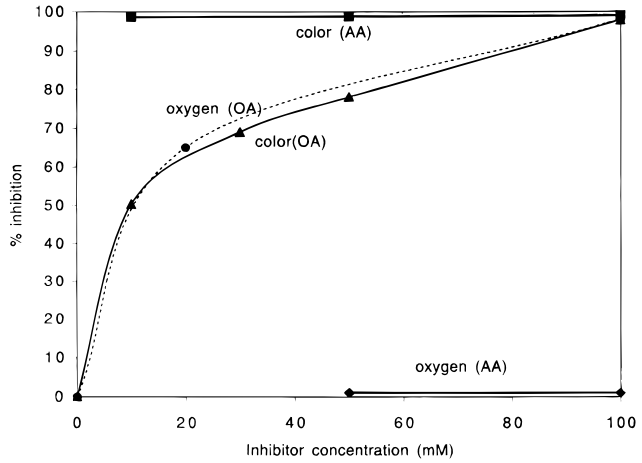

Figure 2. Inhibitory effect of oxalic acid (OA) and ascorbic acid (AA) measured by color change and oxygen uptake.

as shown in Table 1. Kojic acid was selected as a reference since it was reported as a reversible inhibitor by dialysis (Chen et al., 1991b). The PPO activity was not recovered after dialysis in all concentrations of oxalic acid when compared to the control, while kojic acid was reversibly recovered. After adding the various concentrations of cupric chloride to the mixtures, the residual enzyme activity was also measured as shown in Table 2. The enzyme activity of the control was decreased with the addition of cupric chloride because chloride is a noncompetitive inhibitor on PPO (Janovitz-Klapp et al., 1990). However, Enzyme activity in the presence of oxalic acid was increased according to the concentration of cupric chloride, with maximum observed recovery, 59% at 10 mM oxalic acid and 17% at 50 mM oxalic acid. Therefore, when PPO was mixed with oxalic acid for a period of time, the enzyme activity was not recovered via dialysis but only partially recovered when cupric ion was added. Sato (1980b) also reported that no restoration in the activity of spinach PPO was observed when the ammonium oxalate was removed from the PPO-oxalate mixture. Goldstein et al. (1964) reported that inhibition of dopamine-β-hydroxylase by chelating of tropolone was not reversed by Cu^{2+} and only reversed by a dialysis against the buffer. Kahn and Andrawis (1985) reported that the inhibition exerted by chelating of tropolone on PPO was reversed by dialysis or excess Cu^{2+}.

Table 3. Relative Antibrowning Effects of Various Inhibitors in the Catechol-Mushroom PPO System Expressed in I_{50} Value

PPO inhibitors				I_{50} value (mM)
tartaric acid				200
concn (mM)	10	200	300	
% inhibition	1	50	81	
citric acid				150
concn (mM)	100	200	300	
% inhibition	30	66	84	
oxalacetic acid				80
concn (mM)	50	100	200	
% inhibition	24	60	92	
glutathione				3.7
concn (mM)	3	5	10	
% inhibition	29	95	99	
cysteine				2
concn (mM)	1	2.5	10	
% inhibition	26	60	99	
oxalic acid				1.1
concn (mM)	0.5	1.0	10	
% inhibition	2	46	94	
kojic acid				0.7
concn (mM)	0.5	1	10	
% inhibition	46	58	94	

The relative inhibition activity of oxalic acid was compared with known antibrowning agents. The percent inhibition (I) of each inhibitor at different concentrations is shown in Table 3. At the 10 mM concentration, oxalic acid, kojic acid, cysteine, and glutathione showed over 90% inhibition, but citric acid, tartaric acid, and oxalacetic acid showed less than 5% inhibition on catechol−PPO reaction. The I_{50} value in millimolar concentration to inhibit the enzyme activity by 50% was obtained from the graph of percent inhibition vs inhibitor concentration and also shown in Table 3. I_{50} values of inhibitors were found to be 0.7 mM for kojic acid, 1.1 mM for oxalic acid, 2 mM for cysteine, 3.7 mM for glutathione, 80 mM for oxalacetic acid, 150 mM for citric acid, and 200 mM for tartaric acid. Among these tested, oxalic acid and kojic acid are the most potent inhibitors on catechol-PPO reaction. These results are in agreement with our previous study on apple slices that showed that oxalic acid and kojic acid were the best antibrowning agents with the minimal concentration of 0.05% for preventing apple browning (Son et al., 1999).

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