Inhibition of Diphenol oxidase activity of strawberry (*Fragaria* sp) Using L-cysteine and L-glycine

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ABSTRACT

L-glycine and L-cysteine exhibit, strong inhibition of partial purified DIPHENOL OXIDASE at strawberry. The concentration of L-glycine inhibiting Diphenol oxidase activity by 50% (IC₅₀) was 0.5 and 0.4 mM at pH 6.7 and 8, respectively. The inhibition of partial purified Diphenol oxidase activity is pH and inhibitor dependent. Kinetic studies indicate that L-glycine is a uncompetitive inhibitor and L-cysteine is competitive and noncompetitive inhibitor of Diphenol oxidase activity. $V_{\max}$ and K_m for catechol oxidation at pH 6.7 and in presence of L-glycine (1.4M) was $0.09 \Delta A \text{ min}^{-1}$ and 10 mM. $V_{\max}$ for catechol oxidation at pH 8 and in absence of L-glycine was $0.09 \Delta A \text{ min}^{-1}$, with a K_m of 3.5 mM. Kinetics parameters indicated the highest catalytic efficiency (units $\text{mg}^{-1} \text{ prot mM}^{-1}$) with catechol and L-glycine at pH 8: 4, then with L-cystein at pH 8: 1.7, L-glycine at pH 6.7: 1.4 and L-cystein at pH 6.7: 0.25.

Keyword: Inhibition; L-cysteine; polyphenol oxidase; strawberry; *Crataegus* spp

1. INTRODUCTION

Many vegetables and fruits become discolored during storage or processing, an action mediated by the enzyme polyphenol oxidase [Broothaerts et al., 2000]. Diphenol oxidase (tyrosinase, EC 1-14-18-1) is a copper-containing enzyme that is widespread in plants, synthesised early in tissue development and stored in chloroplasts [Van Gelder et al., 1997]. The enzyme is widely distributed in a multitude of organisms from bacteria to mammals [Robb., 1984]. Enzymatic browning is the main function of polyphenol oxidase in fruits and vegetables, and it is often undesirable and responsible for unpleasant sensory qualities and reduction in nutrient quality [Sanchez-Amat et al., 1997]. When cell membrane integrity is disrupted, phenolic substrates encounter the enzyme and are converted to o-quinones in a two-step process of hydroxylation of monophenols to diphenols (monophenolase activity), followed by oxidation of diphenols to o-quinones (Diphenol oxidase activity) [Espin et al., 1998]. Diphenol oxidase has been implicated in the formation of pigments, oxygen scavenging [Trebst et al., 1995] and defense mechanism against plant pathogens, [Mohammadi et al., 2002] and herbivory insects [Constabel et al., 2000]. Phenolic compounds serve as precursors in the formation of physical polyphenolic barriers, limiting pathogen translocation. The quinones formed by Diphenol oxidases can bind plant proteins, reducing protein digestibility and their nutritive value to herbivores [Ryan., 2000]. On the other hand, the oxidation of phenolic substrates by Diphenol oxidase is thought to be the

major cause of the brown coloration of many fruits and vegetables during ripening, handling, storage and processing.

2. MATERIALS AND METHODS

The strawberry used in this study were obtained from Kurdistan of Iran and frozen at -25°C until used. Catechol, polyvinylpyrrolidone (PVPP), catechol, tyrosine were purchased from Merck (Darmstadt, Germany). Acetone, ammonium sulphate, L-cysteine, kojic acid, L-glycine, polyethylene glycol (PEG), phenylmethanesulfonyl fluoride (PMSF), cellulose membrane (76x49mm) and DEAE-cellulose were purchased from Sigma-Aldrich (St. Louis, USA). All chemicals were of analytical grade.

2.1. Enzyme Extraction and Ion Exchange Chromatography

500 grams of strawberry were homogenized in 250 mL of 0.1M phosphate buffer (pH 6.8) containing 10 mM ascorbic acid and 0.5% polyvinylpyrrolidone with the aid of a magnetic stirrer for 1h. The crude extract samples were centrifuged at 30000 g for 20 min at 4°C . Solid ammonium sulphate $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to obtain 30 and 80% $(\text{NH}_4)_2\text{SO}_4$ saturation, respectively. After 1 h, the precipitated proteins for each stage were separated by centrifugation at 30000 g for 30 min. The precipitate was redissolved in a small volume of distilled water and dialyzed at 4°C against distilled water for 24 h with 4 changes of the water during dialysis. The dialysate was applied to a column (2.5 cm x 30 cm) filled with DEAE-cellulose, balanced with 10 mM phosphate buffer, pH 6.8. In order to remove non adsorbed fractions the column was washed with 200 mL of the same buffer at the flow rate of 0.5 mL/min. Then, a linear gradient of phosphate buffer concentration from 20 to 180 mM was applied. 5 mL fractions were collected in which the protein level and diphenol oxidase activity towards catechol as substrate were monitored. The fractions which showed diphenol oxidase activity were combined and were used as enzyme source in the following experiments. Protein concentration measured by lowry method.[Lowry et al., 1951].

2.2. Diphenol oxidase Assay

Enzymatic activity was determined by measuring the increase in absorbance at 420 nm for catechol with a spectrophotometer (6305 JENWAY). The sample cuvette contained 3 ml of substrate catechol in constant concentrations and in presence of different concentration of L-glycine or L-cysteine, prepared in the phosphate buffer. Assays were carried out by addition of 200 μl of extracts to the sample cuvette, and changes in absorbance 420 nm were recorded. The reference cuvette contained just 3 ml of substrate solution. Polyphenol oxidase activity was determined by measuring the amount of quinone produced, using an extinction coefficient of $2450\text{ M}^{-1}\text{cm}^{-1}$ for catechol. Enzyme activity was calculated from the linear portion of the curve. One unit of diphenol oxidase activity was defined as the amount of enzyme that produces 1 micromole of quinone per minute. Assays were carried out at room temperature and results are the averages of at least three assays.

2.3. Inhibition of Diphenol oxidase Activity by L-glycine Concentration and pH

Inhibition of Diphenol oxidase activity was conducted in a disposable cuvette containing 3 mL of the standard reaction mixture. The concentration of L-glycine was 0, 0.2, 0.4, 0.6, 0.9, 1.2, 1.4, 1.8 and 2 M in an phosphate buffered reaction mixture with pH of 6.7 and 8, and Diphenol oxidase activity for the oxidation of catechol at a final concentration of 15 and 45 mM was determined at pH 6.7 and 8 respectively. This concentration for L-cysteine was 0, 0.2, 0.4, 0.8, 1, 2, 3, 5, 8 and 16M in an phosphate buffered reaction mixture with pH of 6.7 and 8.

2.4. Inhibition of Diphenol oxidase Activity by L-cysteine Concentration and pH

Inhibition of Diphenol oxidase activity was conducted in a disposable cuvette containing 3 mL of the standard reaction mixture. The final concentration of L-cysteine was 0.35 and 1.2 M in an phosphate buffered reaction mixture with pH of 6.7 and 0.33 and 1.2 M in an phosphate buffered reaction mixture with pH of 8, and Diphenol oxidase activity for the oxidation of catechol at a final concentration of 15 and 45 mM was determined at pH 6.7 and 8, respectively.

2.5. Kinetic Study

Various concentrations of catechol (25-50 mM) and L-cysteine (0.0, 0.35 and 1.2 mM) were prepared in 0.1 M phosphate buffer (pH 6.7). These various concentrations for catechol were 5, 7, 10, 15 and 20 mM and for L-cysteine were 0.0, 0.33 and 1.2 M at pH 8. Various concentrations of catechol (7, 10, 15 and 20 mM) and L-glycine (0.0, 0.74 and 1.2 M) were prepared in 0.1 M phosphate buffer (pH 6.7). These various concentrations for catechol at pH 8 were 8, 10, 12, 15 and 16 mM and for inhibitor of L-glycine were 0.0, 0.74 and 1.2 M. The reaction mixture and Diphenol oxidase activity assay were the same as those for the standard reaction. The inhibition kinetics of L-glycine and L-cysteine on Diphenol oxidase activity were determined by Lineweaver-Burk plots [Marangoni., 2002].

2.6. Preincubation of L-glycine with Diphenol oxidase or catechol.

Preincubation of L-glycine with Diphenol oxidase was performed by mixing a series of L-glycine solutions (0.4, 0.7 and 1.2 mM) prepared in 0.1 M phosphate buffer (pH 8) with diphenol oxidase extract in a cuvette held at 25 °C for 0, 1, 2, 4, and 5 min. The reaction was initiated by adding 45 mM catechol to the L-glycine and Diphenol oxidase mixture after the tested incubation time. For the preincubation study between L-glycine and catechol, 1 M L-glycine and 15 mM catechol were mixed and held at 25 °C for 5 min. The reaction was initiated by adding Diphenol oxidase to the mixture, and the diphenol oxidase activity was determined following the same procedure as described above.

2.7. Preincubation of L-cysteine with diphenol oxidase or catechol.

Preincubation of L-cysteine with Diphenol oxidase was performed by mixing a series of L-cysteine solutions (0.4, 0.7 and 1 M) prepared in 0.1 M phosphate buffer (pH 6.7) with Diphenol oxidase extract in a cuvette held at 25 °C for 0, 1, 2, 4, and 5 min. The reaction was initiated by adding 15 mM catechol to the L-cysteine and Diphenol oxidase mixture

after the tested incubation time. For the preincubation study between L-cysteine and catechol, 5 M L-cysteine and 45 mM catechol were mixed and held at 25 °C for 5 min. The reaction was initiated by adding Diphenol oxidase to the mixture, and the Diphenol oxidase activity was determined following the same procedure as described above.

3. RESULTS AND DISCUSSION

3.1. Effect of L-glycine and L-cysteine acid on Diphenol oxidase activity in strawberry extract

L-glycine and L-cysteine inhibited the Diphenol oxidase activity detectable with catechol as substrate. The concentration of L-glycine inhibiting Diphenol oxidase activity by 50% (IC₅₀) was 0.5 and 0.4 mM at pH 6.7 and 8, respectively (Figure 1). IC₅₀ for L-cysteine inhibiting Diphenol oxidase activity was 0.4 at pH 6.7 and 0.5 at pH 8 (Table 1).

Table 1. Effect of L-glycine and cysteine on the DIPHENOL OXIDASE activity at pH 6.7 and 8.

Substrate (catechol)	pH 6.7	Inhibitor	IC ₅₀ (M)	Type of inhibition
		Glycine	0.3	Uncompetitive
		Cysteine	0.35	Uncompetitive
	pH 8	Inhibitor	IC ₅₀ (M)	Type of inhibition
		Glycine	0.45	competetive
		Cysteine	0.4	Competetive

Figure 1. L-glycine -mediated inhibition of iphenol oxidase activity as reflected by the oxidation of catechol.

Figure 2. Inverse plots obtained when catechol oxidation was assayed in the presence of different L-glycine concentrations.

3.2. Inhibition Kinetic of L-glycine on DIPHENOL OXIDASE Activity at pH 6.7 and 8

Inhibition of DIPHENOL OXIDASE by L-glycine was determined in the presence of different concentrations of L-glycine for three fixed concentrations of catechol at pH 6.7 and pH 8 (Figure 2). Lineweaver burk plots used to analyze inhibition kinetics show that the extrapolated lines for $1/V$ versus $1/[\text{catechol}]$ are parallel and don't intersect each other near or on the y and x -axis, indicating that L-glycine is a uncompetitive type inhibitor. L-glycine as a uncompetitive inhibitor are thought to bind the the DIPHENOL OXIDASE-catechol complex and not the diphenol oxidase. The effect of L-glycine is to decrease both V_{max} and K_m . A lower K_m corresponds to a higher affinity. The presence of L-glycine as uncompetitive inhibitor increases the affinity of the enzyme for the catechol. An 5-min preincubation of diphenol oxidase with 0.5M L-glycine resulted in a 35% loss in DIPHENOL OXIDASE activity compared to control. Interestingly, preincubation of L-glycine with catechol for 5 min resulted in no additional loss of DIPHENOL OXIDASE activity compared to that without incubation (Figure 3). This finding suggests that L-glycine inhibits DIPHENOL OXIDASE activity by acting directly on the diphenol oxidase-substrate rather than on the enzyme.

3.3. Inhibition Kinetic of L-cysteine on diphenol oxidase Activity at pH 8

Inhibition of DIPHENOL OXIDASE by L-cysteine was determined in the presence of different concentrations of L-cysteine for three fixed concentrations of catechol at pH 8.0. Lineweaver burk plots used to analyze inhibition kinetics show that the extrapolated lines for $1/V$ versus $1/[\text{catechol}]$ intersect each other on the y -axis, indicating that L-cysteine is a

competitive type inhibitor. L-cysteine as a competitive inhibitor can bind at the active site of the enzyme to form an diphenol oxidase-L-cysteine complex. L-cysteine blocks the active site, and catechol as substrate cannot bind until the inhibitor dissociates. Since, L-cysteine and catechol compete for the same site, raising the catechol concentration can eventually overcome the L-cysteine, and V_{max} can be achieved, but L-cysteine raises K_m , indicating that the affinity of diphenol oxidase for catechol is lower in the presence of L-cysteine. To further investigate whether the inhibition of diphenol oxidase activity by L-cysteine is attributable to the inhibitor's effect on diphenol oxidase, the substrate, or both, preincubation of L-cysteine with diphenol oxidase or catechol was carried out before the inhibition reaction started.

Figure 3. Effects of preincubation of glycine with diphenol oxidase or catechol on the inhibition of strawberry diphenol oxidase activity at pH 6.7. diphenol oxidase activity for the oxidation of catechol was determined in a standard reaction mixture buffered with 0.1 M phosphate buffer, after preincubation for 5 min by mixing either diphenol oxidase or catechol (15 mM final concentration) with 0.5M glycine. Activities were expressed as percent relative activity to that determined without glycine or preincubation: no glycine or preincubation (A); 0.5M glycine, no preincubation (B); preincubation of glycine with catechol (C); preincubation of glycine with diphenol oxidase (D).

The vertical bars represent the standard errors of three replicates. An 5-min preincubation of diphenol oxidase with 1M L-cysteine resulted in a 60% loss in diphenol oxidase activity compared to control (without inhibitor) (Figure not shown). Interestingly, preincubation of cystein with catechol for 5 min resulted in no additional loss of diphenol oxidase activity compared to that without incubation . This finding suggests that L-cysteine inhibits diphenol oxidase activity by acting directly on the diphenol oxidase rather than on the substrate.

3.4. Inhibition Kinetic of L-cysteine on DIPHENOL OXIDASE Activity at pH 6.7

Inhibition of diphenol oxidase by L-cysteine was determined in the presence of different concentrations of L-cysteine for three fixed concentrations of catechol at pH 6.7 (not shown). Lineweaver burk plots used to analyze inhibition kinetics show that the extrapolated lines for $1/V$ versus $1/[\text{catechol}]$ intersect each other on the x-axis, indicating that L-cysteine is a noncompetitive type inhibitor. L-cysteine as a noncompetitive inhibitor can bind at an allosteric site on the diphenol oxidase and leave the active site unblocked. Catechol as substrate has an identical affinity for both the L-cysteine diphenol oxidase complex and diphenol oxidase. In presence of L-cysteine as noncompetitive inhibitor of diphenol oxidase, the K_m value is unchanged (100 mM), while $V_{\max}$ is decreased from 0.2 to $0.09 \Delta A \text{ min}^{-1}$. An 5-min preincubation of diphenol oxidase with 1M L-cysteine resulted in a 70% loss in diphenol oxidase activity compared to control. Interestingly, preincubation of cystein with catechol for 5 min resulted in no additional loss of diphenol oxidase activity compared to that without incubation (Figure 4). This finding suggests that L-cysteine inhibits diphenol oxidase activity by acting directly on the diphenol oxidase rather than on the substrate.

Figure 4. Effects of preincubation of cysteine with diphenol oxidase or catechol on the inhibition of strawberry diphenol oxidase activity at pH 6.7. Diphenol oxidase activity for the oxidation of catechol was determined in a standard reaction mixture buffered with 0.1 M phosphate buffer, after preincubation for 5 min by mixing either diphenol oxidase or catechol (15 mM final concentration) with 1M cysteine. Activities were expressed as percent relative activity to that determined without preincubation: no cysteine or preincubation (A); 1M cysteine, no preincubation (B); preincubation of cysteine with catechol (C); preincubation of cysteine with diphenol oxidase (D). The vertical bars represent the standard errors of three replicates.

Table 2. Kinetics parameters for the diphenol oxidase activity at pH 6.7 and pH 8.

pH	Substrate	Inhibitors	V _{max} (Unit/ mg.protein)	K _m (mM)	Catalytic efficiency
6	Catechol	-----	112	22	5
		Glycine (0.74M)	45	19	2.3
		Glycine(1.4 M)	36	17	2.1
7	Catechol	-----	145	9	16.1
		Glycine (0.7 M)	88	8.2	10.7
		Glycine(1.25 M)	62	6.8	9.1
6	Catechol	-----	112	22	5
		Cysteine(0.35M)	58	20	2.9
		Cysteine(1.2 M)	32	19.5	1.6
7	Catechol	-----	145	9	16.1
		Cysteine(0.35 M)	78	7.8	10
		Cysteine (1.6M)	35	7.5	4.6

3.5. Kinetic parameters of DIPHENOL OXIDASE activity in strawberry extract in presence of inhibitors

The Michaelis-Menten constant (K_m) and maximum rate (V_{max}) values for diphenol oxidase activity in strawberry extract were determined by performing activity assays at pH 6.7 and pH 8, in the presence of extract aliquots and various concentrations of either catechol as substrate and various concentrations of L-glycine and L-cysteine as inhibitors. The rate of catechol oxidation to its corresponding o-quinone was measured by monitoring the absorbance increase at 420 nm in a 3-ml reaction mixture containing 0.75 mg extract protein. The maximum rate (V_{max}) for catechol oxidation at pH 6.7 and in absence of L-glycine was $0.25 \Delta A \text{ min}^{-1}$, with a K_m of 25 mM. The catalytic efficiency calculated per milligram protein in the extract was $1.8 \text{ units mg}^{-1} \text{ prot mM}^{-1}$ (Table 2). The maximum rate (V_{max}) and K_m for catechol oxidation at pH 6.7 and in presence of L-glycine (1.4M) was $0.09 \Delta A \text{ min}^{-1}$ and 10 mM, but catalytic efficiency decreased to $1.3 \text{ units mg}^{-1} \text{ prot mM}^{-1}$. The maximum rate (V_{max}) for catechol oxidation at pH 8 and in absence of L-glycine was $0.09 \Delta A \text{ min}^{-1}$, with a K_m of 3.5 mM. V_{max} in presence of L-glycine (1.25M) decreased and reached to $0.07 \Delta A \text{ min}^{-1}$ and K_m decreased to 3.1 mM. Catalytic efficiency at pH 8 in presence of L-glycine decreased from 4.6 to 3.8 $\text{units mg}^{-1} \text{ prot mM}^{-1}$. Data in table 2 shows that catalytic efficiency decreased for catechol oxidation in presence of L-glycine and L-cysteine at pH 6.7 and pH 8.

4. CONCLUSION

This study demonstrates that L-glycine and L-cysteine exhibit, strong inhibition of strawberry diphenol oxidase activity. The inhibition of diphenol oxidase activity is pH and

inhibitor dependent. Kinetic studies via lineweaver-Burk plots indicate that L-glycine is a uncompetitive inhibitor and L-cysteine is competitive and noncompetitive inhibitor of partial purified diphenol oxidase activity. As reported for other plants [Ho K-K., 1999],[Escribano et al., 2002], multiple isoforms of diphenol oxidase were detected in saffron [Saeidian et al., 2007], so we can conclude that diphenol oxidase in strawberry (*Crataegus* spp) maybe have two isoforms, because of different kinetic properties at pH 6.7 and 8.

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