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Inhibitory Effect of Cysteine and Glycine Upon Partial Purified Polyphenol Oxidase of *Pyrus communis*

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Abstract: L-glycine and L-cysteine, exhibit strong inhibition of partial purified PPO of wild pear. The concentration of L-glycine inhibiting PPO activity by 50% (IC_{50}) was 0.8 and 0.5 mM for catechol oxidation and 0.75 and 0.5 mM for pyrogallol oxidation at pH 5 and 7, respectively. IC_{50} for L-cysteine calculated, 0.6 and 0.35 for catechol oxidation and 0.55 and 0.75 mM for pyrogallol oxidation at pH 5 and 7 respectively. The inhibition of partial purified PPO activity is pH and inhibitor dependent. Kinetic studies indicate that L-glycine is a competitive and noncompetitive inhibitor and L-cysteine is competitive and noncompetitive inhibitor of partial purified PPO at pH 5 and 7 in presence of catechol. These inhibitors showed different type of inhibition at pH 5 and 7 in presence of pyrogallol, too. V_{max} and K_m for pyrogallol oxidation at pH 5 and in presence of L-glycine (1M) was 320 unit/mg protein and 6.7 mM. V_{max} for pyrogallol oxidation at pH 7 and in presence of L-glycine was 180 unit/mg protein, with a Km of 5.2 mM. Kinetics parameters indicated the highest catalytic efficiency (units mg^{-1} prot mM^{-1}) with pyrogallol and inhibitors of L-glycine and L-cysteine at pH 5 (47.7 and 34.4) and minimum of catalytic efficiency was earned for inhibitors at pH 7 for catechol oxidation. These date showed that Isoform of PPO at pH 7 has higher sensitivity related to another isoform of PPO at pH 5.

Key words: Inhibition · L-Cysteine · Polyphenol Oxidase · Tomato · *Pyrus communis*

INTRODUCTION

Polyphenol oxidases (PPOs) are copper containing oxidoreductases that catalyze the hydroxylation and oxidation of phenolic compounds in the presence of molecular oxygen. Approximately, nearly 50% of tropical fruits are discarded due to quality defects resulting from enzymatic browning [1]. The browning is mainly catalyzed by the enzyme polyphenol oxidase [2]. Because of the deleterious effect of enzymatic browning on food products, PPO has been extensively studied in a variety of tissues [3, 4]. The enzyme is widely distributed in a multitude of organisms from bacteria to mammals [5]. Enzymatic browning is the main function of PPOs in fruits and vegetables and it is often undesirable and responsible for unpleasant sensory qualities and reduction in nutrient quality [6]. 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 (diphenolase activity) [7]. PPO has been implicated in the formation of pigments, oxygen scavenging [8] and defense mechanism against plant pathogens, [9] and herbivory insects [10]. Phenolic compounds serve as precursors in the formation of physical polyphenolic barriers, limiting pathogen translocation. The quinones formed by PPOs can bind plant proteins, reducing protein digestibility and their nutritive value to herbivores [11]. On the other hand, the oxidation of phenolic substrates by PPO is thought to be the major cause of the brown coloration of many fruits and vegetables during ripening, handling, storage and processing. Our objectives are to comprehend the reaction properties and biochemical characteristics of PPO in plants because an understanding of the essential factors controlling the action of PPO is necessary in an attempt to inhibit or control its activity in fruit and vegetables during processing.

MATERIALS AND METHODS

The wild pears used in this study were obtained from Kurdistan of Iran and frozen at -25°C until used. Catechol,

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polyvinylpyrolidone (PVPP), pyrogallol were purchased from Merck (Darmstadt, Germany). Acetone, ammonium sulphate, L-cysteine, L-glycine, polyethylene glycol (PEG), phenylmethylsulfonyl fluoride (PMSF), cellulose membrane (76x49mm) and DEAE-cellulose were purchased from Sigma-Aldrich (St. Louis, USA). All chemicals were of analytical grade.

Enzyme Extraction: A total of 250 grams of wild pears were homogenized in 150 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 (NH4)2SO4 was added to the supernatant to obtain 30 and 80% $(NH_4)_2SO_4$ 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 hrs with 4 changes of the water during dialysis.

Ion Exchange Chromatography: 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 PPO activity towards catechol as substrate were monitored. The fractions which showed PPO activity were combined and were used as enzyme source in the following experiments. Protein concentration measured by Lowry method [12].

PPO Assay: Enzymatic activity was determined by measuring the increase in absorbance at 420 nm for pyrogallol and 400 nm for catechol with a spectrophotometer (6305 JENWAY). The sample cuvette contained 3 ml of substrate pyrogallol 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 and 400 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 12 $M^{-1}cm^{-1}$ for pyrogallol and 4350 $M^{-1}cm^{-1}$ for catechol. Enzyme activity was calculated from the linear portion of the curve. One unit of PPO 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.

Inhibition of PPO Activity by L-Glycine and L-Cysteine: Inhibition of PPO 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, 0.8, 1, 1.2, 1.6 and 2 M in an phosphate buffered reaction mixture with pH of 5 and 7 and PPO activity for the oxidation of pyrogallol at a final concentration of 10 and 20 mM was determined at pH 5 and 7 respectively. This concentration for L-cysteine was 0,0.1, 0.2, 0.3, 0.35, 0.6, 0.8 1, 1.2 and 2 M in an phosphate buffered reaction mixture with pH of 5 and 7.

Kinetic Study: Various concentrations of pyrogallol (5-15 mM) and L-cysteine (0.0, 0.45, 0.7 and 1 mM) were prepared in 0.1 M phosphate buffer (pH 5). These various concentrations for pyrogallol were 5, 7, 10,12 and 15 mM and for L-cysteine were 0.0, 0.45, 0.7 and 1 M at pH 7. Various concentrations of pyrogallol (5, 7, 10 and 15 mM) and L-glycine (0.0, 0.74 and 1.2 M) were prepared in 0.1 M phosphate buffer (pH 5). These various concentrations for pyrogallol at pH 7 were the same as pH 5. The reaction mixture and PPO activity assay were the same as those for the standard reaction. The inhibition kinetics of L-glycine and L-cysteine on PPO activity were determined by Lineweaver-Burk plots [13].

Preincubation of L-Glycine with PPO or Catechol: Preincubation of L-glycine with PPO was performed by mixing a L-glycine solution (0.25 M) prepared in 0.1 M phosphate buffer (pH 5) with PPO extract in a cuvette held at 25°C for 5 min. The reaction was initiated by adding 25 mM catechol to the L-glycine and PPO mixture after the tested incubation time. For the preincubation study between L-glycine and catechol, 0.25 M L-glycine and 25 mM catechol were mixed and held at 25°C for 5 min. The reaction was initiated by adding partial purified PPO to the mixture and the PPO activity was determined following the same procedure as described above.

Preincubation of L-Cysteine with PPO or Catechol: Preincubation of L-cysteine with PPO was performed by mixing a L-cysteine solution (0.25 M) prepared in 0.1 M phosphate buffer (pH 5) with PPO extract in a cuvette held at 25°C for 5 min. The reaction was initiated by adding

25 mM catechol to the L-cysteine and PPO mixture after the tested incubation time. For the preincubation study between L-cysteine and catechol, 0.25 M L-cysteine and 25 mM catechol were mixed and held at 25°C for 5 min. The reaction was initiated by adding partial purified PPO to the mixture and the PPO activity was determined following the same procedure as described above.

RESULTS AND DISCUSSION

Effect of L-Glycine and L-Cysteine acid on PPO Activity in Partial Purified Pear PPO: L-glycine and L-cysteine inhibited the PPO activity detectable with pyrogallol and catechol as substrates. The concentration of L-glycine inhibiting PPO activity by 50% (IC_{50}) was 0.8 and 0.75 mM at pH 5 in presence of catechol and pyrogallol, respectively (Figs. 1, 2). The concentration of L-glycine inhibiting PPO activity by 50% (IC_{50}) was 0.5 and 0. 5 mM at pH 7 in presence of catechol and pyrogallol, respectively. IC_{50} for L-cysteine inhibiting PPO activity shown in Table 1 (Figs. 3, 4).

Inhibition Kinetic of L-Glycine and L-Cysteine on PPO Activity at pH 5 in Presence of Pyrogallol: Inhibition of PPO by L-glycine was determined in the presence of different concentrations of L-glycine for three fixed concentrations of pyrogallol at pH 5 and pH 7. Lineweaver burk plots used to analyze inhibition kinetics at pH 5 show that the extrapolated lines for $1/V$ versus 1/[pyrogallol] 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 (Figure not shown). L-glycine as a uncompetitive inhibitor binds to PPO-pyrogallol complex to stop PPO from reacting with pyrogallol to form products of quinone. PPO works well at higher pyrogallol and enzyme concentrations that in this condition, substrates of pyrogallol are bonded to enzymes. This binding of the pyrogallol modifies the structure of the PPO making the inhibitor-binding site available. As the binding of L-glycine as inhibitor, K_m and V_{max} decreased and binding affinity of enzyme to substrate increased. In presence of L-glycine as uncompetitive inhibitor of PPO, the K_m value is reached to 6.7 mM, while V_{max} is decreased from 820 to 320 unit/mg protein. An 5-min preincubation of PPO with 0.25 M L-glycine resulted in a 55% loss in PPO activity compared to control. Interestingly, preincubation of L-glycine with catechol for 5 min resulted in no additional loss of PPO activity compared to that without incubation (Fig. 7). This finding suggests that L-glycine inhibits PPO activity by acting directly on both the PPO-substrate and enzyme. L-cysteine showed the same results in this condition, so act as a uncompetitive inhibitor of partial purified PPO.

Inhibition Kinetic of L-Glycine and L-Cysteine on PPO Activity at pH 5 in Presence of Catechol: Inhibition of PPO by L-cysteine was determined in the presence of different concentrations of L-cysteine for three fixed concentrations of catechol at pH 5. Lineweaver burk plots used to analyze inhibition kinetics show at pH 5 that the extra polated lines for $1/V$ versus 1/[catechol] intersect each other on the *y*-axis, indicating that L-cysteine is a competitive type inhibitor, like ascorbate as compietitive inhibitor of *Zyzyphus spina-christi* PPO (19) (Figs. 5). L-cysteine as inhibitor binds to the active site of PPO to form an PPO-L-cysteine complex and competes with the catechol for binding. L-cysteine change active site of enzyme and inhibits the enzyme. In this case, at low [catechol], the inhibitor competes for the active site and effectively lowers the [catechol] at the active site. This lowers the rate at low catechol concentrations and increases the apparent Km, indicating that the affinity of PPO for pyrogallol is lower in the presence of L-cysteine. In presence of L-glycine as competitive inhibitor of PPO, the K_m value is increased and reached to 14.5 mM, while V_{max} nearly unchanged 268 unit/mg protein). An 5-min preincubation of PPO with 0.25 M L-cysteine resulted in a 75% loss in PPO activity compared to control. Interestingly, preincubation of L-cysteine with catechol for 5 min resulted in no additional loss of PPO activity compared to that without incubation (Fig. 6). Incubation of inhibitor and PPO for 5 minute caused 75% inhibition of enzyme, So this inhibition is more than inhibition of enzyme in condition of incubation of catechol and cysteine. This finding suggests that L-glycine inhibits PPO activity by acting directly on the PPO. L-glycine showed the same results in this condition, so act as a competitive inhibitor of partial purified PPO in presence of catechol.

Inhibition Kinetic of L-Glycine and L-Cysteine on PPO Activity at pH 7 in Presence of Catechol: Inhibition of PPO by L-glycine was determined in the presence of different concentrations of L-glycine for three fixed concentrations of catechol at pH 7. Lineweaver burk plots used to analyze inhibition kinetics show that the extrapolated lines for $1/V$ versus 1/[pyrogallol] intersect each other on the *x*-axis, indicating that L-glycine is a

Table 1: Effect of L-glycine and cysteine on the PPO activity at pH 5 and 7

Catechol	pH	Inhibitor	IC_{50} (M)	Type of inhibition
	5	Glycine	0.8	Competitive
		Cysteine	0.6	Competitive
	7	Glycine	0.5	Non-competitive
		Cysteine	0.35	Non-competitive
Pyrogallol	pH	Inhibitor	IC_{50} (M)	Type of inhibition
	5	Glycine	0.75	Un-competitive
		Cysteine	0.55	Un-competitive
	7	Glycine	0.5	Non-Competitive
		Cysteine	0.4	Non-Competitive

Table 2: Kinetics parameters for the PPO activity at pH 6.7 and pH 8

Substrate	pH	Inhibitors	Activity (Vmax) Unit/mg prot	Km (mM)	Catalytic efficiency Unit.mg^{-1} prot. mM^{-1} catechol
5		No Inhibitor	280	9.5	29.4
		cysteine (0.5 M)	275	12	22.9
		Glycine(0.5 M)	268	14.5	18.4
7		No Inhibitor	200	4.7	42.5
		Cysteine (0.5 M)	65	4.8	13.5
		Glycine (0.5 M)	85	4.2	20.2
Pyrogallol	5	No Inhibitor	820	8.7	94.2
		Cysteine (0. 5M)	210	6.1	34.4
		Glycine (0.5 M)	320	6.7	47.7
	7	No Inhibitor	540	5.1	105.8
		Cysteine(0.5 M)	145	4.8	30.2
		Glycine (0.5 M)	180	5.2	34.6

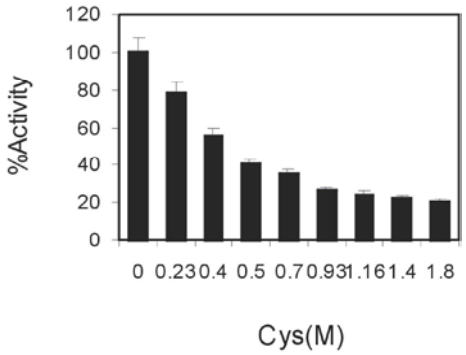

Fig. 1: Cysteine -mediated inhibition of PPO activity as reflected by the oxidation of pyrogallol at pH7

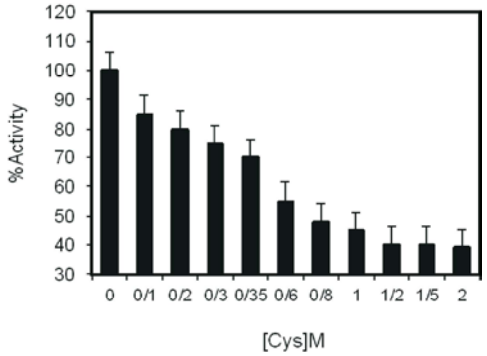

Fig. 3: L-Cysteine -mediated inhibition of PPO activity as reflected by the oxidation of catechol at pH 7

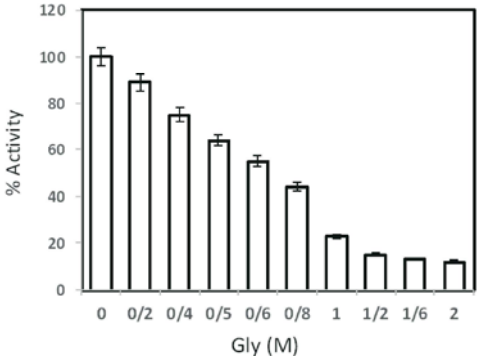

Fig. 2: Cysteine mediated inhibition of PPO activity as reflected by the oxidation of catechol at pH 5

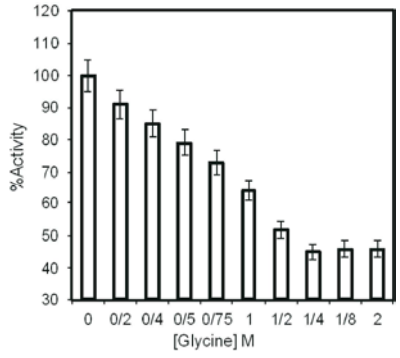

Fig. 4: Glycine -mediated inhibition of PPO activity as reflected by the oxidation of pyrogallol at pH 5

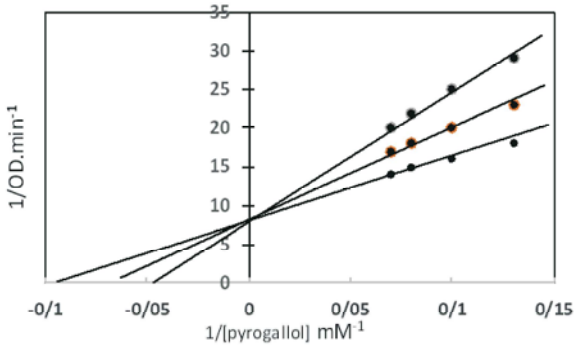

Fig. 5: Inverse plots obtained for catechol oxidation in the presence of different L-Cysteine concentrations at pH 5

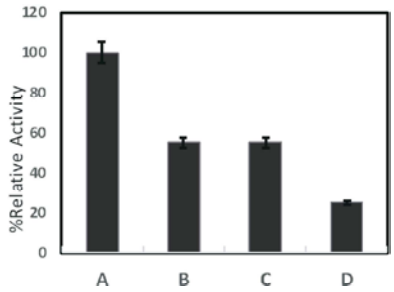

Fig. 6: Effects of preincubation of glycine with PPO or catechol on the inhibition of pear PPO activity at pH 5. PPO activity for the oxidation of catechol was determined in a standard reaction mixture buffered with 0.1 M phosphate buffer, after preincubation for 5 min by mixing either PPO or catechol (25 mM final concentration) with 0.25 M glycine. Activities were expressed as percent relative activity to that determined without glycine or preincubation: no glycine or preincubation (A); 0.25M glycine, no preincubation (B); preincubation of glycine with catechol (C); preincubation of glycine with PPO (D). The vertical bars represent the standard errors of three replicates

noncompetetive type inhibitor (Figure not shown). L-glycine as a noncompetetive inhibitor binds to PPO somewhere other than the active site. This changes the enzyme's three-dimensional structure so that its active site can still bind pyrogallol as substrate with the usual affinity, but is no longer in the optimal arrangement to stabilize the transition state and catalyze the reaction. Pyrogallol as substrate has not an identical affinity for both the L-glycine-PPO complex and PPO. L-glycine as a noncompetitive inhibitor lowers the V_{max}. Thus, PPO simply cannot catalyze the reaction with the same

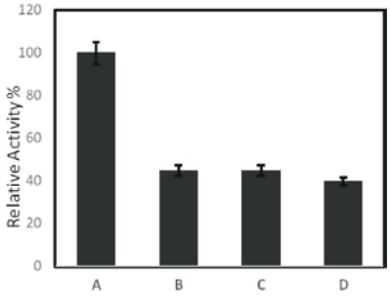

Fig. 7: Effects of preincubation of cysteine with PPO or pyrogallol on the inhibition of pear PPO activity at pH 5. PPO activity for the oxidation of pyrogallol was determined in a standard reaction mixture buffered with 0.1 M phosphate buffer, after preincubation for 5 min by mixing either PPO or pyrogallol (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 pyrogallol (C); preincubation of cysteine with PPO (D). The vertical bars represent the standard errors of three replicates

efficiency as the uninhibited enzyme. In this condition, noncompetitive inhibition cannot be overcome by raising the pyrogallol concentration like competitive inhibition by cysteine and glycine at pH 5. In presence of L-glycine as noncompetitive inhibitor of PPO, the K_m value is nearly unchanged (4.2 mM), while V_{max} is decreased from 200 to 85 unit/mg protein. An 5-min preincubation of PPO with 1M L-glycine resulted in a 70% loss in PPO activity compared to control. Interestingly, preincubation of glycine with pyrogallol for 5 min resulted in no additional loss of PPO activity compared to that without incubation (Figure not shown). This finding suggests that L-cysteine inhibits PPO activity by acting directly on the PPO rather than on the substrate. Inhibition Kinetic of L-glycine and L-cysteine on PPO Activity at pH 7 in presence of pyrogallol showed noncompetitive inhibition (Figure not shown).

Kinetic Parameters of Partial Purified PPO Activity of Wild Pear in Presence of Inhibitors: The Michaelis-Menten constant (K_m) and maximum rate (V_{max}) values for partial purified PPO activity of wild pear were determined by performing activity assays at pH 5 and pH 7, in the presence of various concentrations of either pyrogallol and catechol as substrates and various concentrations of L-glycine and L-cysteine as inhibitors. The rate of

pyrogallol and catechol oxidation to its corresponding o-quinone was measured by monitoring the absorbance increase at 420 and 400 nm in a 3-ml reaction mixture containing 0.85 mg protein. The maximum rate (V_{max}) for pyrogallol oxidation at pH 5 and in absence of L-glycine was reported (18) 820 unit/mg protein, with a Km of 8.7 mM. The catalytic efficiency calculated was 94.2 units mg^{-1} prot mM^{-1} (Table 2). The maximum rate (V_{max}) and Km for pyrogallol oxidation at pH 5 and in presence of L-glycine (1M) was 320 unit/mg protein and 6.7 mM, but catalytic efficiency decreased to 47.7 units mg^{-1} prot mM^{-1}. These data for pyrogallol at pH 7 was 180 unit/mg protein, 5.2 mM and 34.6 units mg^{-1} prot mM^{-1}. The maximum rate (V_{max}) for pyrogallol oxidation at pH 7 and in absence of L-glycine and cysteine was reported 540 unit/mg protein, with a Km of 5.1 mM. V_{max} in presence of L-glycine (1M) decreased and reached to 180 unit/mg protein and Km nearly unchanged (5.2 mM). Catalytic efficiency at pH 7 in presence of L-glycine decreased from 105.8 to 34.6 units mg^{-1} prot mM^{-1}. Data in table 2 shows that catalytic efficiency decreased for pyrogallol oxidation in presence of L-glycine and L-cysteine at pH 5 and pH 7. Kinetics parameters for catechol oxidation by partial purified PPO of wild pear calculated and listed in Table 2.

CONCLUSION

This study demonstrates that L-glycine and L-cysteine exhibit, strong inhibition of partial purified pear PPO activity. These results are the same of inhibitory effect of cysteine on PPO of Sour Cherry (*Prunus cerasus* L. CV. CAB) Pulp [20]. The inhibition of PPO activity is pH and inhibitor dependent. Kinetic studies via lineweaver-Burk plots indicate that L-glycine and L-cystein showed same inhibition, So, They are uncompetetive inhibitor at pH 5 in presence of pyrogallol, competitive inhibitors at pH 5 in presence of catechol and noncompetitive inhibitors of partial purified PPO at pH 5 and pH 7 in presence of catechol and pyrogallol. Parameters of kinetics of partial purified PPO of wild pear showed different properties, So catalytic efficiency of enzyme decreased 1.3 and 1.6 times at pH 5 for catechol oxidation, 3.1 and 2.1 times at pH 7 for catechol oxidation, 2.7 and 2 times at pH 5 for pyrogallol oxidation and 3.5 and 3 times at pH 7 for pyrogallol oxidation in presence of cysteine and glycine, respectively. The parameter of IC50 showed that for catechol oxidation in presence of glycine at pH 5 was maximum (0.8mM) and for catechol oxidation in presence of cysteine at pH 7 was minimum (0.35 mM). As reported for other plants [14, 15], multiple isoforms of PPO were detected in saffron [16, 17], so we can conclude from our results that PPO in Wild pear (pyrus communis) may be to have two isoforms, because of different kinetic properties at pH 5 and 7.

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