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Behaviour of polyphenol oxidase of *pyrus communis* in presence of Detergents and Chaotropic Agents

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ABSTRACT

Polyphenol oxidase (EC 1.14.18.1) is ubiquitous plant enzyme that catalyze the O-dependent oxidation of mono- and o-diphenols to o-quinones. These quinones are reactive species that are able to covalently modify and cross-link a wide variety of cellular nucleophiles via a 1,4 addition mechanism, leading to the formation of polymeric brown or black pigments, which are responsible for significant postharvest losses of fruits andvegetables. Browning of damaged tissues in fresh fruits and vegetables results mainly from the oxidation of phenolic compounds to quinones by PPO. The activation of PPO by sodium dodesyl sulphat investigated, but reports on the effect of some detergents and chaotropic agents on activity of PPO are scarce. Here the effect of detergents and chaotropic agent on activity of enzyme in soluble and in partial purified fraction of PPO of Wild pear was investigated. Sarkosyl acts as a activator of PPO for oxidation of catechol and pyrogallol, although has inhibitory effect in concentrations over 1 mM. Other ionic, Nonionic detergents and Chaotropic agents acted as inhibitors of PPO in soluble and in partial purified fraction. Inhibitory effect of Urea, GnHcl, NP-40 and Sodium cholate in presence of catechol is more than these effect in presence of pyrogallol, but this inhibitory for Triton X-100 in presence of pyrogallol is more than catechol. The same effects were obtained on the partially purified enzyme. Results identified and confirmed the differences in structure and conformation of enzyme in soluble and in partial purified fraction for oxidation of different substrates.

Introduction

Polyphenol oxidase (PPO) (EC 1.14.18.1) is a copper containing enzyme that catalyze the O-dependent oxidation of mono- and o-diphenols to o-quinones [1, 2]. These quinones are reactive species that are able to covalently modify and cross-link a wide variety of cellular nucleophiles via a 1,4 addition mechanism, leading to the formation of polymeric brown or black pigments, which are responsible for significant postharvest losses of fruits andvegetables [3, 4, 5, 6, 7, 8]. The involvement of copper as a prosthetic group of PPO is essential for its activity [9, 10]. Monophenolase activity, also called hydroxylase or cresolase, is always coupled to diphenolase activity, also termed catecholase or oxidase. However, diphenolase activity is not always preceded by hydroxylase activity [11]. PPO from different plant tissues shows different substrate specificities and degree of inhibition. Therefore, characterization of the enzyme could help to develop more effective methods in controlling browning of plants and products. One unusual characteristic of this enzyme is its ability to exist in an inactive or latent state [12]. PPO can be released from latency, or activated by a variety of treatmentsor agents including acid and base shock, urea [13], polyamines [15], anionic detergents such as SDS [16], proteases [17] and fatty acids [18].

Materials And Methods

Chemicals

The wild pears used in this study were obtained from Kurdistan of Iran and frozen at -25 °C until used. Catechol, polyvinylpyrolidone (PVPP), pyrogallol, sodium cholate were purchased from Merck (Darmstadt, Germany). Acetone, ammonium sulphate, polyethylene glycol (PEG), phenylmethylsulfonyl fluoride (PMSF), nonaaethylene glycol octylphenol ether (Triton x-100), nonaethylene glycol octylphenyl ether (NP-40), urea, guanidine chloride (GnHcl) ,cellulose membrane (76x49mm) and DEAE-cellulose were purchased from Sigma-Aldrich (St. Louis, USA). Sarkosyl was purchased from Fluka; All chemicals were of analytical grade.

Enzyme Extraction and PPO Partial Purification

220 grams of wild pears were homogenized in 250 mL of 0.1M phosphate buffer (pH 6.8) containing 10 mM ascorbic acid and 0.5% polyvinyl pyrrolidone with the aid of a magnetic stirrer for 1h. The crude extract samples were centrifuged at 10000 g for 20 min at 4ºC. Solid ammonium sulphate $(NH4)_2SO4$ was added to the supernatant to obtain 30 and 80% $(NH4)_2SO4$ 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 4ºC against distilled water for 24 h with 4 changes of the water during dialysis.

One hundred milliliters of extract prepared as described was brought up to 30-80% (NH4)2SO4 and after extensive dialysis against 0.1 M phosphate buffer, pH 6.8 and 0.02% PMSF was fractionated by passage through a DEAE-cellulose column equilibrated with the same phosphate buffer and elution with a 0-500 mM NaCl gradient. PPO contamining fractions were eluted at low NaCl concentrations. PPO activity were assayed on the partially purified enzyme described above.

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Protein contents of the enzyme extracts were determined according to lowry method using bovine serum albumin as a standard [14].

Enzymatic activity assays

Partial purified Polyphenol oxidase activity was determined spectrophotometrically by following, at a specific wavelength, the increase in absorbance due to the oxidation of a selected substrate to its corresponding o-quinone. Namely, the increase in absorbance was followed at 420 and 400 nm in order to monitor the oxidation of, respectively, pyrogallol and catechol. Assays were conducted at room temperature (~ 22–25 ˚C), in a 3-ml reaction mixture prepared as follows: to 2.9–2.97 ml of 0.1 M phosphate buffer, pH 6.7, containing the appropriate amount of substrate prepared in the same buffer, an aliquot (75 µl) of pear extract was added. 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). In order to correct for substrate autoxidation, the reaction mixture, was placed in the sample cuvette while the reference cuvette contained buffer and the substrate. Enzyme activity was calculated from the linear portion of the curve. Appropriate aliquots of 5 mM detergents and agents prepared in 0.1 M phosphate buffer, pH 6.8, were added to the reaction mixture just before addition of the pear extract. The final volume of the reaction mixture was always 3 ml.

Effect of Detergents and Chaotropic Agents

The enzymatic activity were also measured in the presence of increasing concentrations of ionic detergents (Sodium cholate and sarkosyl), Nonionic detergents (Triton x-100 and NP-40) and Chaotropic agents (Urea and GnHcl). The concentrations used in each case were kept within the range where no aggregation was observed and were determined as a function of the sensitivity of the enzymatic activity. Enzymatic activity of PPO assayed in extract and in partially purified PPO, separately. The PPO Activity Assayes was assayed in the presence of Ionic Detergents in 0.1 M phosphate buffer at pH 6.7 with 20 mM catechol and 15 mM of pyrogallol as the substrates, respectively, in the presence of different concentrations of sarkosyl and sodium cholate. The concentration ranges remained below the critical micelle concentration (CMC) for each detergents. The PPO Activity assays in the presence of Non Ionic Detergents was assayed in 0.1 M phosphate buffer at pH 6.7 with 20 mM catechol and 15 mM of pyrogallol, respectively, as the substrates, in the presence of different concentrations of Triton x-100 and Np-40. The concentration ranges remained below the critical micelle concentration(CMC) for each detergents. The activity assayes in the presence of chaotropic agents was assayed in 0.01 M phosphate buffer at pH 6.8 with 20 mM catechol and 15 mM of pyrogallol as the substrates in the presence of different concentrations of urea or GnHcl. PPO activity assayed at extract and partially purified PPo, separately.

Results

Assays conduct with small cherry peares crude extract

Polyphenol oxidase activity in presence of catechol and pyrogallol assayed as described in materials and methods, were detectable in peares extract. Under our experimental conditions, no lag period was observed in the expression of either activity.

Effect of Ionic detergents

The addition up to 0.5 mM sarkosyl to the reaction mixture resulted up to a 45% increase in the activity when catechol was used as the substrate at pH 6.7 (Figure 1A). This increase was 64% when pyrogallol was used as the substrate. Higher detergent concentration up to 1 mM caused a 245% increase in PPO activity with catechol as the substrate, wherease, 324% increase in activity was observed in the presence of pyrogallol. Over the concentration range (1mM), sarkosyl had inhibitory effect on PPO activity in presence of catechol and pyrogallol, although higher concentrations of sarkosyl, cloudiness prevented further investigations with these substrate(table 1). In the presence of sodium cholate at pH 6.7, a decrease in PPO activity was observed when catechol and pyrogallol was used as the substrate, separately. The inhibition increased with increasing detergent concentration, until a plateau was reached. In the presence of sodium cholate, the PPO activity decreased progressively to 11% of the control. The detergent concentration increased progressively to 0.5 mM in presence of catechol, So that, PPO activity reached to 35%, so, showed 65% Inhibition of PPO. In the presence of higher sodium cholate concentrations (1 mM), the PPO activity was reduced to 11% of the control (Figure 2A). A decrease of 36 % was observed on PPO activity when pyrogallol was used as substrate at 0.5 mM sodium cholate (Figure 2A). However, higher detergent concentrations could be used in the presence of pyrogallol. It was observed that PPO activity was reduced by 68% in 1 mM sodium cholate (table 1)

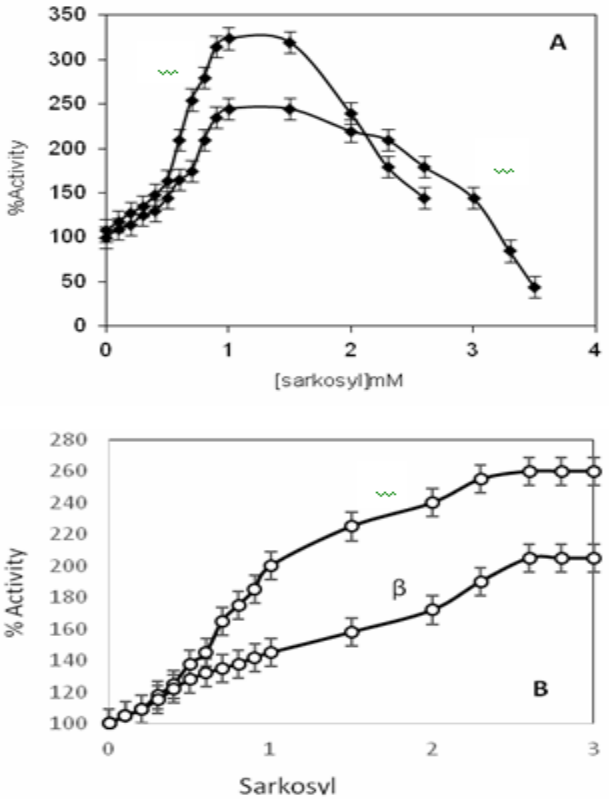

Figure 1. Effect of sarkosyl concentrations on wild pear polyphenol oxidase activity in soluble (A) and partial purified PPO activity(B) in presence of catechol 20 mM (α) and pyrogallol 15 mM (β). The reaction medium at 25 °C contained 0.1*M* sodium phosphate buffer, pH 6.8 and increasing concentrations of sarkosyl (0 to 3 m*M*).

Effect of Nonionic detergents

The addition of Triton x-100 or NP-40 to the reaction mixture at pH 6.7 led to a decrease in the PPO activity, whether catechol or pyrogallol was used as the substrate. The extent of inhibition, however varied with the detergent used. With catechol as the substrate, up to 45% inhibition was observed in the presence of 1 µM of triton x-100. 55% inhibition was observed in the presence of 1 µM TX-100 for oxidation of

pyrogallol (Figure 3A). Over the same ranges of concentrations (3 µM for triton x-100), activity of PPO reached to 35% for catechol and pyrogallol oxidation. Activity of PPO decreased in presence of NP-40 as detergent and catechol and pyrogallol as substrates. So, up to 55% inhibition was observed in the presence of 1 µM of NP-40 in presence of catechol and 45% inhibition was observed for oxidation of pyrogallol (Figure 6A). Inhibition of PPO reached to 65% and 55% in presence of 3 µM NP-40 for oxidation of catechol and pyrogallol, respectively. Over the same ranges of concentrations (3 µM for triton x-100), no effect was observed on PPO activity (table 2).

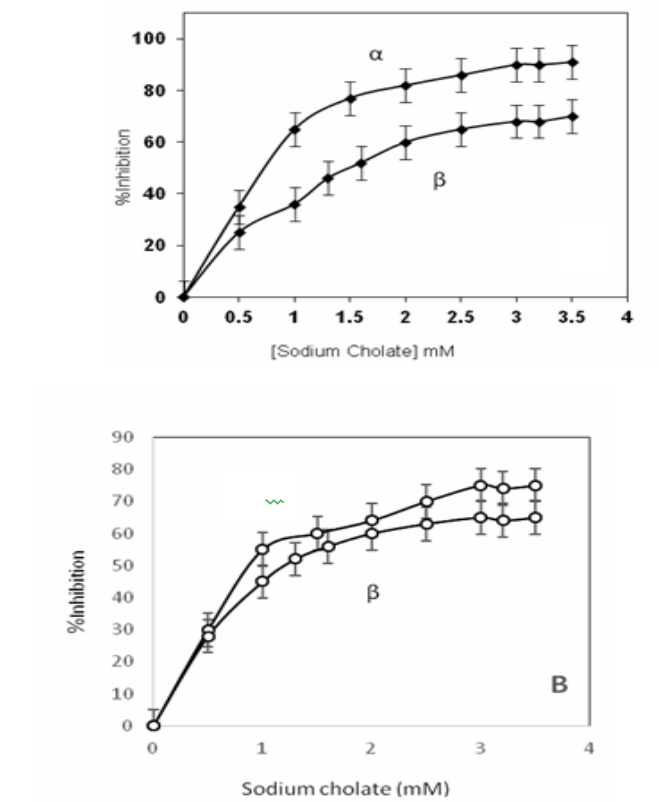

Figure 2. Effect of sodium cholate concentrations on wild pear polyphenol oxidase activity in soluble (A) and partial purified PPO activity(B) in presence of catechol 20 mM (α) and pyrogallol 15 mM (β). The reaction medium at 25 °C contained 0.1*M* sodium phosphate buffer, pH 6.8 and increasing concentrations of sodium cholate (0 to 3.5 m*M*).

Effect of chaotropic Agents

The addition of increasing of urea to the assay mixture at pH 6.7 resulted in increasing inhibition of the PPO activity, so that 35% inhibition was reached with 10 mM urea and increasing of urea concentration up to 100 mM caused only 68% inhibition in PPO activity in presence of catechol; no further inhibition was observed for up to 300 mM urea (Figure 5A). When the PPO activity was assayed in the presence of urea for oxidation of pyrogallol, up to 25% inhibition was observed in 10 mM urea; a further increase in urea concentration resulted in more inhibition so that 50% inhibition of the PPO activity was observed in 100 mM urea (Figure 5A and table 3).

Up to 35% and 78% inhibition of PPO activity was observed for catechol oxidation in presence of 10 and 100 mM GnHcl, respectively. On the other hand, up to 10% inhibition of the PPO activity was observed for pyrogallol oxidation at 10 mM GnHcl; a further increase in GnHcl to 100 mM led to as much as 45% inhibition of PPO activity (Figure 4A and table 3). Over the same ranges of concentrations (300 mM for urea and GnHcl), no effect was observed on PPO activity.

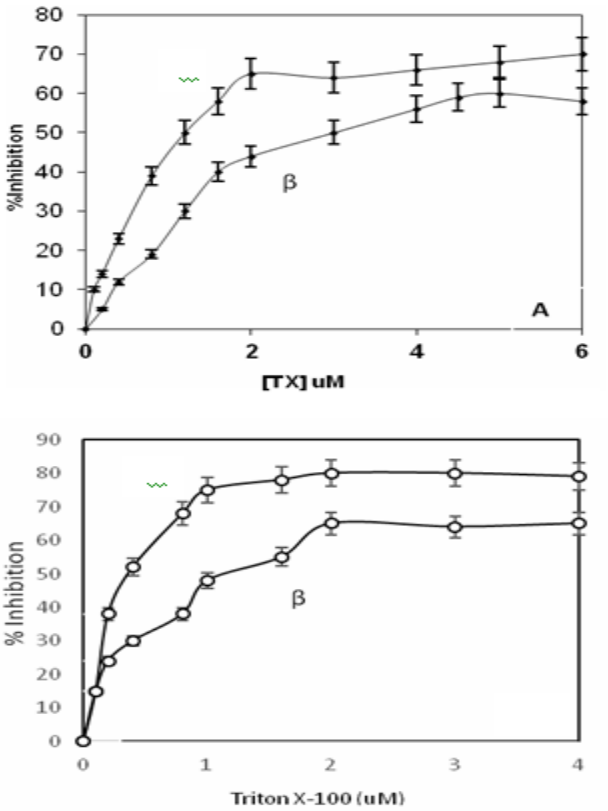

Figure 3. Effect of Triton x-100 concentrations on wild pear polyphenol oxidase activity in soluble (A) and partial purified PPO activity(B) in presence of catechol 20 mM (α) and pyrogallol 15 mM (β). The reaction medium at 25 °C contained 0.1M sodium phosphate buffer, pH 6.8 and increasing concentrations of Triton x-100.

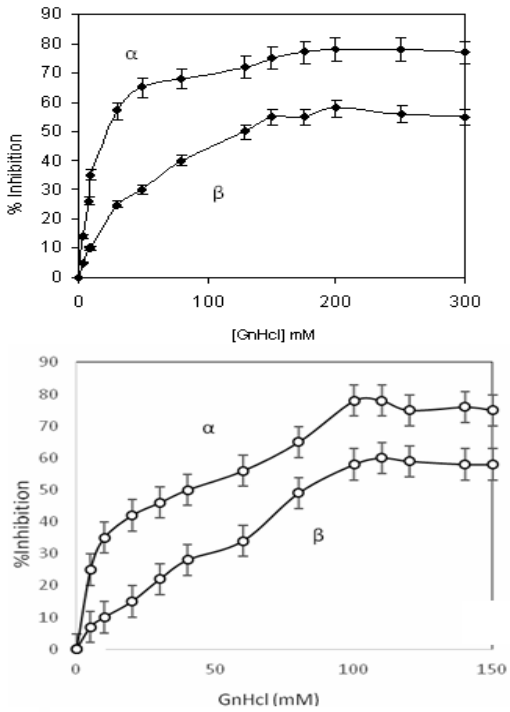

Figure 4. Effect of guanidine hydro chloride (Gn.Hcl) concentrations on wild pear polyphenol oxidase activity in soluble (A) and partial purified PPO activity(B) in presence of catechol 20 mM (α) and pyrogallol 15 mM (β). The reaction medium at 25 °C contained 0.1M sodium phosphate buffer, pH 6.8 and increasing concentrations of Gn.Hcl

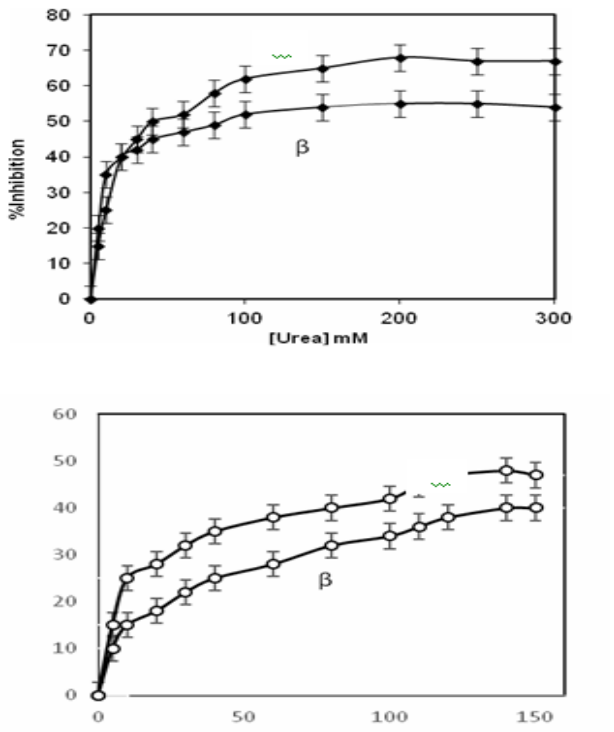

Figure 5. Effect of Urea concentrations on wild pear polyphenol oxidase activity in soluble (A) and partial purified PPO activity(B) in presence of catechol 20 mM (α) and pyrogallol 15 mM (β). The reaction medium at 25 °C contained 0.1 M sodium phosphate buffer, pH 6.8 and increasing concentrations of urea.

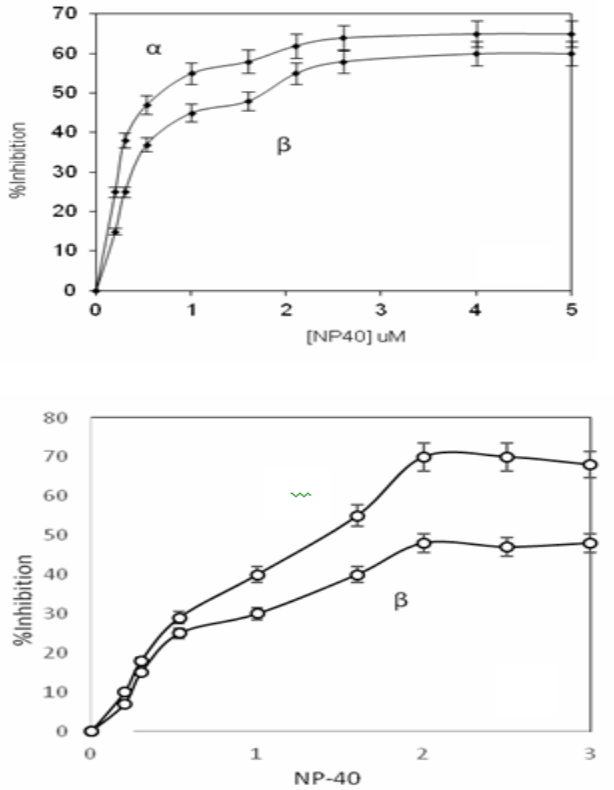

***Figure* 6. Effect of NP-40 concentrations on wild pear polyphenol oxidase activity in soluble (A) and partial purified PPO activity(B) in presence of catechol 20 mM (α) and pyrogallol 15 mM (β). The reaction medium at 25 °C contained 0.1M sodium phosphate buffer, pH 6.8 and increasing concentrations of Triton x-100 and NP-40.**

Assays conducted with partial purified PPO 0f wild pear

The partially purified PPO contained as described in the materials and methods exhibited both activity for oxidation of catechol as a dihydroxy phenol and pyrogallol as trihydroxy phenol. Investigation of PPO activity was done immediately after collecting of fractions because after storage, the residual activity decreased. The effect of ionic detergents on the activity of partial purified PPO was nearly similar to that found with the extract. For investigation of PPO activity of partial purified PPO a different detergent concentration wes used. The partial purified PPO of *pyrus communis* showed a lower sensitivity to sodium cholate, being inhibited at different detergent concentration. 0.05 mM sodium cholate caused 30% and 25% inhibition of enzyme in presence of catechol and pyrogallol respectively and 2 mM cholate caused 60% inhibition of enzyme for catechol oxidation and 55% inhibition of partial purified PPO for pyrogallol oxidation (table 4). While 2 mM sodium cholate caused 80% and 60% inhibition of enzyme in the extract, respectively. The effect of nonionic detergents on the PPO activity of partial purified enzyme was not similar to that observed with the extract with some differences in sensitivities. Tritonx-100 inhibited the enzymatic activity in the extract more than partially purified fraction for pyrogallol oxidation , but in presence of catechol, Tritonx-100 inhibited the enzymatic activity in the extract lower than partially purified fraction (Table 2 and 5). Tx-100 in low concentration (1 mM) inhibited PPO activity for oxidation of pyrogallol to a lower extent in the in the partially purified enzyme than oxidation of catechol. The extent of inhibition of PPO activity increased after partial purification related to extract. With NP-40, PPO activity was inhibited to a greater extent in the extract than in the partially purified enzyme in presence of catechol , but this inhibition for partial purified PPO is greater than extract at high concentration of NP-40 for pyrogallol oxidation. In comparision of oxidation of catechol and pyrogallol, Np-40 showed higher degree of inhibition of PPO in presence of pyrogallol (Figure 6B, table 5). These results earned with urea and GnHcl as the same of Triton x-100 and NP-40. But the extent of inhibition of PPO activity decreased after partially purification related to extract (table 6), So with sodium cholate, PPO activity was inhibited to a lower extent in the partial purified enzyme than in the extract (Figure 7, table 4). Activation of PPO activity increased 4 time and 1.1 time for catechol oxidation at 1 and 3 mM sarkosyl, respectively. These result showed that partial purified enzyme activation is more than extract at lower concentration of sarkosyl.

Table 1: Effect of ionic detergents on PPO of pyrus communis in soluble at pH 6.7 (A, Activatory and I, Inhibitory)

Ionic Detergents					
sarkosyl			Sodium Cholate		
concentration	Catechol oxidation	Pyrogallol oxidation	concentration	Catechol oxidation	Pyrogallol oxidation
0.5 mM	45 % A	64 % A	0.5 mM	65 % I	36 % I
1 mM	245 % A	324 % A	1 mM	89 % I	68 % I

Table 2: Effect of nonionic detergents on PPO of pyrus communis in soluble at pH 6.7 (A, Activatory and I, Inhibitory)

Non Ionic Detergents					
TX-100			NP-40		
concentration	Catechol oxidation	Pyrogallol oxidation	concentration	Catechol oxidation	Pyrogallol oxidation
1 uM	45% I	55% I	1uM	55% I	45% I
3 uM	65 % I	65 % I	3 uM	65 % I	55% I

Table 3: Effect of Chaotropic agents on PPO of pyrus communis in soluble at pH 6.7 (A, Activatory and I, Inhibitory)

Chaotropic Agents					
Urea			GnHcl		
concentration	Catechol oxidation	Pyrogallol oxidation	concentration	Catechol oxidation	Pyrogallol oxidation
10 mM	**35 % I**	**25 % I**	10 mM	**35 % I**	**10 % I**
100 mM	**68 % I**	**50 % I**	100 mM	**78 % I**	**45 % I**

Table 4: Effect of ionic detergents on partial purified PPO of pyrus communis at pH 6.7 (A, Activatory and I, Inhibitory)

Ionic Detergents					
sarkosyl			Sodium Cholate		
concentration	Catechol oxidation	Pyrogallol oxidation	concentration	Catechol oxidation	Pyrogallol oxidation
1 mM	180% A	14% A	1mM	30 % I	25 % I
3 mM	260% A	220 % A	2 mM	60 % I	55 % I

Table 5: Effect of non ionic detergents on partial purified PPO of pyrus communis at pH 6.7 (A, Activatory and I, Inhibitory)

Non Ionic Detergents					
TX-100			NP-40		
concentration	Catechol oxidation	Pyrogallol oxidation	concentration	Catechol oxidation	Pyrogallol oxidation
1 mM	**75% I**	**48% A**	1mM	**30% I**	**40 % I**
4 mM	**80% I**	**65 % A**	4 mM	**48 % I**	**70 % I**

Table 6: Effect of Chaotropic agents on partial purified PPO of pyrus communis at pH 6.7 (A, Activatory and I, Inhibitory)

Chaotropic Agents					
Urea			GnHcl		
concentration	Catechol oxidation	Pyrogallol oxidation	concentration	Catechol oxidation	Pyrogallol oxidation
10 mM	25 % I	15 % I	10 mM	35 % I	10 % I
150 mM	48 % I	40 % I	150 mM	78 % I	58 % I

Discussion

The present investigation was conducted on extracts obtained from pear. The extract prepared as described in the Materials and Methods consisted of the soluble fraction of the fruit homogenate and the PPO activity reported here refer to the soluble enzyme. Each assay was performed at least in triplicate and each experiment was repeated three times. The results were expressed as means of the values obtained and the standard deviations of the means were calculated and shown on the figures. PPO retains its intriguing property of being able to exist in a latent form which it can be released by a variety of treatments, such as exposure to activators that are reputed to inactive enzymes [20]. Sarkosyl would activate wild pear PPO activity for oxidation of catechol as a dihydroxy phenol and pyrogallol as a trihydroxy phenol and exhibited different sensitivities toward the various detergents and agents used. PPO activity for oxidation of catechol was sensitive to detergent/agent concentrations than the activity of PPO for pyrogallol oxidation , So the result is the same of results that earned for small cherry PPO activity against detergents and chaotropic agents (saeidian, 2007). Our results showed that among the detergents/agents tested in this study, only sarkosyl would activate PPO while other detergents inhibited the enzyme activity. Assuming that activation results from the binding of discrete detergent molecules to the enzyme in order to trigger some local conformational changes. The inhibition observed with sodium cholate may be due to steric hindrance upon binding of this bulkier detergent to the enzyme. Nonionic detergents were larger than sodium cholate and may cause inhibition upon binding to the enzyme by preventing access of the substrate to the active site because of steric hindrance [19]. The results for partial purified PPO showed that enhanced activation was observed with sarkosyl that may be due to the loss of some inhibitors present in the extract, alternatively, it may be due to conformational changes to the enzyme that may be particularly sensitive in this species of plants. The present study shows that PPO in *pyrus communis* are activated by sarkosyl but not by urea and other detergents. Interestingly, while no more than 50% and 68% inhibition was observed in the presence of up to 100 mM urea for oxidation of pyrogallol and catechol respectively. Similarly, GnHcl caused no more than 58 and 78% inhibition of PPO for oxidation of pyrogallol and catechol. Thus activity of soluble PPO of pyrus communis was sensitive to chaotropic agents and nonionic detergents for oxidation of catechol as the same as oxidation of pyrogallol. These finding indicated probably a difference in the conformational and in the microenvironment of the respective active site in the enzyme. Our results showed that partial purification caused PPO to be more activated because of probably changing in conformation of enzyme or converting latent form of PPO to active form.

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