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Effect of Sodium dodecyl sulphate on partial purified polyphenol oxidase activity in Red and Green tomatoes (*Solanum Lycopersicum)*

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ABSTRACT

In order to better understand how to prevent enzymatic greenning, it is important to understand kinetic properties of polyphenol oxidase. we investigated the effect of SDS on the rate of catechol oxidation by small cherry tomato partial purified PPO. PPO activity increased with increasing SDS concentration. The most effective concentration of SDS was 0.8, 1 and 1.25 mM in according to pH and type of substrate, where the measured activity was 0.074 and 0.247 units/mg.protein at pH 6.7 and 0.159 and 0.118 unit/mg.protein at pH 8 for catechol and pyrogallol, respectively. The activation of field small cherry PPO increased linearly with the SDS concentration up to 1 – 1.5 mM and decreased thereafter. The activity of small cherry PPO was also enhanced 1.7-fold by exposure to SDS at pH 8.0 in presence of catechol, 1.8-fold by exposure to SDS at pH 8.0 in presence of pyrogallol, 2.3-fold by exposure to SDS at pH 6.7 in presence of catechol and 2.6-fold by exposure to SDS at pH 6.7 in presence of pyrogallol. Therefore sodium dodesyl sulphate is an activator of polyphenol oxidase that can probably change latent form of enzyme to active form, so increases the activity of polyphenol oxidase.

Key word: Polyphenol Oxidase, Catechol, Pyrogallol, SDS, *Solanum Lycopersicum*

INTRODUCTION

Polyphenol oxidase (PPO; EC 1.14.18.1), a member of type III copper proteins, catalyses ortho-hydroxylation of monophenols (cresolase activity) and oxidation of ortho-diphenols to orthodi-quinones (catecholase activity) at the expense of molecular oxygen. The resulting highly reactive quinones auto polymerize to form bgreenn polyphenolic catechol melanins, a process thought to protect damage to plants from pathogens and insects (Kessler and Baldwin, 2002). The enzyme can be found not only in different fungal, mammalian, avian and insect species but also in a variety of plant species (Moore and Flurkey, 1990). In plants, PPO is located in the chloroplast thylakoid membranes and often exists in multiple forms. An unusual and intriguing characteristic of the enzyme is its ability to exist in either a latent and/or an active form (Mayer and Harel, 1979). PPO can be released from latency or activated by acid and base shock (Kenten, 1958), detergents (Jiang et al. 2003), urea (Swain et al. 1966) and proteases (Robinson and Dry, 1992). SDS as an activating agent is intriguing because very few enzymes are known to be activated by SDS in contrast with the many that are inactivated by it. Kenten (1958) has reported that the activation of crude broad bean leaf PPO by SDS occurred below 1 mM SDS. Extending these observations further,

Robb et al (Robb et al. 1964) observed that this activation process is reversible and that prolonged incubation in the presence of SDS leads to a loss of activity. Laveda et al (2001) demonstrated the total reversibility of the SDS activation of latent peach PPO by SDS entrapment with cyclodextrins. Some authors have suggested that PPO plays a role in plant resistance against diseases (Melo et al. 2006) and against insect herbivory (Felton et al. 1992). Li and Steffens (2002) obtained direct evidence of such a role for PPO in plants. The biochemical and kinetic properties of many PPOs from fruits and vegetables have been investigated because their associated bgreenning reactions affect food flavor and quality.

MATERIALS AND METHODS

Materials and Reagents

The green and red tomatoes used in this study were obtained from Kurdistan of Iran (Baneh) and frozen at -25 °C until used. Catechol, polyvinylpyrolidone (PVPP), pyrogallol, tyrosine were purchased from Merck (Darmstadt, Germany). Acetone, ammonium sulphate, L-cysteine, kojic acid, 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

200 grams of tomatoes 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 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.

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 Determination

Protein contents of the enzyme extracts were determined according to lowry method using bovine serum albumin as a standard (Lowry et al. 1984).

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 Small cherry tomato 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. 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 12 $M^{-1}cm^{-1}$ for catechol. Enzyme activity was calculated from the linear portion of the curve. One unit of PPO was defined as the amount of enzyme producing a change in absorbance of 0.001 min^{-1}. Results were average of three different experiments. Appropriate aliquots of 5 mM SDS prepared in 0.1 M phosphate buffer, pH 6.7 and pH 8, were added to the reaction mixture just before addition of the small cherry tomato extract. The final volume of the reaction mixture was always 3 ml. The pH activity curve was determined using a citrate phosphate-borate buffer system (range 2–10) at a concentration of 0.1 M.

SDS Activation of PPO

The SDS solutions were prepared in 0.1M sodium phosphate buffer (pH 6.7 and pH 8). The enzyme assay solution contained different concentrations of the buffered SDS solutions and substrates (catechol and pyrogallol) in sodium phosphate. The sample cuvette contained 3 ml of substrate (pyrogallol or catechol) in constant concentrations and in presence of different concentration of SDS (below the critical micelle concentration), prepared in the phosphate buffer.

RESULTS

PH activity profile of PPO activity in small cherry tomato extract

Polyphenol oxidase activity in small cherry tomato extract was assayed at various pHs ranging from 2 to 10, using either pyrogallol and catechol as substrate. Two pH optima were observed, respectively at 6.7 and 8 for both substrates No activity was detectable at pHs 2 and 10, regardless of the substrate (saeidian, 2013).

Effect of SDS on PPO activity at pH 6.7 in presence of catechol

Since PPO was found in many fruits and vegetables in an inactive or latent form (Fraignier et al. 1995) that can be activated by a variety of treatments, including exposure to detergent (Moore and Flurkey, 1990). we investigated the effect of SDS on the rate of catechol oxidation by small cherry tomato partial purified PPO. The results presented in Fig. 1 show that, PPO activity increased with increasing SDS concentration. The most effective concentration of SDS was 1.5 mM where the measured activity was 0.074 units/mg.protein. At SDS concentrations between 0 to 0.6 mM, the increase in activity is 16% of the maximum activation. However, a very sharp linear increase in PPO activity from 0.6 to 1.5 mM with 84% of the maximum activation at 0.6 mM SDS was observed. A further increase in the SDS concentration led to a near linear decrease in the PPO activity. Maximum activation was achieved in 10 min, further incubation with SDS led to a decrease in PPO activity (Figure 1).

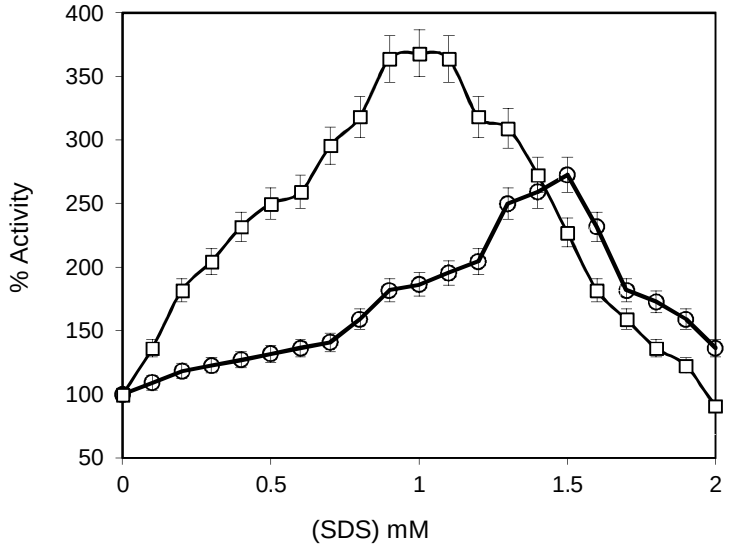

Fig. 1 Effect of increasing concentrations of SDS on the rate of pyrogallol (10 mM, ●) and catechol (35 mM, ○) oxidation by small cherry tomato extract, at pH 6.7.

The rate of catechol oxidation to its corresponding o-quinone was measured by monitoring the absorbance increase at 400 nm in a 3-ml reaction mixture containing 0.7 mg extract protein. Figure 2 shows the effect of catechol concentration on its oxidation rate. Unlike the hyperbolic plots shown in Figs. 3, 5 and 6, the plot in Fig. 2 was sigmoidal, exhibiting a ‘‘lag’’ phase. Thus for catechol concentrations up to 120 mM, the reaction rate did not exceed 0.009 unit/mg protein, but it increased promptly thereafter to reach a maximum rate of 0.063 unit/mg protein at 36 mM catechol. Km and catalytic efficiency could not be calculated, because of the sigmoid shape of the plot. Substrate inhibition was observed for catechol concentrations exceeding 35 mM; the reaction rate was reduced by 85% at 80 mM p-cresol. Furthermore, substrate inhibition was observed. When the effect of catechol concentration on its oxidation rate was investigated in the presence of 1 mM SDS, the plot obtained was hyperbolic and no longer sigmoidal (figure 2). The maximum rate observed under these conditions was of 0.144 Unit/mg.protein, with a Km of 18 mM. The catalytic efficiency calculated per milligram protein in the extract was of 0.008 units mg^{-1} prot mM^{-1} (Table 1). This represented a 52% increase in Vmax compared to the results obtained with catechol in the absence of SDS. Furthermore, substrate inhibition was considerably reduced: only 20% reduction in the reaction rate was observed at 80 mM catechol while in the absence of SDS, the reaction rate was reduced by 75% at 80 mM catechol.

Table 1: Catalytic efficiency for the PPO activity detected in small cherry tomato at pH 6.7 and pH 8 in presence of pyrogallol and catechol as substrates and SDS.

pH	Substrate	SDS	Catalytic efficiency (units mg^{-1} prot mM^{-1})
6.7	catechol	-SDS	-------
		+ SDS (1 mM)	0.008
	pyrogallol	-SDS	1.86
		+ SDS (0.8 mM)	4.9
8	catechol	-SDS	0.006
		+ SDS (0.8 mM)	0.01
	pyrogallol	-SDS	31.03
		+ SDS (1 mM)	55.86

Effect of SDS on PPO activity at pH 6.7 in presence of pyrogallol

The effect of increasing SDS concentration on the activity of small cherry PPO in presence of pyrogallol is represented in Figure 1. PPO activity increased with increasing SDS concentration. The most effective concentration of SDS was 1mM where the measured activity was 0.247 units/mg. protein. At SDS concentrations between 0 to 1mM, the increase in activity is 350%. However, a very sharp linear increase in PPO activity from 0 to 0.3 mM with 50% of the maximum activation at 0.3 mM SDS was observed. A further increase in the SDS concentration led to a near linear decrease in the PPO activity. Maximum activation was achieved in 10 min, further incubation with SDS led to a decrease in PPO activity (Figure not shown). When the effect of pyrogallol concentration on its oxidation rate was investigated in the absence of SDS at pH 6.7, the plot obtained was hyperbolic (Fig. 3).

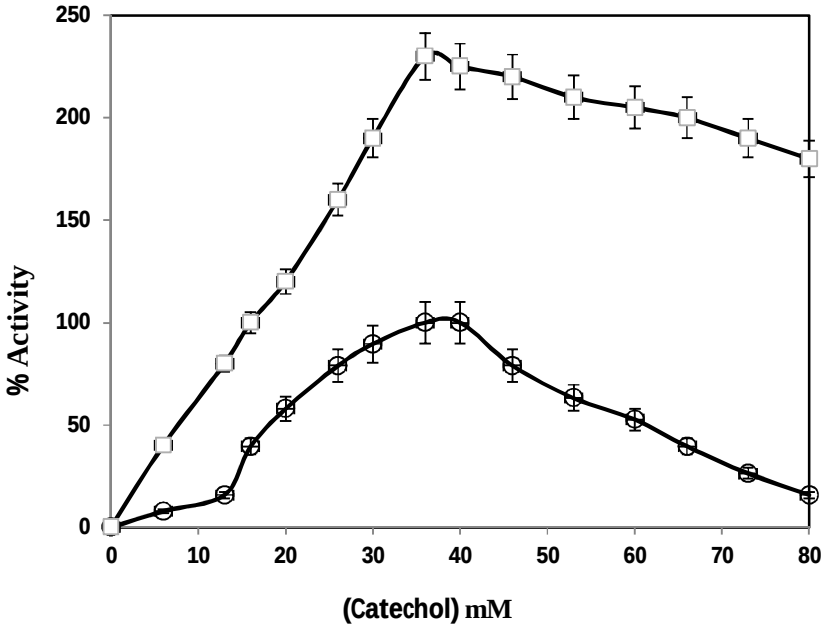

Fig. 2 Effect of catechol concentration on its oxidation rate in the presence of 1 mM SDS (□) and in the absence of SDS (○), at pH 6.7

The maximum rate observed under these conditions was of 11.173 Unit/mg.protein, with a Km of 6 mM. The catalytic efficiency calculated per milligram protein in the extract was of 1.86 units mg^{-1} prot mM^{-1} (Table 1). Furthermore, substrate inhibition was observed. When the effect of pyrogallol concentration on its oxidation rate was investigated in the presence of 0.8 mM SDS, the plot obtained was hyperbolic (Fig. 3). The maximum rate observed under these conditions was of 16.290 Unit/mg.protein, with a Km of 3.3 mM. The catalytic efficiency calculated per milligram protein in the extract was of 4.9 units mg^{-1} prot mM^{-1} (Table 1). This represented a 40% increase in Vmax compared to the results obtained with pyrogallol in the absence of SDS. Furthermore, substrate inhibition was considerably reduced: only 14% reduction in the reaction rate was observed at 100 mM pyrogallol while in the absence of SDS, the reaction rate was reduced by 45% at 100 mM pyrogallol.

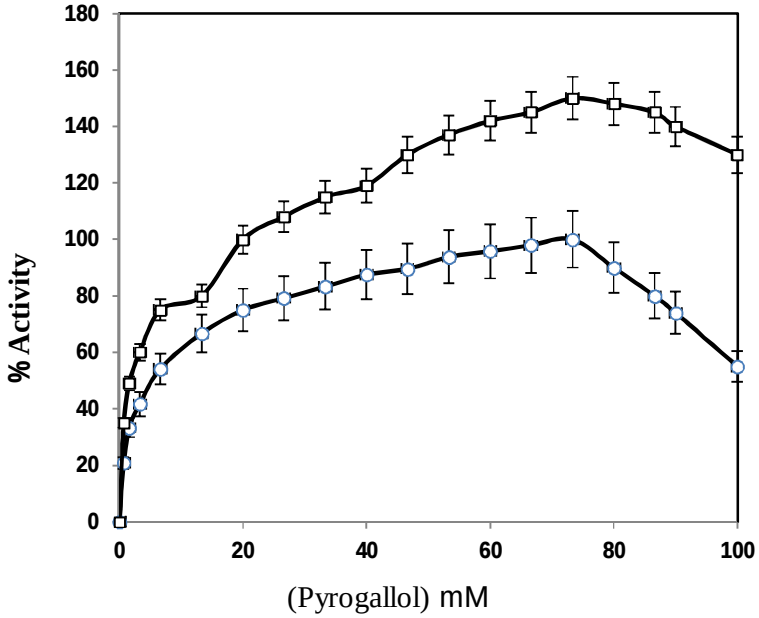

Fig. 3 Effect of pyrogallol concentration on its oxidation rate in the presence of 0.8 mM SDS (□) and in the absence of SDS (○), at pH 6.7

Effect of SDS on PPO activity at pH 8 in presence of catechol

The effect of increasing SDS concentration on the activity of small cherry PPO in presence of catechol is not represented. PPO activity increased with increasing SDS concentration. The most effective concentration of SDS was 1 mM where the measured activity was 0.159 Unit/mg.protein. A linear increase in PPO activity from 0.1 to 1 mM with maximum activation at 1 mM SDS was observed. A further increase in the SDS concentration led to a near linear decrease in the PPO activity. Maximum activation was achieved in 15 min, further incubation with SDS led to a decrease in PPO activity (Figure not shown). When the effect of catechol concentration on its oxidation rate was investigated in the absence of SDS at pH 8, the plot obtained was hyperbolic (Fig. 4). The maximum rate observed under these conditions was of 0.178 Unit/mg.protein, with a Km of 27 mM. The catalytic efficiency calculated per milligram protein in the extract was of 0.006 units mg^{-1} prot mM^{-1} (Table 1). Furthermore, substrate inhibition was observed. When the effect of catechol concentration on its oxidation rate was investigated in the presence of 0.8 mM SDS, the plot obtained was hyperbolic (Fig. 4). The maximum rate observed under these conditions was of 0.242 Unit/mg.protein, with a Km of 22 mM. The catalytic efficiency calculated per milligram protein in the extract was of 0.01 units mg^{-1} prot mM^{-1} (Table 1). This represented a 35% increase in Vmax compared to the results obtained with catechol in the absence of SDS. Furthermore, substrate inhibition was considerably reduced: only 34% reduction in the reaction rate was observed at 90 mM catechol while in the absence of SDS, the reaction rate was reduced by 72% at 90 mM catechol.

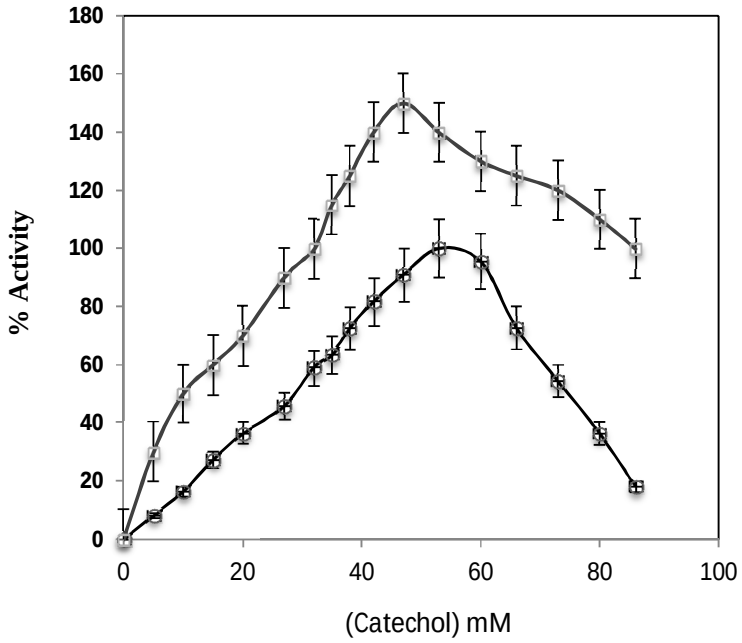

Fig. 4 Effect of catechol concentration on its oxidation rate in the presence of 0.8 mM SDS (□) and in the absence of SDS (○), at pH 8.

Effect of SDS on PPO activity at pH 8 in presence of pyrogallol

The effect of increasing SDS concentration on the activity of small cherry PPO in presence of pyrogallol is not represented. PPO activity increased with increasing SDS concentration. The most effective concentration of SDS was 1.5 mM where the measured activity was 0.118 Unit/mg.protein. At SDS concentrations between 0 to 0.7 mM, the increase in activity is 26% of the maximum activation. However, a sharp linear increase in PPO activity from 0.7 to 1.5 mM with 84% of the maximum activation at 1.5 mM SDS was observed. A further increase in the SDS concentration led to a near linear decrease in the PPO activity. Maximum activation was achieved in 15 min, further incubation with SDS led to a decrease in PPO activity. When the effect of pyrogallol concentration on its oxidation rate was investigated in the absence of SDS at pH 8, the plot obtained was hyperbolic (Fig. 5). The maximum rate observed under these conditions was of 46.55 Unit/mg.protein, with a Km of 1.5 mM. The catalytic efficiency calculated per milligram protein in the extract was of 31.03 units mg^{-1} prot mM^{-1} (Table 1). Furthermore, substrate inhibition was observed. When the effect of pyrogallol concentration on its oxidation rate was investigated in the presence of 1 mM SDS, the plot obtained was hyperbolic, too (Fig. 5). The maximum rate observed under these conditions was of 111.73 Unit/mg.protein, with a Km of 2 mM. The catalytic efficiency calculated per milligram protein in the extract was of 55.86 units mg^{-1} prot mM^{-1} (Table 1). This represented a 60% increase in Vmax compared to the results obtained with pyrogallol in the absence of SDS. Furthermore, substrate inhibition was considerably reduced: only 37% reduction in the reaction rate was observed at 30 mM catechol while in the absence of SDS, the reaction rate was reduced by 80% at 30 mM catechol.

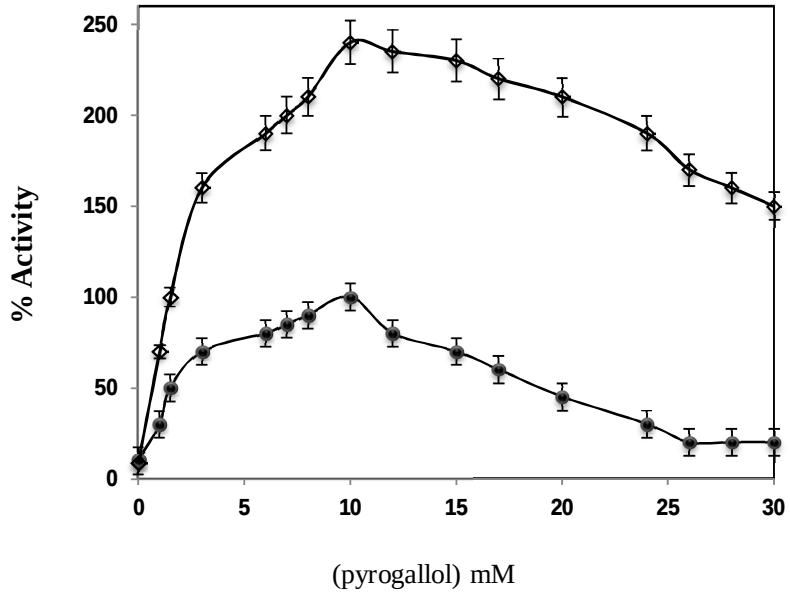

Fig. 5 Effect of pyrogallol concentration on its oxidation rate in the presence of 1 mM SDS (□) and in the absence of SDS (●), at pH 8.

DISCUSSION

In order to better understand how to prevent enzymatic bgreenning, it is important to understand kinetic properties of polyphenol oxidase. Increases in fruit and vegetable markets projected for the future will not occur if enzymatic bgreenning is not understood and controlled. So our results showed that SDS has activating effect on polyphenol oxidase in presence of both dihydroxy and trihydroxy phenols, So that, this activating effect at pH 6.7 is more than activating effect at pH 8, same to that reported for saffron PPO (Saeidian et al. 2007). The reason for increase of activity of PPO is that probably polyphenol oxidase exists in latent form, therefore in presence of SDS, binds to the protein chain with its hydrocarbon tail, exposing normally buried regions of latent form of polyphenol oxidase and changes its conformation to active forms in low concentrations of SDS. But with more increase in concentration of SDS, It would be a denaturant for PPO, So the detergent wraps around the polypeptide backbone and coating the protein chain with surfactant molecules. Therefore in high concentration of SDS, PPO activity gradually decreased and in more concentration, PPO activity probably will reach to zero because of denaturing of enzyme. Activation of small cherry PPO occurred in the presence of SDS (Figure 1 and 2). The concentration of SDS required for maximal activation is substrate and pH dependent so, is 0.8 mM for catechol in pH 8, 1 mM for catechol at pH 6.7, 0.8 mM for pyrogallol at pH 6.7 and 1 mM for pyrogallol at pH 8, in contrast to that reported for plant PPOs. The SDS concentrations required for maximum PPO activity are below the determined CMC of SDS. The activation of field small cherry PPO increased linearly with the SDS concentration up to 1 – 1.5 mM and decreased thereafter (Figure 1 and 2). Moore and Flurkey (1990) observed that broad bean leaf PPO was activated by SDS in a sigmoidal manner below the CMC and attributed it to a co-operative interaction between SDS binding and activation. The activity of small cherry PPO was also enhanced 1.7-fold by exposure to SDS at pH 8.0 in presence of catechol, 1.8-fold by exposure to SDS at pH 8.0 in presence of pyrogallol, 2.3-fold by exposure to SDS at pH 6.7 in presence of catechol and 2.6-fold by exposure to SDS at pH 6.7 in presence of pyrogallol. As reported for other plants, multiple isoforms of PPO were probably detected in small cherry tomato.

CONCLUSION

Therefore sodium dodesyl sulphate is an activator of polyphenol oxidase that can probably change latent form of enzyme to active form, so increases the activity of polyphenol oxidase. The pH activity profiles and SDS activation profiles suggested the presence of more than one isoforms of PPO at pH 6.7 and 8, suggesting them to be at least two PPO isoenzymes that we designated PPOI and PPOII. At pH 6.7, PPOI was much less active with catechol as substrate (0.063 units mg^{-1} prot) than with pyrogallol (11.173 units mg^{-1} prot). At pH 8, PPOII was also much less active with catechol as substrate (0.178 units mg^{-1} prot) than with pyrogallol (46.55 units mg^{-1} prot).

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