Demonstration and Modification of the pH Profile of Polyphenol Oxidase in Solanum Lycopersicum

Shahryar Saeidian

***Abstract*—Polyphenol oxidase (EC.1.14.18.1) catalyzes the hydroxylation and oxidation of phenols to o-quinones leading to browning in plants. For polyphenol oxidase activity two pH optimum were observed, respectively at 6.7 and 8, that probably belong to at least two isoenzyme, so we named them ISOIPPO and ISOIIPPO respectively. These isoenzymes have different kinetics properties. Activation of PPO by SDS occurred below 1.3 mM SDS. ISOIPPO was much more active (86 times) with pyrogallol as substrate (4.9 unit/mg protein) in comparison of catechol (0.057 unit/mg protein) in presence of SDS. This comparison is 143 times in absence of SDS (3.3 and 0.023 unit/mg protein for pyrogallol and catechol, respectively). SDS activation probably alters both its enzymatic and physical characteristics. ISOIIPPO activity gradually increased during development of tomatoes from green to ripe, but ISOIPPO activity is nearly constant during development. PPO are believed to play physiological roles both in preventing insects and microorganisms from attacking plant and as part of the wound response of plant by its phenolic content, so these roles are more important for ISOIIPPO than ISOIPPO during changes from green to red tomatoes.**

***Index Terms*—Solanum lycopersicum, pH, catechol, pyrogallol.**

I. Introduction

Browning reactions in fruits and vegetables are recognized as a serious problem in the food industry. In fruits, the principal enzyme responsible for the browning reactions is polyphenol oxidase (PPO). PPO (EC 1-14-18-1) is a copper containing enzyme which uses molecular oxygen to catalyze the o-hydroxylation of monophenols to o-diphenols and their further oxidation to colored and highly reactive o-quinones. These o-quinones readily polymerize and/or react with endogenous amino acids and proteins to form complex brown pigments. Because such browning decreases their marketability, the enzyme has been extensively studied [1]-[4] Enzymatic browning is the main function of PPOs in fruits and vegetables, and it often is undesirable and responsible for unpleasant sensory qualities and losses in nutrient quality [5]. 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 [6]. PPO can be released from latency, or activated by a variety of treatments or agents including acid and base shock, urea [7], polyamines [8], anionic detergents such as SDS [9], proteases [10] and fatty acids [11].

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II. Materials and Methods

A. Chemicals

PPO substrates and SDS were obtained from Sigma. All other chemicals were of analytical grade.

B. Extract Preparation Sub Cellular Fractionation

Small cherry tomatoes were gathered from Kurdistan (Baneh) in Iran and used throughout these studies. Extracts were prepared from tomatoes weighting each between 5 and 12 g by homogenization in phosphate buffer 0.1 M, pH 7 in presence of PMSF 2 %. After centrifugation at 3,000 xg for 10 min, then at 35,000 xg for 30 min, a clear, transparent supernatant termed "crude extract" was obtained and used for our studies.

C. Protein Determination

Protein concentration of the various extracts and solutions was determined by the dye-binding method of lowery [12] using bovine serum albumin as standard.

D. Effect of pH

Polyphenol oxidase activities were determined spectrophotometrically at 25℃ by measuring the appearance of reaction products in the medium. The activity of the enzyme was determined in the pH range 3-10 by using a citrate-phosphate-borate buffer 0.1 M. The optimum pH for the PPO activity of extracts was obtained in presence of pyrogallol (4 mM) and catechol (40 mM) as substrates. 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 or catechol) in constant concentrations. Assays were carried out by addition of 200 µl of extracts to the sample cuvette, and changes in absorbance (420 nm 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 o-quinone produced, using an extinction coefficient of 12 $M^{-1}cm^{-1}$ for pyrogallol and using an extinction coefficient of 3450 $M^{-1}cm^{-1}$ 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 o-dopaquinone per minute. Assays were carried out at room temperature and results are the averages of at least three assays and the mean

and standard deviations were plotted.

E. SDS Activation of PPO

PPO was pre-incubated at various concentrations of SDS prepared in phosphate buffer 0.1 M, pH 8 for 5 min, following which PPO was assayed at pH 8 as described above. The enzyme assay solution contained 3 ml of the constant concentration of catechol in sodium phosphate buffer. Relative activity was plotted against SDS concentration. Blanks were performed by omitting extract from samples in presence and absence of SDS, and rates were negligible. Additionally, darkening of substrate solution (catechol or pyrogallol) was tested without enzyme, both in presence and absence of detergents and was also negligible during the time of the assay. Activities were calculated from the increase in absorbance during the linear phase of the reaction. Extractions and assays were performed at least three times for each SDS concentration and means and standard deviations were calculated. Other conditions are detailed in the text and the captions of Fig.1.

F. Effect of SDS on pH Profile

The effect of pH on the optimal activity of SDS-activated PPO was evaluated using phosphate buffer 0.1 M and pH 6.7 and 8 seperately in extracts of green and ripe tomatoes. The optimum pH for the PPO activity of extracts was obtained in presence of pyrogallol (4 mM) and catechol (40 mM) as substrates, seperately. The enzyme assay solution contained 3 ml of the constant concentration of catechol or pyrogallol in addition to SDS at constant concentration (1 mM) in sodium phosphate buffer. Relative activity was plotted against pH concentration.

III. Results

A. pH Profile of PPO Activity in Solanum Lycopersicum

pH is a determining factor in the expression of enzymatic activity; it alters the ionization states of amino acid side chains or the ionization of the substrate. Polyphenol oxidase activity in solanum lycopersicum extract was assayed at various pHs ranging from 2 to 10, using catechol and pyrogallol as substrates for green and ripe small cherry tomatoes. Depending on the condition and substrate, either one or two pH optimum was observed, respectively at 6.7 and 8. The optima at 6.7 and 8 were found in green extract (Fig. 2 (a)) and optimum at 8 was found in ripe condition (Fig. 3 (a)). No activity was detectable at pHs 2 and 10, regardless of the condition. Fig. 2 (a) shows the pH activity profile obtained for catechol at green condition and Fig. 3 (a) shows the pH activity profile obtained for catechol at ripe condition; one peak was identifiable at 8. The pH profile obtained with pyrogallol as substrate in green condition was similar to that obtained with catechol at green condition (Fig. 4 (a)) and the pH profile obtained with pyrogallol as substrate in row condition was similar to that obtained with catechol at row condition (Fig. 5 (a)). Results of pH profiles of PPO in green and ripe solanum lycopersicum led to two peaks at 6.7 and 8 that probably belong to at least two isoenzymes, so we named them ISOIPPO for optimum pH at 6.7 and ISOIIPPO for optimum pH at 8.

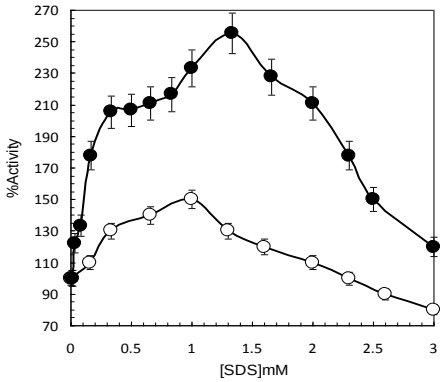

Fig. 1. Effect of different concentration of SDS on polyphenol oxidase activity (ISOIPPO) of green small cherry tomatoes in presence of (a) catechol 40 mM (filled circles) and (b) pyrogallol 4 mM (open circles) as substrate in temperature room.

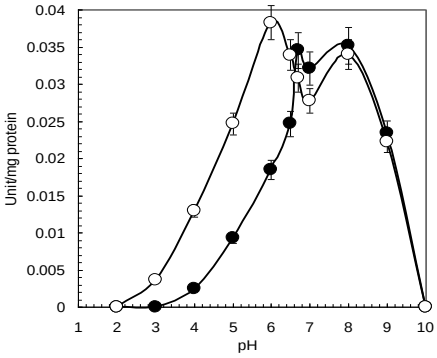

Fig. 2. pH profile of polyphenol oxidase activity of green small cherry tomato in (a) absence (filled circles) and (b) presence (open circles) of SDS (1mM). Polyphenol oxidase activity was determined in 0.1 M citrate-phosphate-borate buffer system in the presence of catechol 40mM.

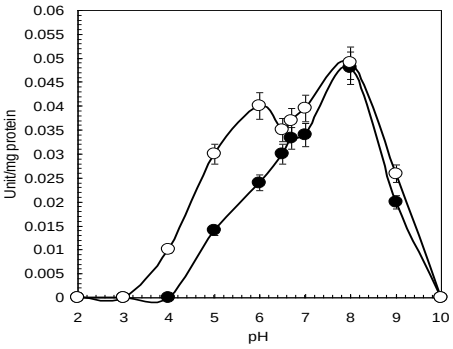

Fig. 3. pH profile of polyphenol oxidase activity of ripe small cherry tomato in (a) absence (filled circles) and (b) presence (open circles) of SDS (1mM). Polyphenol oxidase activity was determined in 0.1 M citrate-phosphate-borate buffer system in the presence of catechol 40mM.

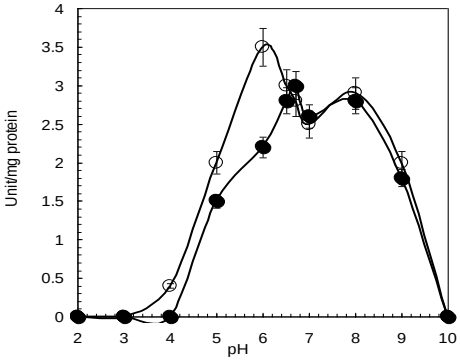

Fig. 4. pH profile of polyphenol oxidase activity of ripe small cherry tomato in (a) absence (filled circles) and (b) presence (open circles) of SDS (1mM).

Polyphenol oxidase activity was determined in 0.1 M citrate-phosphate-borate buffer system in the presence of pyrogallol 4mM.

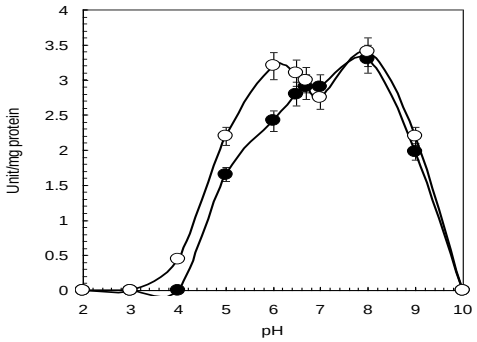

Fig. 5. pH profile of polyphenol oxidase activity of green small cherry tomato in (a) absence (filled circles) and (b) presence (open circles) of SDS (1mM). Polyphenol oxidase activity was determined in 0.1 M citrate-phosphate-borate buffer system in the presence of pyrogallol 4mM.

B. Effect of SDS on ISOIPPO Activity in Ripe Solanum Lycopersicum

The effect of SDS concentration on the enzymatic activity at a saturating concentration for each substrate was studied. The soluble ISOIPPO activity showed hyperbolic behavior with the hydrophobic substrates, catechol and pyrogallol. Since PPO was found in many fruits and vegetables in an inactive or latent form that can be activated by a variety of treatments, including exposure to detergent [13], we investigated the effect of SDS on the rate of catechol and pyrogallol oxidation by small cherry tomatoes extract. Furthermore, the maximum activation for the both substrates was obtained at the same SDS concentrations (1 mM to 1.3 mM). The results presented in Fig. 1 (a) for green condition and pH 6.7 (ISOIPPO) shows that in concentration ranging 0 to 1.3 mM; SDS would progressively increases the oxidation rate of 40 mM catechol up to 2.55 times. In presence of higher SDS concentrations, catechol oxidation rate decreased. The most effective concentration of SDS was 1.3 mM where the measured activity was 0.057 unit/mg protein. A very sharp linear increase in PPO activity was observed in range of 0 to 1.3 mM of SDS. A further increase in the SDS concentration leads to a linear decrease in the PPO activity. The results presented in Fig. 2 (b) for green condition and pH 6.7 (ISOIPPO) shows that in concentration ranging 0 mM to 1 mM; SDS would progressively increase the oxidation rate of 4mM pyrogallol up to 1.5 times. In presence of higher SDS concentrations, pyrogallol oxidation rate decreased. The most effective concentration of SDS was 1 mM where the measured activity was 4.9 unit/mg proteins. A sharp linear increase in PPO activity was observed in range of 0 mM to 1 mM of SDS. A further increase in the SDS concentration leads to a linear decrease in the PPO activity. In order to investigate the effect of SDS on pH profile of ISOIPPO and ISOIIPPO, we used 1 mM of SDS throught the experiments.

C. Effect of SDS on pH Activity Profile of PPO Activity in Solanum Lycopersicum

A pH variation in the absence and presence of SDS was carried out on the soluble PPO for all the selected substrates. Polyphenol oxidase activity in solanum lycopersicum extracts in both condition of green and ripe small cherry tomatoes were assayed at various pH ranging using either catechol and pyrogallol as substrate and in presence of SDS 1 mM as an activator of PPO. At this detergent concentration the enzyme was more active than absence of SDS. The concentration Of SDS is below the cmc (critical micelle concentration) for the detergent at the experimental conditions [13], [14]. In every condition, two pH optima were observed, respectively at 6 and 8. The optima at 6 and 8 were found for all substrates. No activity was detectable at pH 2 and 10 regardless of the substrate. Fig. 3 (b) and Fig. 4 (b) shows the pH activity profile obtained for catechol and pyrogallol respectively, in ripe extracts, so two peaks were identifiable at 6 and 8 in presence of SDS. In presence of SDS and ripe condition, the enzyme exhibited two pH optimums in 6 and 8, but in absence of SDS at least one pH optima were observed at 8. A minor peak was observed at approximately 6.7, so in presence of SDS, activity of enzyme increased at pH 6 in the reaction medium. This activation by SDS at various pHs is not dependent on the substrate, because the pH profile obtained with pyrogallol as substrates was similar to that obtained with catechol in both condition of green and ripe small cherry tomatoes extracts. Fig. 2 (b) and Fig. 5 (b) shows the pH activity profile obtained for catechol and pyrogallol respectively, in row (green) extracts, so two peaks were identifiable at 6 and 8 as optima pH in presence of SDS. Two pH optima were observed in absence of SDS in contrast to ripe condition. The increase in PPO activity of small cherry tomatoes in green and ripe conditions is associated with a shift in the pH optimum from 6.7 to 6 (Fig. 2-Fig. 5). The activity of ISOIPPO at pH 6 is 25% more than native form at ripe condition in presence of catechol and 40 % more than native form in presence of pyrogallol. The activity of ISOIPPO at pH 6 is 52% more than native form at green condition in presence of catechol and 37 % more than native form in presence of pyrogallol. The activity of ISOIIPPO at pH 8 is constant in presence and absence of SDS and is not dependent on the substrate, so SDS showed no effect on ISOIIPPO activity. The observed shift in the pH optima from 6.7 to 6 for ISOIPPO could be probably related to the displacement of sensitive pKa values at the catalytic site, so make a detergent-induced pH profile acidic shift. Susana and et al at 2007 reported that anionic detergents activated PPO at pH below critical micelle concentration (cmc) that this behaviour is due to a detergent-induced pH profile alkaline shift [15].

IV. Discussion

pH profile of solanum lycopersicum PPO led to two peaks at 6.7 and 8 that probably belong to at least two isoenzyme that exhibited differential substrate utilization and SDS activation profile, suggesting them to be two PPO isoenzymes that we designated ISOIPPO and ISOIIPPO. SDS stimulated ISOIPPO and ISOIIPPO activity with catechol and pyrogallol as substrate. ISOIIPPO was more active with pyrogallol and catechol in ripe tomatoe extract than green condition, but ISOIIPPO was less active with pyrogallol and catechol in green tomatoes extract than ripe condition. This comparison showed that ISOIIPPO activity gradually increased during development of the small cherry tomatoes from green to ripe but ISOIPPO activity is nearly

constant during development. This is maybe for these reason that PPO are believed to play physiological roles both in preventing insects and microorganisms from attacking plant and as part of the wound response of plant by its phenolic content, so these roles are more important for ISOIIPPO than ISOIPPO during changes from green to red tomatoes. ISOIPPO was much more active (86 times) with pyrogallol as substrate (4.9 unit/mg protein) in comparison of catechol (0.057 unit/mg protein) in presence of SDS. This comparison is 143 times in absence of SDS (3.3 and 0.023 unit/mg protein for pyrogallol and catechol, respectively). Results show that kinetics properties of two isoenzymes are different and it depends on condition of development and substrate too. Activation of crude small cherry tomato PPO by SDS occurred below 1.3 mM SDS that reported by kenten for broad bean PPO below 1mM SDS. SDS activation probably alter both its enzymatic and physical characteristics, suggesting a limited conformational change, due to binding of small amounts of SDS, which could induce or initiate the activation of latent PPO [13] However, convincing structural and experimental details of the conformational changes at the molecular level are yet to be elucidated. These studies have demonstrated that the activation by SDS accompanied probably by localized secondary and tertiary structural changes, leading to an elevated *k* cat and a marked shift in the pH optimum from 6.7 to 6.0. Further, these conformational changes are similar for SDS-activated PPOs, suggesting a common feature in the activation phenomena [16], [17]. In according to our results, we predict that the active site of the enzyme probably is not affected by SDS and that a stepwise conformational change favors the access of hydrophobic substrate such catechol for PPO in green condition and favor the access of pyrogallol for ISOIIPPO. Therefore, as can be observed in Figs and results, for the PPO, the behavior of the enzyme activation and the SDS concentration at which maximum activity was reached was different for each substrate and for each isoenzyme. This property confirmed the different nature of ISOIPPO and ISOIIPPO and existence of two Isoenzymes. Differential activation of PPO mediated by SDS may be of relevance in the control of PPO activity since each isoenzyme is able to express activity toward a specific substrate better related to another isoenzyme while remaining latent to others. These results strongly suggest that the conformational change needed for the enzyme to express its maximum activity toward a substrate is dependent on the substrate nature, with the binding of more SDS molecules (and higher SDS concentrations) for the more hydrophilic substrates being necessary.

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