Optimum Temperature and Thermal Stability of Crude Polyphenol Oxidase In Green Small Cherry Tomatoe (Solanum Lycopersicum)

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ABSTRACT: Polyphone oxidase (PPO) (EC.1.14.18.1) catalyzes the hydroxylation of monophenols to o-diphenols and the oxidization of o-diphenols to o-quinones leading to browning in plants. Polyphenol oxidase activity was detected in green small cherry tomato (Solanum Lycopersicum) cultivated in Kurdistan (Iran). In this study, the effect of temperature on activity in presence of catechol and pyrogallol was investigated. For polyphenol oxidase activity in green small cherry tomatoes extract two pH optimum were observed, respectively at 6.7 and 8, that probably belong to at least two isoenzyme, so we named them ISOIPPO for optimum pH at 6.7 and ISOIIPPO for optimum pH at 8. Activity of isoenzymes of PPO in presence of pyrogallol was optimum after incubation at 55°C. Maximum activity of ISOIPPO is 240% and for ISOIIPPO is 157%. Activity increased to 150%, 180%, 200% and 110% after 70 minute incubation at 27, 40, 55 and 70°C for ISOIPPO. This increase in activity for ISOIIPPO is 135%, 157%, 185% and 100% after 70 minute incubation at 27, 40, 55 and 70°C. Activity was constant even after 80 minute incubation at 70°C. Incubation at high temperature (90°C) was accompanied with decrease of activity to 50% and 28% for ISOIPPO and ISOIIPPO, respectively.

Keyword: Pyrogallol, Polyphenol oxidase, Solanum lycopersicum, Temperature

INTRODUCTION

Many vegetables and fruits become discolored during storage or processing, an action mediated by the enzyme polyphenol oxidase (PPO) (Broothaerts et al., 2000). PPO (tyrosinase, EC 1-14-18-1) is a copper containing enzyme, widespread in plants that is synthesized early in tissue development and stored in chloroplasts (Van Gelder et al., 1997). The enzyme is a copper protein widely distributed in multitude of organisms from bacteria to mammals (Robb, 1984). Enzymatic browning is the main function of PPOs in fruits and vegetables, and it is often undesirable and responsible for unpleasant sensory qualities and losses in nutrient quality (Sanchez-Amat and Solano, 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 (diphenolase activity) (Espin et al., 1998). These highly reactive quinones polymerize with other quinones, aminoacids and proteins to produce colored compounds, and nutrient quality and attractiveness is reduced (Matheis and Whitaker, 1984). 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. Our objective was to characterize PPO activity with temperature from greensmall cherry tomato cultivated in Kurdistan of Iran.

MATERIALS AND METHODS

For extract preparation, green small cherry tomato was 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 g for 10 min, then at 35,000 g for 30 min, a clear, transparent supernatant termed “crude extract” was obtained and used for our studies. Protein concentration was determined by the Lowry method (Lowry et al, 1951).
Effect of pH

pH profile of polyphenol oxidase activities was determined spectrophotometrically at 25 °C by measuring the appearance of reaction products in the medium. The activity of the enzyme in greensmalacherry tomatoes was determined in the pH range 3-10 by using a citrate-phosphate-borate buffer 0.1 M. The optimum pH for PPO activity of extracts was obtained in presence of pyrogallol (4mM) and catechol (40mM) 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). Assays were carried out by addition of 200 µl of extracts to the sample cuvette, and changes in absorbance 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.

Effect of Temperature on ISOIPPO and ISOIIPPO activity in green small cherry tomatoes

PH profile of green solanum lycopersicum PPO led to two peak at 6.7 and 8 that probably belong to at least two isoenzyme, so we named them ISOIPPO for optimum pH at 6.7 and ISOIIPPO for optimum pH at 8. PPO activity of isoenzymes was assayed at two pH optimum of 6.7 and 8. For determining the optimum temperature values of the enzyme, ISOIPPO and ISOIIPPO activity were measured separately, at constant temperatures (27, 45, 55 and 70°C separately) using catechol (40mM) and pyrogallol (4mM). The effect of temperature on the activity of ISOIPPO and ISOIIPPO was tested by heating the crude extract to the appropriate temperatures in different times. The desired temperatures were provided by using a Memmert model ST/70 temperature controller attached. After different times (0 -100 minute) at a same temperature, enzyme cooled in ice and was added and the reaction was followed spectrophotometrically at given time intervals as described above.

Heat Inactivation of PPO

The thermal denaturation of the isoenzymes was studied at different temperature (30 - 90 °C) and constant concentration of pyrogallol 4 mM and catechol 40 mM. 200 µl of crude extracts solution in a test tube was incubated at the required temperature for fixed time intervals. At the end of the required time interval, the test tube was cooled in an ice bath. The activity of the ISOIPPO and ISOIIPPO were then determined.

RESULTS AND DISCUSSION

pH profile of PPO activity in greensolanum lycopersicum

pH is a determining factor in the expression of enzymatic activity; it alters the ionization states of amino acid side chains or substrate. For polyphenol oxidase activity, two pH optimum were observed, respectively at 6.7 and 8. No activity was detectable at pHs 2 and 10, regardless of the condition. Figure 1 shows the pH activity profile obtained for catechol. The results for pyrogallol was similar to catechol (not shown).

Effect of temperature on ISOIPPO activity

Time courses at 27 ºC showed an increase of ISOIPPO activity so, after 80 minute incubation, activity reached to 153% (Fig.2). Time course at 45, 55 and 70 ºC has the similar condition, so increase in time of incubation accompanied with more increase in activation. After 80 minute incubation of ISOIPPO in 45, 55 and 70 ºC, activity reached to 180, 200 and 116% respectively. We can conclude that increase in temperature from 27 to 45 and then to 55 ºC change probably structure of ISOIPPO to ordered shape, so that activity of enzyme in 45 and 55 ºC increased and in more temperature(70ºC) enzyme slowly denature and activity decreased related to 27, 45 and 55 ºC. Activity of ISOIPPO after 80 minute incubation at 55 ºC is 1.3, 1.1 and 1.7 times more than 27, 45 and 70 ºC.

Effect of temperature on ISOIIPPO activity

Time courses at 27 ºC showed a rapid increase of ISOIIPPO activity so after 80 minute incubation, activity reached to 140% related to 5 minute incubation (Fig.3). During this time course from 5 minute to 80 minute incubation, activity gradually increased, so whatever we increased time of incubation, activity of ISOIIPPO became more increased. Time course at 45, 55 and 70 ºC has the similar condition, so increase in time of incubation accompanied with more increase in activation. After 80 minute incubation of ISOIIPPO in 45, 55 and 70 ºC, activity reached to 160, 185 and 101 % respectively. We can conclude that increase in temperature from 27 to 45 and then to 55 ºC change probably structure of ISOIIPPO to ordered shape, so that activity of enzyme in 45 and 55 ºC increased and more temperature(70ºC) accompany with denaturation of enzyme and activity decreased related to 27, 45 and 70 ºC.
55 °C. Activity of ISOIPPO after 80 minute incubation at 55 °C is 1.1, 1.2 and 1.8 times more than 27, 45 and 70 °C. Activity of ISOIPPO after 80 minute incubation at 45 °C is 1.1 and 1.8 time more than 27 and 70 °C.

**Optimum Temperature and Thermal Inactivation**

PPO in presence of pyrogallol showed fluctuations in activity with increasing temperature even as high as 90 °C. The plot for pyrogallol demonstrated that the enzyme was very thermostable between 25 and 75 °C. It is reported that optimum temperature values are 40 °C for Chinese cabbage (Nagai and Suzuki, 2001) using catechol as substrate and 15 °C for Dog rose (Sakiroglu et al, 1996) using pyrogallol as substrate and 35 °C for saffron polyphenol oxidase using catechol as substrate (saeidian et al, 2007). Our results showed optimum temperature is 55 °C for both ISOIPPO and ISOIIPPO. Like most chemical reactions, with increase of temperature from 27 °C, gradually, activity of ISOIPPO increased so; we reached to maximum of activity at 55 °C (240%) (Fig.4). With more increase in temperature from 55 °C to 90 °C, activity of ISOIPPO decreased gradually and reached to 46 % at 90 °C. The drop in percentage residual activity at high temperatures can actually be due to the unfolding of the tertiary structure of the enzyme to form the secondary structure. Activity of ISOIPPO even in 90 °C was observed and is 50% related to control (27 °C). These results showed that ISOIPPO bear high temperatures. A ten degree Centigrade rise from 45 to 55°C in temperature after 5 minute incubation will increase the activity of ISOIPPO from 173 to 240%. Variations in reaction temperature as small as 5 degrees from 50 to 55 °C introduce increase of 40% in the activity and from 70 to 75 °C introduce decrease of 46% in the activity. Rate of ISOIIPPO increases as the temperature is raised. With increase of temperature from 27 °C, gradually, activity of enzyme increased. We reached to maximum of activity at 55 °C (157%). With more increase in temperature from 55 °C to 90 °C, activity of ISOIIPPO decreased gradually and reached to 28 % at 90 °C. Activity of ISOIIPPO even in 90 °C is 28% of control. These results showed that ISOIIPPO bear high temperatures. A ten degree Centigrade rise from 45 to 55°C in temperature after 5 minute incubation will increase the activity of ISOIIPPO from 128 to 157%. Variations in reaction temperature as small as 5 degrees from 50 to 55 °C introduce increase of 15% in the activity and from 70 to 75 °C introduce decrease of 57% in the activity. The thermal stability profile for ISOIIPPO, presented in the form of the residual percentage activity, is shown in figure 5.

**CONCLUSION**

For ISOIPPO and ISOIIPPO, this is complicated by the fact that activity of enzymes is adversely affected by high temperatures. As shown in figure 4 and 5, the reaction rate increases with temperature to a maximum level, then abruptly declines with further increase of temperature. Because this enzyme rapidly become denatured at temperatures above 55°C in contrast to most of enzymes that denatured at temperatures above 40 °C. ISOIPPO is very resistant to high temperature related to ISOIIPPO, so that ISOIPPO remain 50% of its activity at 90 °C, but for ISOIIPPO is 28%. Maximum activity of ISOIPPO is more than ISOIIPPO so, ISOIPPO is 240% but for ISOIIPPO is 157%. These results showed that potential of activation for ISOIIPPO is very more than ISOIPPO. The observed increase in activity of small cherry PPO by heating could, in part, be due to a releasing of latent PPO. Vamos-Vigyazo, L. (1981) reported the presence of latent PPO in apple peel extracts. Lee et al. (1991) indicated that heating at 60 °C activated latent PPO in cocoa bean, but no activation was observed at higher temperatures. This is unusual characteristic of this enzyme that exist in an inactive or latent state (Gandia-Herrero et al, 2004). PPO can be released from latency, or activated by a variety of treatments or agents including polyamines (Jimenezatienzar et al, 1991), anionic detergents such as SDS (Santosh et al, 2006; saeidian et al, 2007), proteases (Laveda et al, 2001) and fatty acids (Golbeck and Cammarata, 1981). It has been noted that heat stability of the enzyme may be related to ripeness of the fruit and molecular forms of the enzyme, and in some cases it is also dependent on pH (Zhou and Feng, 1991). Rapid decrease in activity in high temperature after optimum temperature might be due to involvement of disulfide bond in the active site or in three dimensional conformation of the enzyme. There are several other reports that describe high temperature liability of PPO from other sources to the same temperature range. The drop in percentage residual activity at high temperatures can actually be due to the unfolding of the tertiary structure of the enzyme to form the secondary structure (Lowrenco et al, 1990).

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Figures

Figure 1. The pH dependence of polyphenol oxidase activity of green small cherry tomato (*Solanum Lycopersicum*). Activity was determined in 0.1 M citrate-phosphate-borate buffer system in the presence of 40 mM catechol.

Figure 2. Effect of time of incubation on activity of ISOIPPO in extract of small cherry tomato (*Solanum Lycopersicum*) in presence of pyrogallol (4 mM) [45 °C (●) and 55 °C (○), 27 °C (◇), 70 °C (▲)].

Figure 3. Effect of time of incubation on activity of ISOIPPO in extract of small cherry tomato (*Solanum Lycopersicum*) in presence of pyrogallol (4 mM) [55 °C (●) and 45 °C (○), 27 °C (◇), 70 °C (▲)].
Figure 4. Effect of temperature on activity of ISOIPPO in extract of green small cherry tomato \((\text{Solanum Lycopersicum})\) in presence of catechol 40 mM.

Figure 5. Effect of temperature on activity of ISOIPPO in extract of green small cherry tomato \((\text{Solanum Lycopersicum})\) in presence of catechol 40 mM.

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