World Journal of Agricultural Sciences 7 (4): 444-449, 2011
ISSN 1817-3047

Partial Properties of Polyphenol Oxidase in Sour Cherry (*Prunus cerasus* L. CV. CAB) Pulp

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Abstract: The activity of polyphenol oxidase (PPO) in sour cherry fruit are determined by spectrophotometer. The effects of optimum pH, substrate concentration, temperature, substrates and inhibitors on PPO are studied and the reaction kinetic equation is established. The results showed that the optimum conditions are pH7.5, 0.1 M substrate and 20°C for PPO assay. However, the PPO is inactivated in boiling water for 80s. The K_m, V_{max} and kinetic equation are 3.5 mM, 50 U/min and $1/V = 0.0688/[S]+19.915$ using catechol as substrate, respectively. Of the inhibitors tested, *L*-cysteine and ascorbic acid are the most effective.

Key words: Sour cherry · Polyphenol oxidase (PPO) · Characteristic · Substrate · Inhibitor

INTRODUCTION

Sour cherry (*Prunus cerasus* Ledeb) is one of the more healthful edible berries, it contains significant levels of polyphenolids (anthocyanins and other flavonoids), as well as the alkaloid, melatonin [1]. Sour cherry can be used for dry fruits, powders from individually quick frozen (IQF) cherry, frozen cherries, juices and juice concentrates[2-5]. Enzymatic browning is the important factor that affects the processing quality of sour cherry. And polyphenol oxidase (PPO, EC 1.14.18.1) is the main cause of enzymatic browning of sour cherry processing products [6, 7]. PPO uses molecular oxygen to *o*-diphenols and their further oxidation to coloured and highly reactive *o*-quinones. These *o*-quinones readily polymerize and/or react with endogenous amino acids and proteins to form complex brown pigments[6]. PPO has been widely investigated in various plant tissues [8-11]. However little is known on the properties of PPO in sour cherry (*Prunus cerasus* Ledeb. cv. CAB) pulp. In this work, the properties of PPO in sour cherry, including optimum temperature, optimum pH, substrate specificity and inhibitors were studied.

MATERIALS AND METHODS

Plant Materials and Crude Enzyme Preparation: Mature sour cherry fruits (*Prunus cerasus* Ledeb. cv. CAB) were obtained from Beijing Academy of Agriculture and Forestry Science, China. Five grams of pulp was homogenized with 5-mL 0.1-M sodium phosphate buffer (pH 7.5) containing 1% polyvinylpolypyrrolidone and then centirifuged at 12,000×g for 20 min at 4°C. The supernatant was collected for the enzyme assay.

Standard Reaction for Assay of PPO Activity: PPO activity was determined by the method of Liu *et al*. [12]. The standard reaction mixture contained 1.0 mL of 0.1-M catechol, 1.0 mL 0.1-M phosphate buffer (pH 7.5) and 0.1 mL of extract and was incubated for 5 min at 30°C or as indicated in the Results and Discussion section. By measuring the increase in absorbance at 420 nm with a UV-spectrophotometer (TU-1810, Beijing Puxi General Instrument Co., Beijing, China), the PPO activity was expressed as one unit = 0.001 ΔA420/min/g fresh weight. The PPO activity with pyrogallic acid as substrate was estimated by measuring the increase in absorbance at 320 nm.

Assay for Substrate Specificity, Optimum pH, Substrate Concentration and Temperature of PPO: For substrate specificity, PPO activity was assayed with various potential substrates, including catechol, 1,4-hydroquinone, resorcinol, phloroglucinol and pyrogallic acid. Relative enzymatic activity was calculated as a percentage of the highest activity.

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PPO was measured with the standard reaction mixture buffer previously described at a pH range of 3.6–9.0; with 0.1 M of pH3.6–5.6 acetate buffer, 0.1 M of pH6.0–8.0 phosphate buffer and 0.1 M of pH8.6–9.0 Tris-HCl buffer, respectively.

PPO was measured with the standard reaction mixture buffer previously described at substrate concentration range of 0.02–0.16 M for catechol. And measured the crude enzyme solution range of 0.2–1.8 mL.

For assay the effect of temperature on PPO activity, 2 mL of the standard reaction mixture buffer, in Eppendorf tubes (Sigma Chemical Co., St. Louis, MO), was incubated in a water bath at temperatures of 10–70□ for 5 min, with 0.2–1.8 mL of crude enzyme solution added and shaken well, respectively, immediately measured the increase in absorbance. In addition, 1 mL of the crude enzyme solution are respectively heated for 10–80 s, taken out and immediately cooled by immersing in an ice bath and measured the increase in absorbance.. Sour cherry pulp are blanchinged, respectively kept warm for 3 min when the core temperature reached 10-80°C. After blanching, the crude enzyme solution is extracted, with 2 mL standard reaction mixture buffer added and shaken well, immediately measured the variation of absorbance.

Michaelis constant (K_m) and maximum velocity (V_{max}) of PPO in sour cherry pulp was determined using catechol as substrate according to the method of Lineweaver and Burk [13].

Various potential inhibitors of PPO were assayed by the standard reaction as previously described by adding inhibitors at concentrations indicated in the Results and Discussion section.

RESULTS AND DISCUSSION

Substrate Specificity: Substrate specificity has a significant effect on PPO activity. The substrate specificity of the crude PPO is given in Table 1. As shown in Table 1, according to the activity assay of PPO in sour cherry with catechol as the standard, PPO relative activity of 1,4-hydroquinone and gallic acid are,respectively 11.4% and 70.1%, while the resorcinol and phloroglucinol cannot be used for the substrate assay. The affinity of enzyme for substrate by means of K_m indicates that catechol and gallic acid have a better affinity for PPO in sour cherry, the K_m values are 3.5 mM and 41.5 mM respectively, which is similar to the results of PPO activity of mango [8]. The K_m values of substrates indicate the affinity of substrate for enzyme, so the optimal substrate for PPO in sour cherry obtained from the experiments is catechol. Kumar *et al*. [6] Studied the substrate specificity of 'Barbados' cherry (*Malpighia glabra* L.) and found that catechol, guaiacol, dopamine, pyrogallic acid and caffeic acid can be used for the substrate assay and the affinity of diphenols substrate was better than triphenol substrate. Also, researchers used tannin, catechol, chlorogenic acid, tyrosine, ferulic acid, etc. as the substrate assay and found that the optimal substrate for different fruits and vegetables materials were different [6, 8, 14], where most were appropriately catechol, dopamine, etc., few were triphenol compounds [15].

pH Optima: After Sour cherry PPO reaction system conducting in the buffer solution (pH3.6-9.0), PPO activity measured is shown in Fig. 1. Different pH values have significant effects on the PPO activity of sour cherry. There are two activity peaks at pH5.0 and pH7.5 and the activity is higher at pH7.5 than pH5.0, so pH7.5 is chosen to determine PPO activity of sour cherry; the activity is lower when pH<4.0. In addition, Colak *et al*. [7] found that the Optimum pH for cherry cultivars 'Globigemmis' and 'Oxygemmis' (*Laurocerasus officinalis* Roem.) was 7.0, Kumar *et al*.[6] found that the Optimum pH for 'Barbados' cherry (*Malpighia glabra* L.) was 7.2, which was similar to the experimental results. The appearance of two activity peaks, indicates that PPO in sour cherry may have two different conformations and pomegranate [14] and many other fruits all have a similar situation. The study of Ayaz *et al*. [10] shows that the optimal pH value for PPO in the different fruits or the same fruit at different developmental

Table 1: Substrate specificity of PPO in sour cherry

Substrate	PPO relative activity /%	K_m/mM	V_{max} /(U/min)	V_{max}/ K_m/(U/mM/min)	R^2
Catechol	100.0	3.5	50.0	14.3	0.9901
1,4-hydroquinone	11.4				
Resorcinol	0.0				
Pyrogallic acid	70.1	41.5	52.6	1.3	0.9536
Phloroglucinol	0.0				

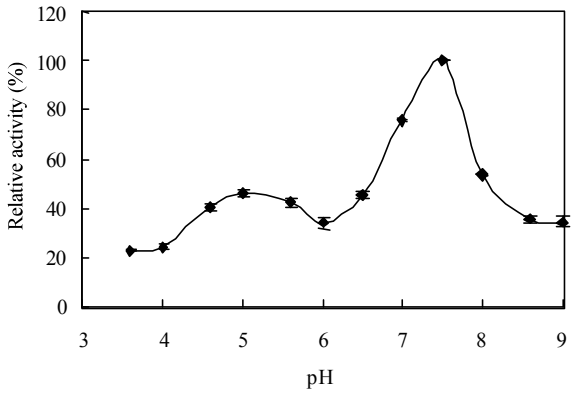

Fig. 1: PPO activity as a function of assay pH from sour cherry pulp

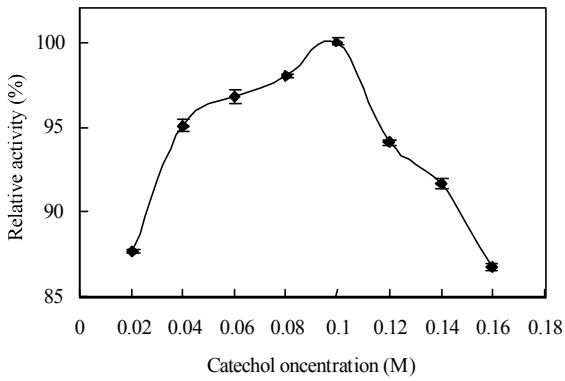

Fig. 2: PPO activity as a function of assay substrate concentration from sour cherry pulp

stages are different. The optimum pH values for PPO in many kinds of fruit are mostly concentrated in between pH4.0–8.0 [6-15] and PPO activity can be inhibited in strong acid or alkali environment. Because PPO is a copper enzyme, in the lower pH of acidic conditions, the dissociation of cofactor Cu^{2+} inhibits the enzyme activity; in the higher pH of alkaline conditions, Cu^{2+} is spun off to become insoluble cupric hydroxide, which also causes enzyme inactivation [14]. Therefore, in the processing of sour cherry, adjusting the pH value can inhibit PPO activity and reduce enzymatic browning.

Substrate Concentration Optima: The effect of substrate concentrations on PPO reaction velocity of sour cherry is shown in Fig. 2. It shows that the substrate concentration has a significant effect on PPO reaction velocity of sour cherry. When the substrate concentration is less than 0.1 M, the activity increases with the concentration increasing, the reaction velocity also increases; when the substrate concentration is more than 0.1 M, the activity decreases

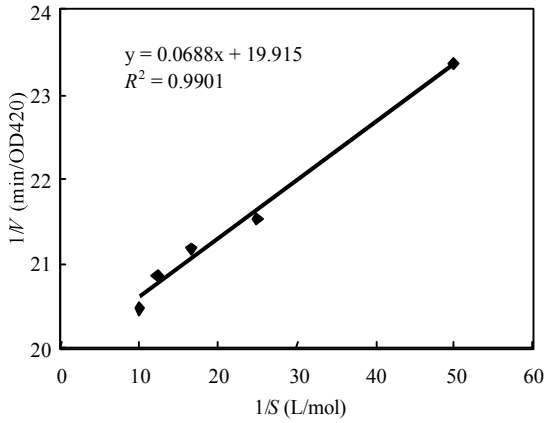

Fig. 3: $1/V \sim 1/[S]$ chart of the enzymatic reaction of PPO in sour cherry pulp

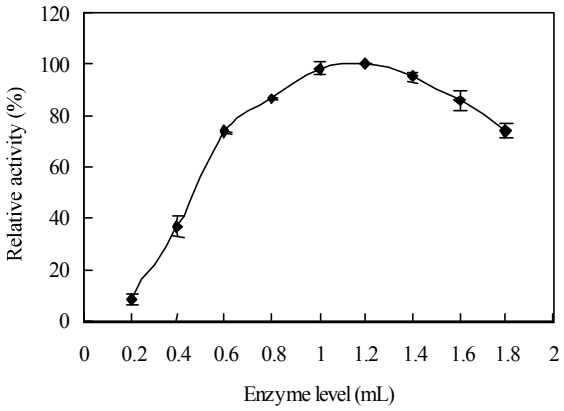

Fig. 4: Effect of enzyme level on reaction velocity

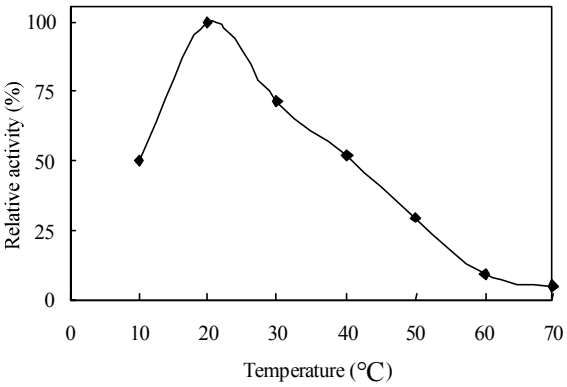

Fig. 5: Effect of reaction temperature on PPO activity of sour cherry pulp

with the concentration increasing, the reaction velocity also correspondingly decreases. During the experiment it can be seen, when the substrate concentration is more than 0.2 M, the enzyme and the substrate show turbidity after mixing and the absorbance value cannot be measured by enzyme activity.

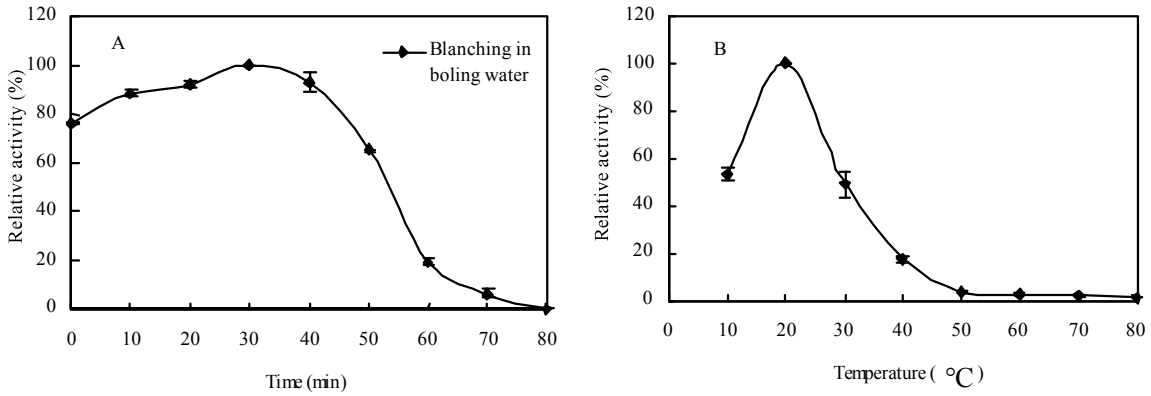

Fig. 6: Effects of blanching on PPO activity of sour cherry pulp

According to Linewaver-Burk equation [16], with $1/V$ as the ordinate, $1/[S]$ as the abscissa (Fig.3), the enzymatic kinetic equation of PPO in sour cherry is fitted with: $1/V = 0.0688/[S]+19.915$, where $R^2 = 0.9901$, indicating that PPO in sour cherry has a good affinity for catechol. And for the enzymatic reaction of PPO in sour cherry, Michaelis constant: K_m = 3.5 mM; maximum reaction velocity: V_{max} = 50 U/min.

Enzyme Level Optima: The effect of the enzyme level of PPO in sour cherry on the reaction velocity is shown in Fig.4, when the enzyme level is lower, the reaction velocity increases with the enzyme solution increasing; when the enzyme level reaches 1.2 mL, the increase of reaction velocity is not evident and shows the declining trend, which may be related to the of enzyme product and competitive inhibition of enzyme [16]. Therefore, the optimum enzyme level of PPO in sour cherry is 1.0–1.2 mL.

Effect of Temperature: The temperature has double effects on PPO activity. And the temperature increases not only accelerate the catalytic reaction velocity, but also promote enzyme protein denaturation, which is the comprehensive reaction of two antagonisms [8]. Fig. 5 shows that the optimum temperature of PPO activity of sour cherry is 20°C; when the temperature is higher than 30°C, the enzyme activity significantly decreases; when the temperature reaches 50°C, PPO activity decreases by 84.7%; when the temperature reaches 60°C, PPO activity decreases by 96.8%. The results are different from 40–50°C obtained from Colak *et al*. [7], indicating that the PPO thermal stability of different types of cherries are different. The optimal temperatures of PPO activity between different species are quite different, roughly between 20–60°C [6-15].

The crude enzyme solution and pulp of sour cherry are blanched with different temperatures and different times and found that the thermal stability of sour cherries PPO is lower. When enzyme solution is blanched in boiling water, enzyme activity first shows an increase temporarily with the heating time increasing; the activity decreases rapidly when the heating time is greater than 40 s; the sour cherry PPO activity are all inactivated when the heating time reached 80 s(Fig.6A). Fig.6B shows that PPO activity is the highest when sour cherry pulp is blanched in water bath (20°C) for 3 min, which is consistent with the optimum temperature obtained from sour cherry PPO above. PPO activity significantly decreases with the blanching temperature increasing, PPO activity decreases by 96.3% when blanched at 50°C for 3 min (Fig. 6B). Thus it can be seen, the thermal stability of PPO in sour cherry is lower and the temperature regulation method is a very effective measure to inhibit PPO activity. With respect to the temperature on the inhibition of PPO activity, there are many related studies at home and abroad [9, 11].

Effect of Inhibitor: As shown in Fig. 7, after adding inhibitor, PPO activity are significantly lower than the control, indicating that the four inhibitors have better inhibitory effects on PPO activity of sour cherry and the inhibitory effects increases with the inhibitor concentration increasing. When *L*-cysteine concentration added reaches 0.4 mM, PPO activity decreases by 97.4% (Fig. 7A); When ascorbic acid concentration added reach 0.6 mM, PPO activity decreases 97.5% (Fig. 7B); when sodium sulfite concentration added reaches 2.5 mM, PPO activity decreases by 95.2% (Fig. 7C); when citric acid concentration added reaches 1 M, PPO activity decreases by 97.8% (Fig. 7D). In the case of the same inhibitor concentration, the inhibitory effects on PPO activity of

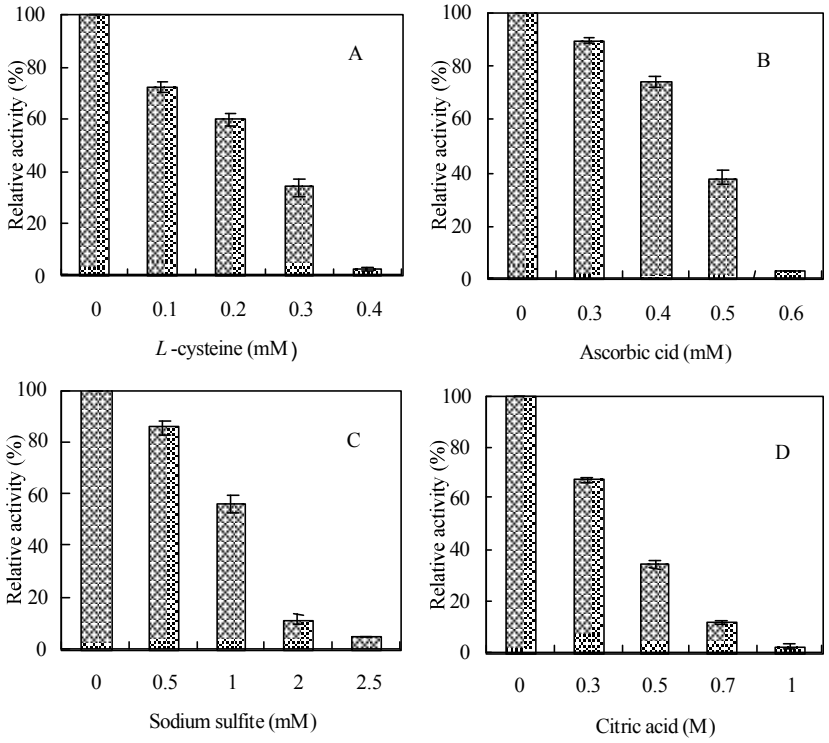

Fig. 7: Effects of several inhibitors on PPO activity of sour cherry pulp

sour cherry are in the sequence of *L*-cysteine > ascorbic acid > sodium sulfite > citric acid, in which the levels of *L*-cysteine and ascorbic acid are least, but the effects are best. The results are consistent with large number of researches [4~15]. Another study shows that glutathione, glycine, EDTA-Na_2, sodium chloride, acetic acid, metal ions, some natural extracts [8, 14] and part of the compound inhibitors also have inhibitory effects on PPO. EDTA-Na_2 is also introduced to be an inhibitor during the experiments, but a concentration of 10 mM cannot have inhibitory effect on PPO in sour cherry. The results are consistent with Kumar *et al*. [6] and Colak *et al*. [7], indicating that EDTA-Na_2 is not an ideal activity inhibitor of PPO in sour cherry. Addition of the inhibitors needs to be strictly controlled with reference to countries and relevant standards, while pollution of the material should be avoided and some safe inhibitors should be chosen. In food processing, pH can be combined with temperature and a variety of inhibitors to use in combination, so as to control the production of PPO enzymatic browning.

CONCLUSIONS

Our study showed that catechol and gallic acid could be the substrate assay for PPO activity of sour cherry pulp, the affinity and activity of catechol is better. For PPO in sour cherry, the optimum substrate is catechol; the optimum reaction pH is 7.5; the optimum substrate concentration is 0.1 M; the optimal volume of crude enzyme solution for the determination is 1.2 mL; the optimum temperature is 20□, the enzyme are all inactive when blanched in boiling water for 80 s. With catechol as substrate, the kinetic equation for PPO enzymatic reaction of sour cherry is $1/V = 0.0688/[S]+19.915$, $K_m = 3.5$ mM, $V_{max} = 50$ U/min. Four inhibitors of *L*-cysteine, ascorbic acid, citric acid and sodium sulfite show varying degrees of inhibition on PPO in sour cherry, the levels of *L*-cysteine and ascorbic acid are the least, but the results are best.

ACKNOWLEDGMENT

This research was supported by the Institute of Pomology and Forestry, Beijing Academy of Agriculture and Forestry Science of China.

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