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Full Length Research Paper

Biochemical properties of oxidases of Yali pear

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The biochemical properties of polyphenol oxidase (PPO), peroxidase (POD) and ascorbate peroxidase (APX) from Yali pear were investigated. The optimum pH and temperature of three enzymes was 5.6, 4.0, 7.0 and 20, 40, 50°C, respectively. Enzyme kinetics results showed that the Michaelis constant (Km) and maximum velocity (Vmax) of PPO for catechol were 0.22 M and 1111 U/ml/min. The Km and Vmax values of POD for guaiacol were 0.14 M and 1429 U/ml/min. The Km of APX for ascorbic acid and H_2O_2 were 0.41 and 0.083 mM, respectively, and the Vmax of APX was 455 and 208 U/ml/min for ascorbic acid and H_2O_2, respectively. The inhibitory effects of the four inhibitors (ascorbic acid, citric acid, L-cysteine and phytic acid) on each enzyme were different, suggesting that the composite inhibitor is more appropriate for processing of Yali pear.

Key words: Yali pear, polyphenol oxidase, peroxidase, ascorbate peroxidase, biochemical property.

INTRODUCTION

Yali pear (*Pyrus bertschneideri* Reld) belongs to super white pear. It has been grown for more than 2000 years and is popular in China (Chen et al., 2006). In addition to its being consumed fresh, Yali pear can be processed for high value-added products, such as pear juice, perry, pear vinegar, preserved pear and canned pear. However, enzymatic browning during processing impairs sensory properties and marketability of the product (Franck et al., 2007). Enzymatic browning during fruit and vegetable processing mainly arises from the oxidation of phenolic compounds (Lamikanra and Watson, 2001; Mayer and Harel, 1979). The main enzymes responsible for the browning reaction are polyphenol oxidase (PPO), peroxidase (POD) (Gónzalez-Barrio et al., 2005; Vamos-Vigyazo, 1981) and ascorbate peroxidase (APX) (Wu et al., 1995). The activities of their enzymes, together with concentrations of phenolic compounds, pH, temperature and oxygen availability of the tissue, determine browning rate (Benjar and Athapol, 2006).

Polyphenol oxidase (PPO; EC 1.14.18.1) is responsible for the enzymatic browning occurring in many plants (Zawitowski et al., 1991) and has been investigated in numerous sources, for example, in apples (*Malus* sp.) (Espin et al., 1995), pears (*Pyrus* sp.) (Hwang et al., 1996) and grapes (*Vitis* sp.) (Nunez-Delicado et al., 2003). The involvement of peroxidase (POD; EC 1.11.1.7) in browning has been repeatedly reported (Richard-Forget and Gauillard, 1997; Degl'Innocenti et al., 2005). POD catalyses the oxidative coupling of phenolic compounds using H_2O_2 as the oxidizing agent (Kay et al., 1967), although the oxidation is limited by the availability of electron acceptor compounds such as superoxide radicals, hydrogen peroxide, and lipid peroxides (Richard-Forget and Gauillard, 1997).

POD also could enhance PPO-mediated browning reactions (Toivonen and Brummell, 2008). Ascorbate peroxidase (APX; EC 1.11.1.11) can remove H_2O_2 using ascorbic acid as electron donor (Asada, 1999). When browning occurred in Yali pear fruit cores, APX activety reached the peak while PPO activity changed a little

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Abbreviations: PPO, Polyphenol oxidase; **POD,** peroxidase; **APX,** ascorbate peroxidase; **PVPP,** polyvinylpolypyrrolidone; **EDTA,** ethylene diamine-tetra acetic acid.

#These authors contributed equally to this work.

(Guan, 1994). The objective of this study was to characterize PPO, POD and APX from Yali pear grown in China for better understanding of enzymatic browning mechanism. Optimal pH, temperature optimum, thermal stability, kinetic parameters and degrees of inhibition by general inhibitors were investigated.

MATERIALS AND METHODS

Yali pears were harvested at commercial maturity from Yangxin Yali pear production base at Binzhou, Shandong province, China, and stored at 0 to 4°C for 15 days. Polyvinylpolypyrrolidone (PVPP, insoluble) and L-cysteine were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals used in this experiment were analytical reagent grade and purchased from local chemical suppliers in China.

Preparation and activity assay of crude PPO

PPO preparation was obtained according to the method of Sun et al. (2009). Pear flesh with core removed (15 g) were homogenized at 4°C in phosphate buffer (30 ml, 0.1 M, pH 6.8) containing 1.25 g of PVPP. The homogenate was kept at 4°C for 10 min and then filtrated through cheesecloth. The filtrate was centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was crude PPO preparation. Freshly PPO preparation (0.5 ml) in phosphate buffer (4 ml, 0.1 M, pH 6.8) with the presence of catechol solution (0.5 ml, 0.5 M) was measured at 420 nm at 30°C using a spectrophotometer for PPO activity (Umit Unal, 2007). One unit of PPO activity was defined as a change of 0.001 in absorbance per minute.

Preparation and activity assay of crude POD

POD was extracted and determined using the method described by Ofelia et al. (2008). Pear flesh samples (15 g) were homogenized in phosphate buffer (30 ml, 0.1 M, pH 7.0) with 2 mM ethylene diamine-tetra acetic acid (EDTA) and 1.25 g of PVPP. The following procedure was the same as the extraction of PPO preparation. POD activity was determined in the mixture of POD preparation (2 ml), phosphate buffer (1.8 ml, pH 7.0), guaiacol solution (1 ml, 50 mM) and H_2O_2 (0.2 ml, 0.88 M) for the increase in absorbance at 470 nm at 30°C using a spectrophotometer (Ofelia et al., 2008). One unit of POD activity was defined in same way as PPO.

Preparation and activity assay of crude APX

The extraction and determination of APX were conducted in a method modified from Zhou et al. (2003). Pear flesh samples (15 g) were homogenized in phosphate buffer (30 ml, 0.1 M, pH 7.8) containing 10 mM EDTA and 4 mM ascorbic acid. Then the same procedure as PPO extraction was taken. The supernatant obtained (0.1 ml) was immediately added to the mixture of phosphate buffer (2 ml, 0.1 M, pH 7.0), ascorbic acid (1.5 ml, 1 mM) and H_2O_2 (0.3 ml, 1 mM), and measured for the decrease in absorbance at 290 nm at 25°C. Absorbance under non-enzymatic oxidation and H_2O_2-independent oxidation was used for correction. APX activity unit was defined in same way as PPO.

Optimum pH

Each enzyme activity was d etermined under standard conditions using phosphate buffers with a range of pH from 3.0 to 7.8. Relative enzymatic activity was described as percentage of the maximum activity (Montero et al., 2001). The optimal pH obtained for each enzyme was used in all other assays in this study.

Optimal temperature and thermal stability

Each enzyme activity was measured in a temperature range of 10 to 70°C using a water bath to determine the optimum temperature (Sun et al., 2009). The temperature stability of each enzyme was determined with enzyme solutions incubated at different temperatures (60, 70, 80 and 90°C) for 5, 10, 15, 20 and 25 min, respectively. The residual enzyme activity was calculated by comparison with unheated enzyme.

Kinetic parameters

Michaelis constant (Km) and maximum velocity (Vm) were determined in a range of concentrations of substrates for each enzyme. PPO activities were measured using catechol (from 0.04 to 0.2 M) as substrate. The activities of POD were measured using guaiacol and hydrogen peroxide (H_2O_2) as the substrates at a concentration varying from 0.01 to 0.2 M and from 0.18 to 1.24 M, respectively. APX activities using ascorbic acid (from 0.1 to 2.5 mM) and H_2O_2 (from 0.1 to 2.5 mM) as the substrates were determined. Km and Vmax values of each enzyme were calculated from a plot of 1/V *vs.* 1/S by the method of Lineweaver and Burk (1934).

Effects of inhibitors

The inhibitory effects of ascorbic acid (0.01 to 0.2 M), citric acid (0.1 to 2 M), L-cysteine (0.01 to 0.2 M) and phytic acid (0.002 to 0.1 M) on PPO, POD and APX activity were determined as relative residual activity and calculated in the following equation:

Residual activity = A_i/A_0.

Where A_0 was initial enzyme activity (without inhibitor) and A_i was enzyme activity with inhibitor (Umit Unal, 2007).

Statistical analysis

The variance of the data was analyzed using DPS 7.05. All experiments were performed in triplicate (n = 3), and the results represented as mean ± standard deviations (SD) of three replicated determinations.

RESULTS

Optimal pH

The activities of PPO, POD and APX of Yali pear were measured at different pHs to find pH optimum (Figure 1). PPO activity increased with elevation of pH value from 3.0 to 5.6 and sharply declined from pH 5.6 to 7.8, showing the optimum pH of PPO was 5.6. The maximum activity was at a pH of approximately 4.0 for POD. In the case of APX, there were two peaks, with higher activity at neutral than at acidic pH (Figure 1). Enzyme activity greatly declined with acidic degree.

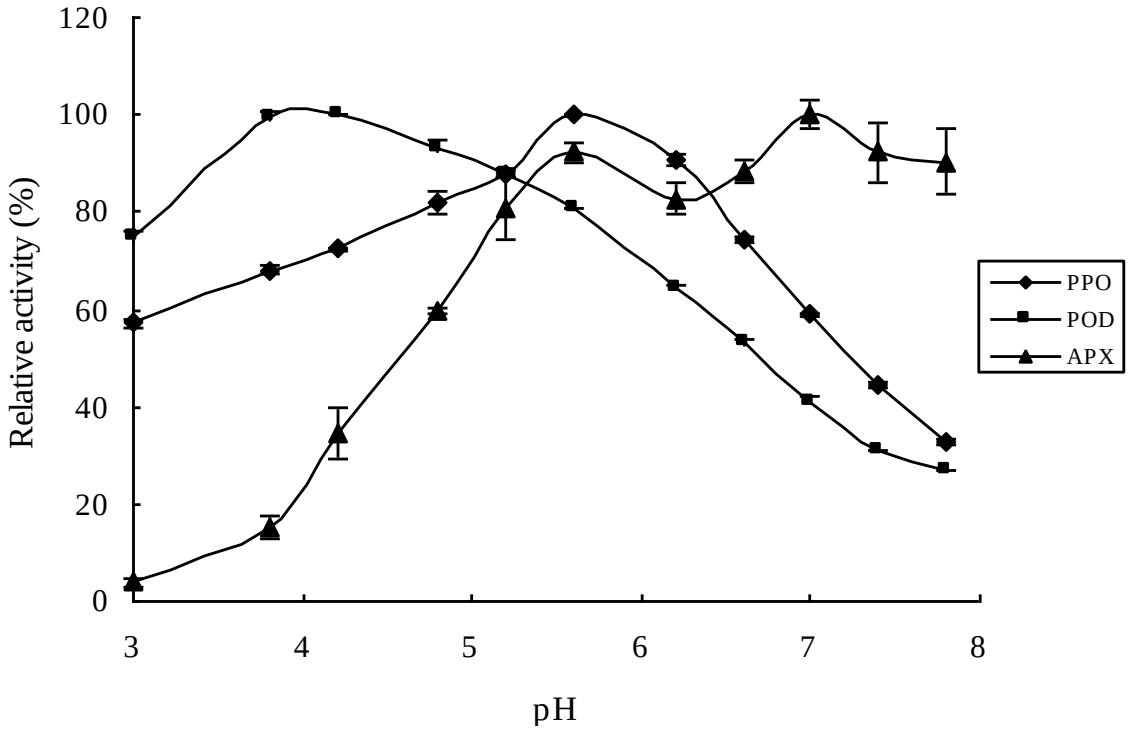


Figure 1. Effects of pH on the activities of PPO, POD and APX of Yali pear. Each enzyme activity was assayed using the standard reaction mixture and the pH values of buffer were different (3, 3.8, 4.2, 4.8, 5.2, 5.6, 6.2, 6.6, 7, 7.4, 7.8).

Temperature optimum

Yali pear PPO had the maximum activity at 20°C and lost only 22% of activity when the temperature dropped to 10°C (Figure 2). When the temperature increased from 20 to 70°C, the enzyme activity declined rapidly. The optimal temperature for POD was 40°C. The POD activity gradually declined under and above 40°C (Figure 2). At 70°C, POD activity was only 8.5% of the maximum activity, but remained 55.5% at 10 °C. With APX, the optimum temperature was 50°C. At 10 and 70°C, the APX activities remained 33.9 and 75.5%, respectively (Figure 2).

Thermal stability

The thermal stability profile of PPO, POD and APX, presented as residue activity after pre-incubation at the specified temperature, are presented in Figure 3. Activities of these three enzymes declined greatly in the first 5 min and slowly after 5 min. After incubation for 25 min, the PPO residual activity remained about 45.0% at 80°C and 8.5% at 90°C (Figure 3a). In case of POD, the residual activity remained about 15% at 60°C after incubation for 25 min, and 6% at other temperatures (Figure 3b). As to APX, the residual activity increased after 10 min incubation at 80°C and remained 30% after 25 min at 90°C (Figure 3c).

Kinetic parameters

The correlation coefficient (R^2), and the regression curve equation was obtained by the analysis of the Lineweaver–Burk double reciprocal plot (Figure 4). The analysis of the Lineweaver–Burk curves revealed that Yali pear POD showed a low affinity to H_2O_2. The Km and Vmax values of each enzyme for the corresponding substrate were calculated from the regression curve equation. The Km and Vm values of PPO for catechol were 0.22 M and 1111 U/ml/min, POD for guaiacol 0.14 M and 1429 U/mL · min, respectively. In the case of APX, the Km and Vmax values were 0.41 mM and 455 U/ml/min for ascorbic acid, and 0.083 mM and 208 U/ml/min for H_2O_2, respectively.

Effects of inhibitors

The activities of the three enzymes all decreased as the concentration of ascorbic acid increased (Figure 5a), but the concentration for full inhibition differed and was 0.002 M for POD, and 0.008 M for PPO and APX. Citric acid showed differential effects on inhibition of three enzymes

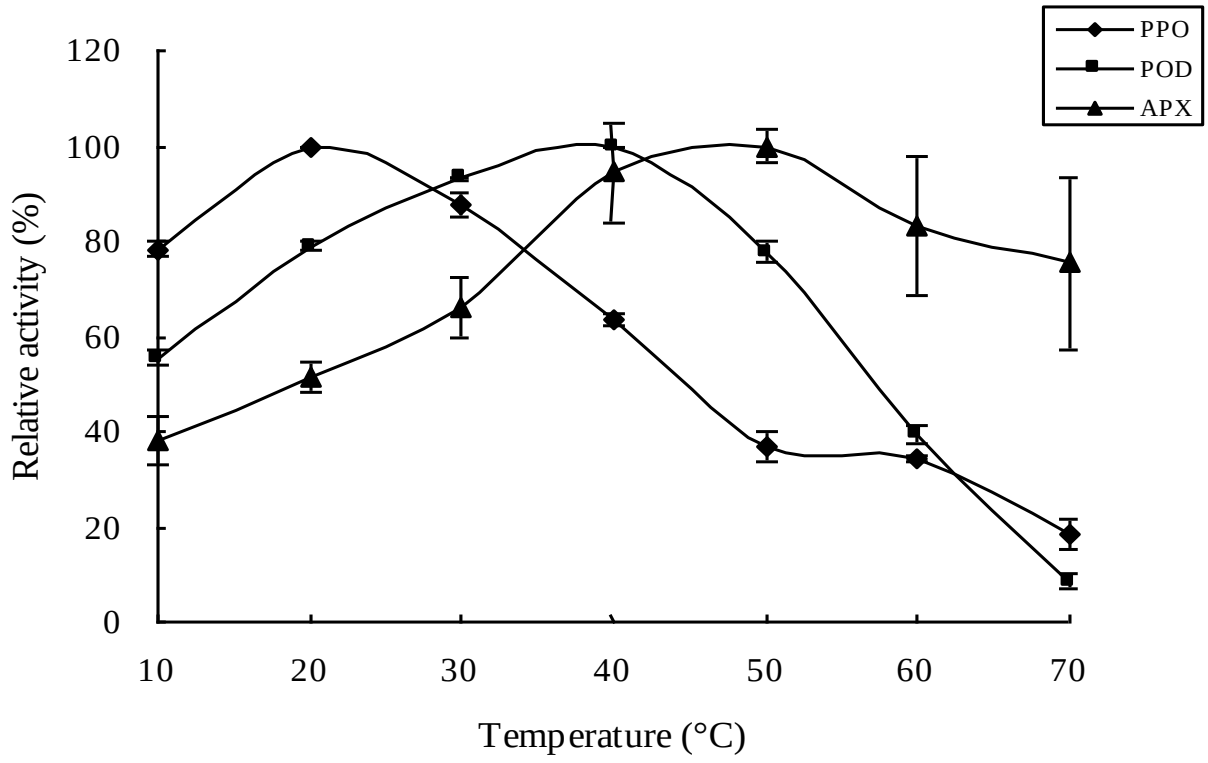


Figure 2. Effects of temperature on the activities of PPO, POD and APX of Yali pear. Substrate solution was incubated for 5 min in water bath at different temperatures (ranged from 10 to 70°C at 10°C interval).

(Figure 5b). APX activity was fully inhibited by 0.8 M citric acid, but PPO and POD residual activities were still 40.6 and 8.1%, respectively. The order of inhibitory effect was APX>POD>PPO. The PPO activity was fully inhibited by L-cysteine at concentration of only 0.008 M (Figure 5c). However, the concentration increased to 0.02 M, POD and APX residual activities were still 18.7 and 28.1%, respectively. Full inhibition of POD activity by phytic acid was achieved at the concentration of 0.05 M, and APX at 0.1 M (Figure 5d). However, POD activity was the minimum at 0.02 M. When the concentration of phytic acid was over 0.02 M, the activity of POD was improved.

DISCUSSION

The optimum pH has been found to depend on plant variety, nature of phenolic substrates and extraction methods (Duangmal and Owusu-Apenten, 1999). The optimal pH for butter lettuce PPO was 5.5 with catechol as substrate (Gawlik-Dziki et al., 2008), but it was 6.8 using 4-methylcatechol. Pear pH is normally lower than 4.7. Thus, PPO and POD might play more important roles than APX in enzymatic browning of Yali pear, which agrees with the previous report on some other plants (Vamos-Vigyazo, 1981). POD may be most likely to cause enzymatic browning of Yali pear. The PPO, POD and APX of Yali pear are sensitive to high values of pH; thereby pH adjustment may be an effective practice to control enzymatic browning.

It is reported that the optimum temperature value are 35°C for butter lettuce PPO (Gawlik-Dziki et al., 2008). The optimal temperature of mulberry PPO for 4-methylcatechol and pyrogallol oxidation was 20 and 45°C for catechol (Arslan et al., 2004). By the temperature optima of Yali pear PPO, POD and APX, it can be concluded that the enzymatic browning of Yali pear at lower temperature may be caused mainly by PPO and POD, and APX plays a role at higher temperature. The thermal stability profile of Yali pear PPO, POD and APX indicated that the thermal inactivation could be described by a biphasic first-order decay process (Padron et al., 1975), which might reflect the existence of isoenzymes with different thermal properties.

Previous study report that PODs are the most heat stable enzymes in plants (Ofelia et al., 2008) is in contrast to the result in this study. Fortea et al. (2009) also found table grape POD lost > 90% of relative activity after only 5 min of incubation at 75°C. As seen from the thermal stability profile of the three enzymes, using heat treatment to control enzymatic browning, a temperature more than 90°C should be chose and elevated quickly to avoid quality loss caused by high temperature. A wide range of safety chemical compounds has been proposed to inhibit

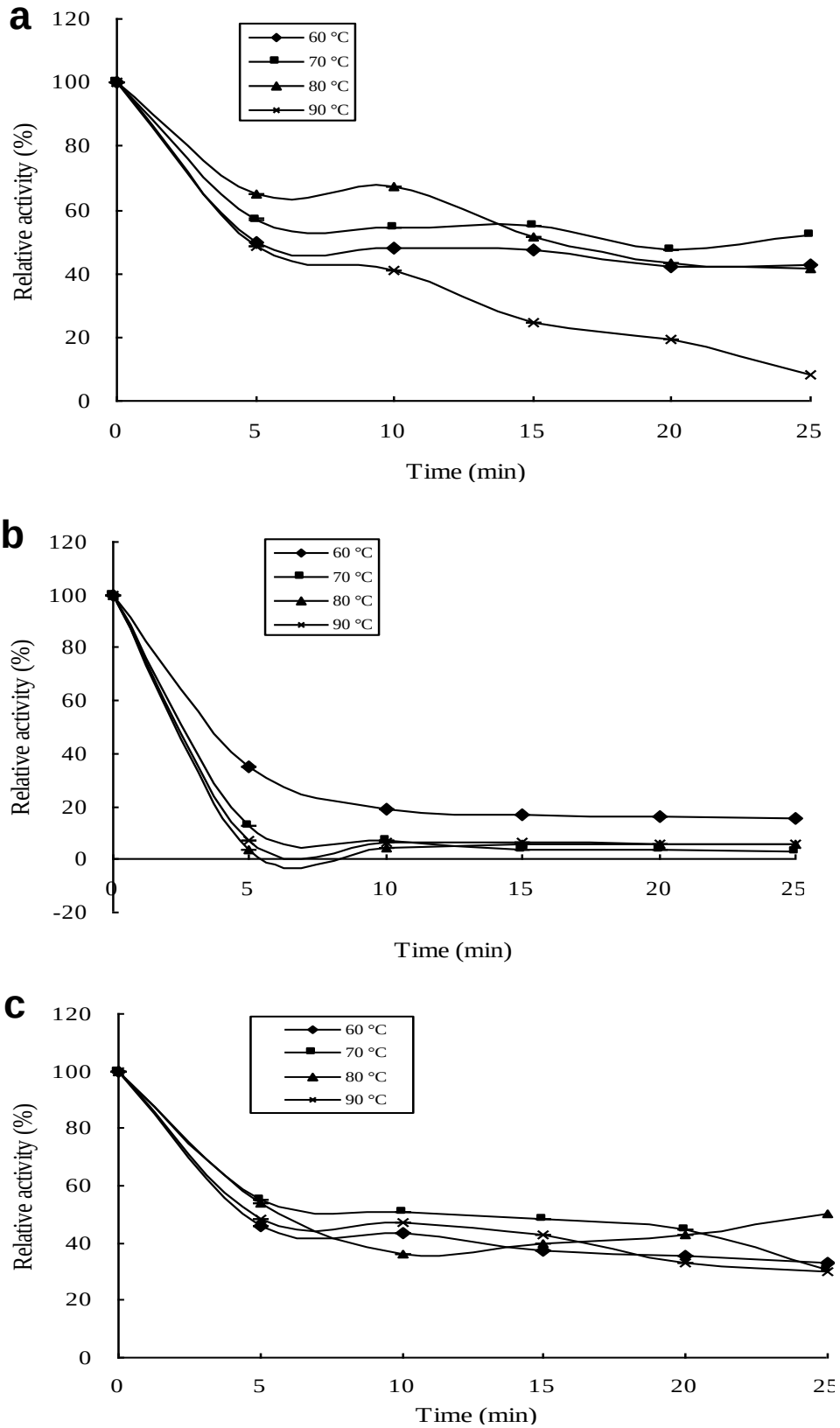
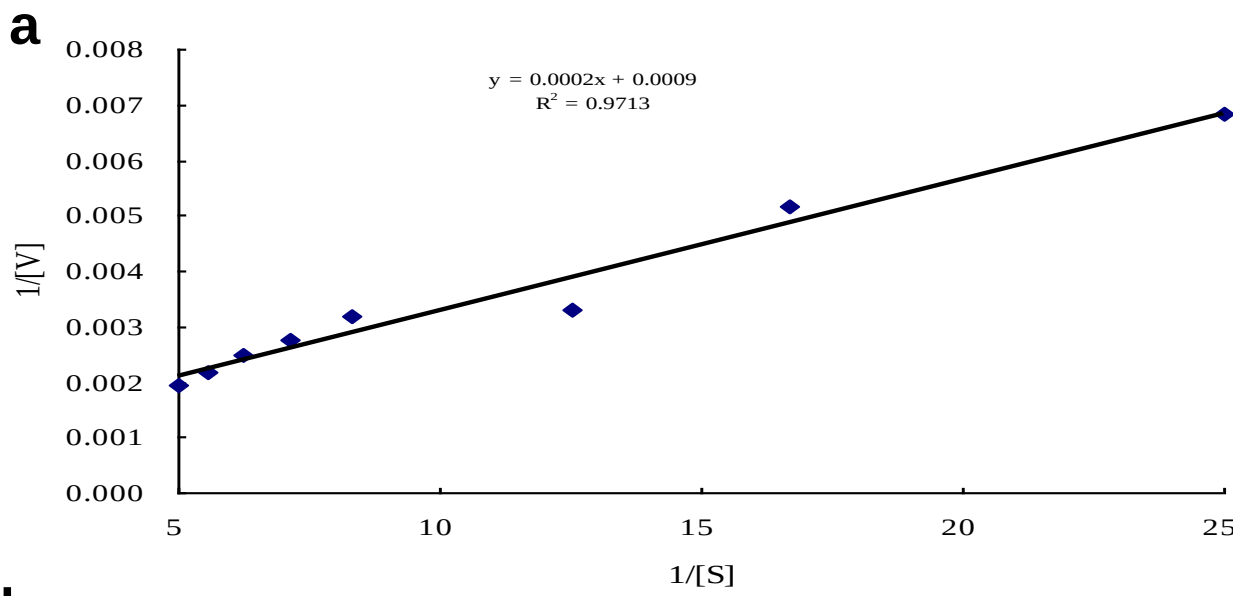
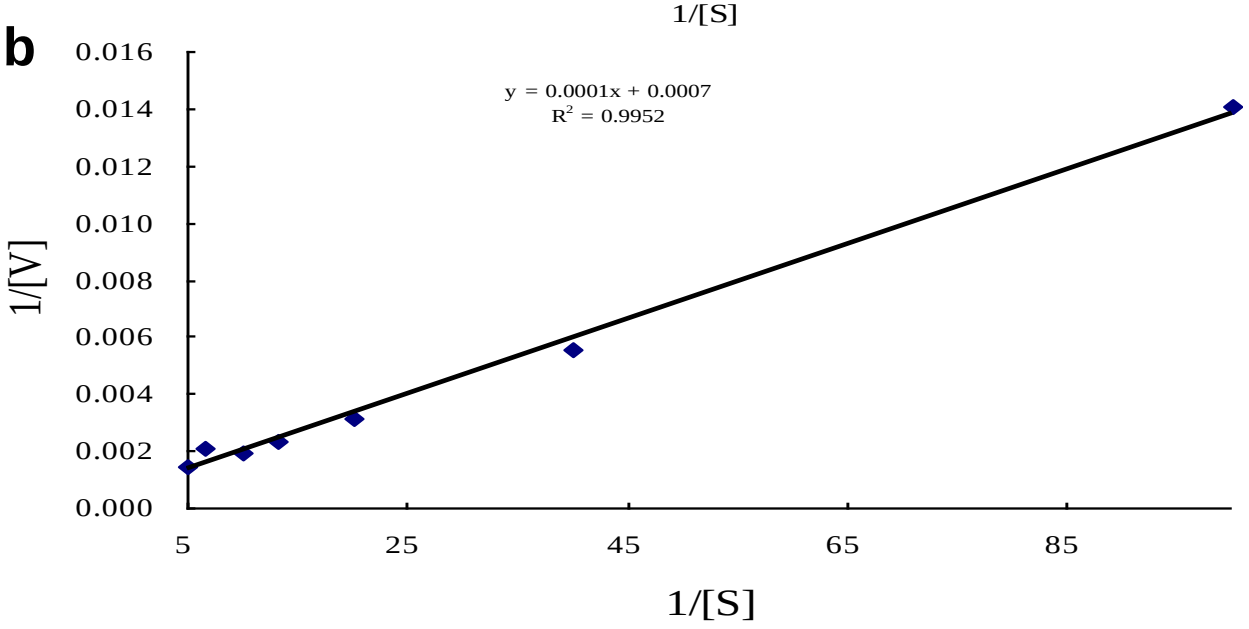
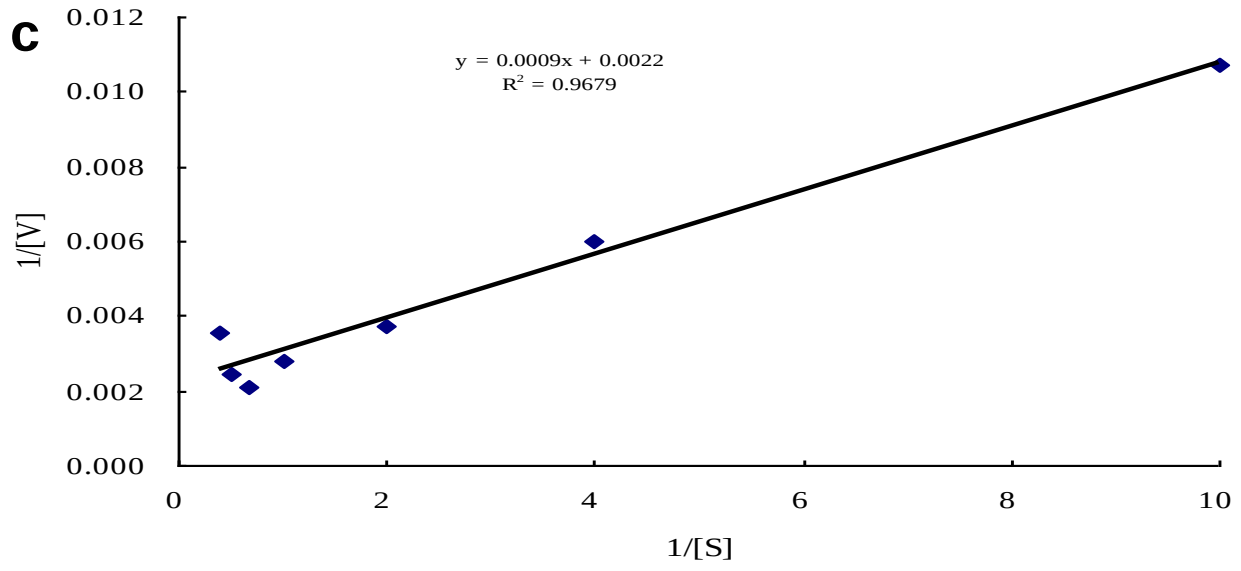


Figure 3. Thermal stabilities of PPO (a), POD (b) and APX (c). The enzyme solutions were incubated in water bath at different temperatures (60, 70, 80 and 90°C) using incubation time from 5 to 25 min.


a
y = 0.0002x + 0.0009
R² = 0.9713
1/[V]
1/[S]
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
5
10
15
20
25


b
y = 0.0001x + 0.0007
R² = 0.9952
1/[V]
1/[S]
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
5
25
45
65
85


c
y = 0.0009x + 0.0022
R² = 0.9679
1/[V]
1/[S]
0.012
0.010
0.008
0.006
0.004
0.002
0.000
0
2
4
6
8
10

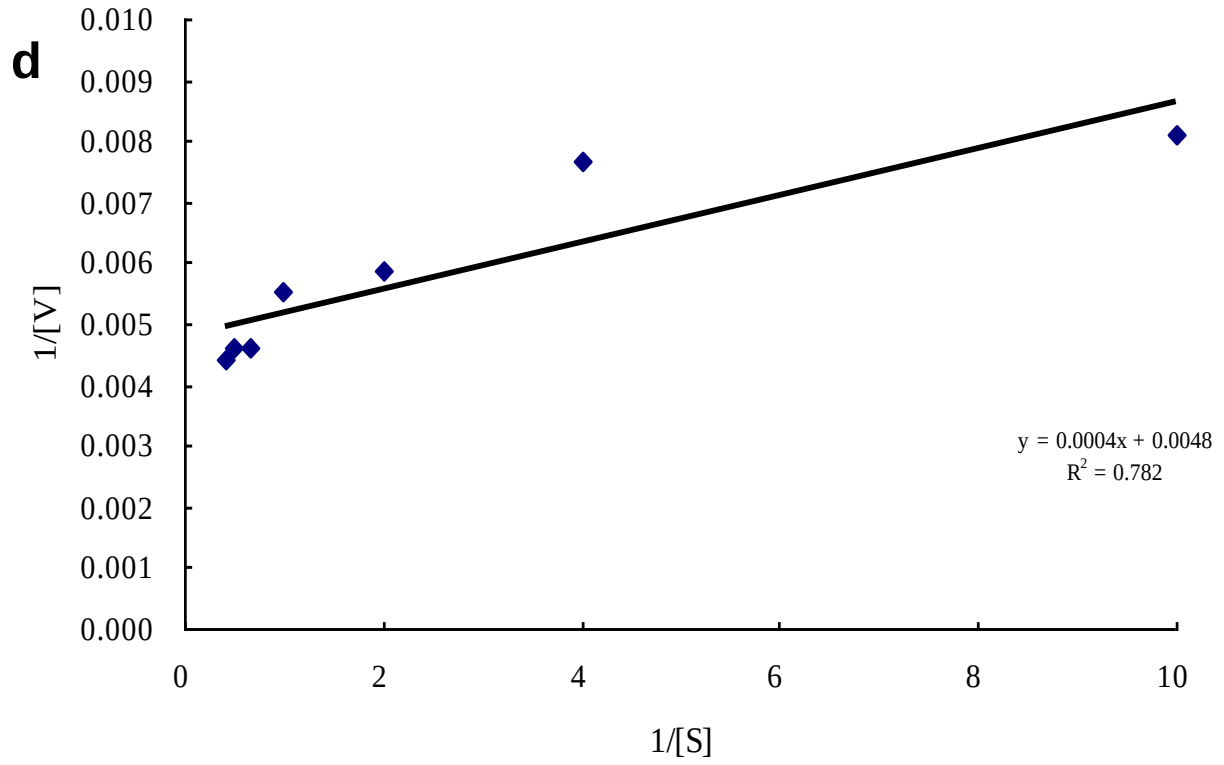
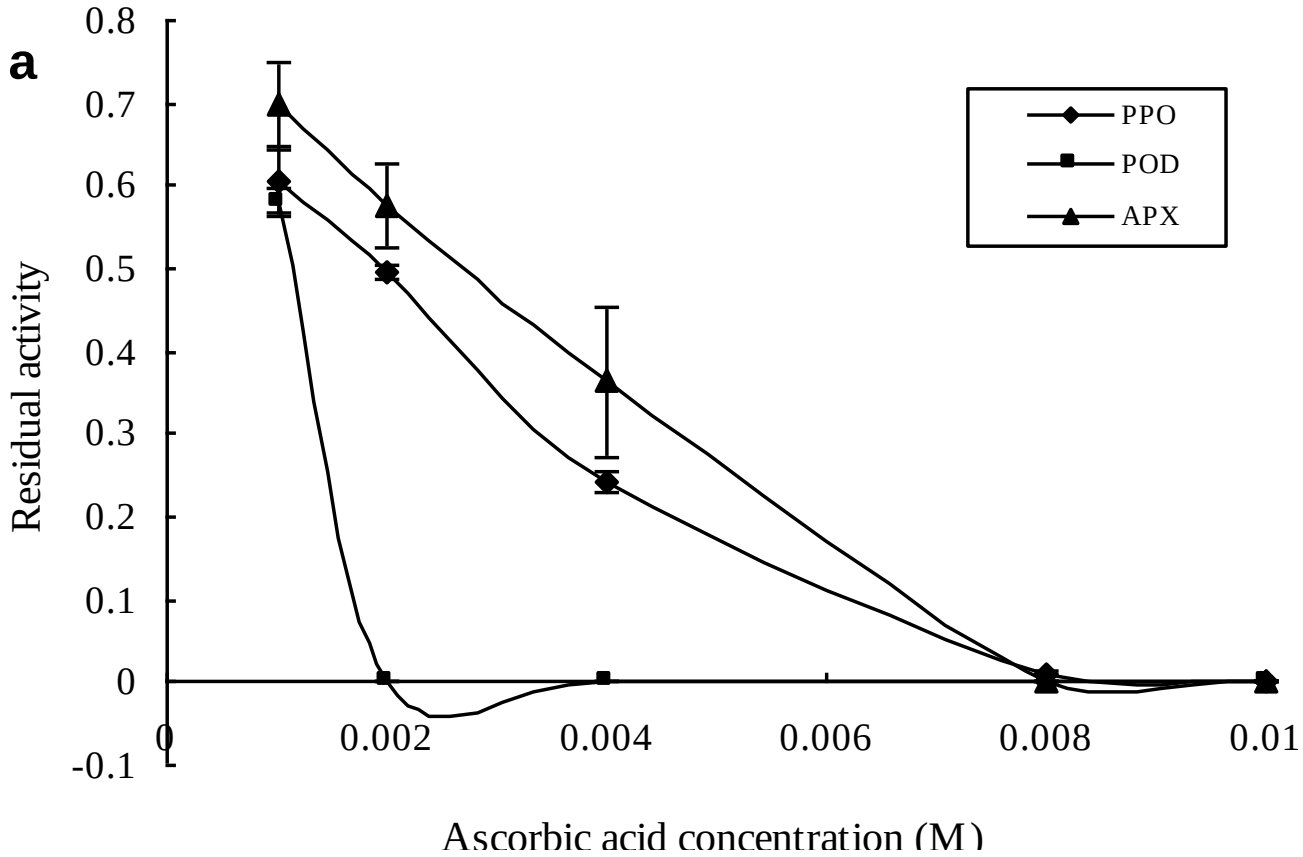
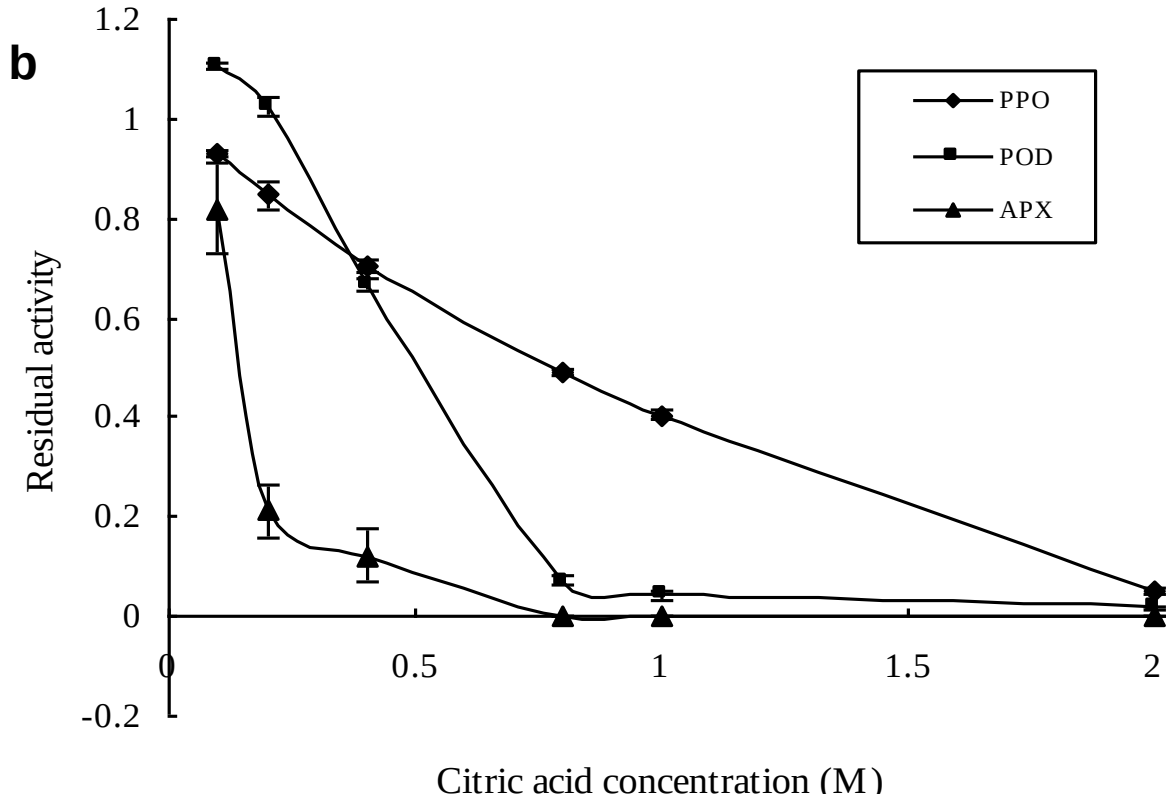
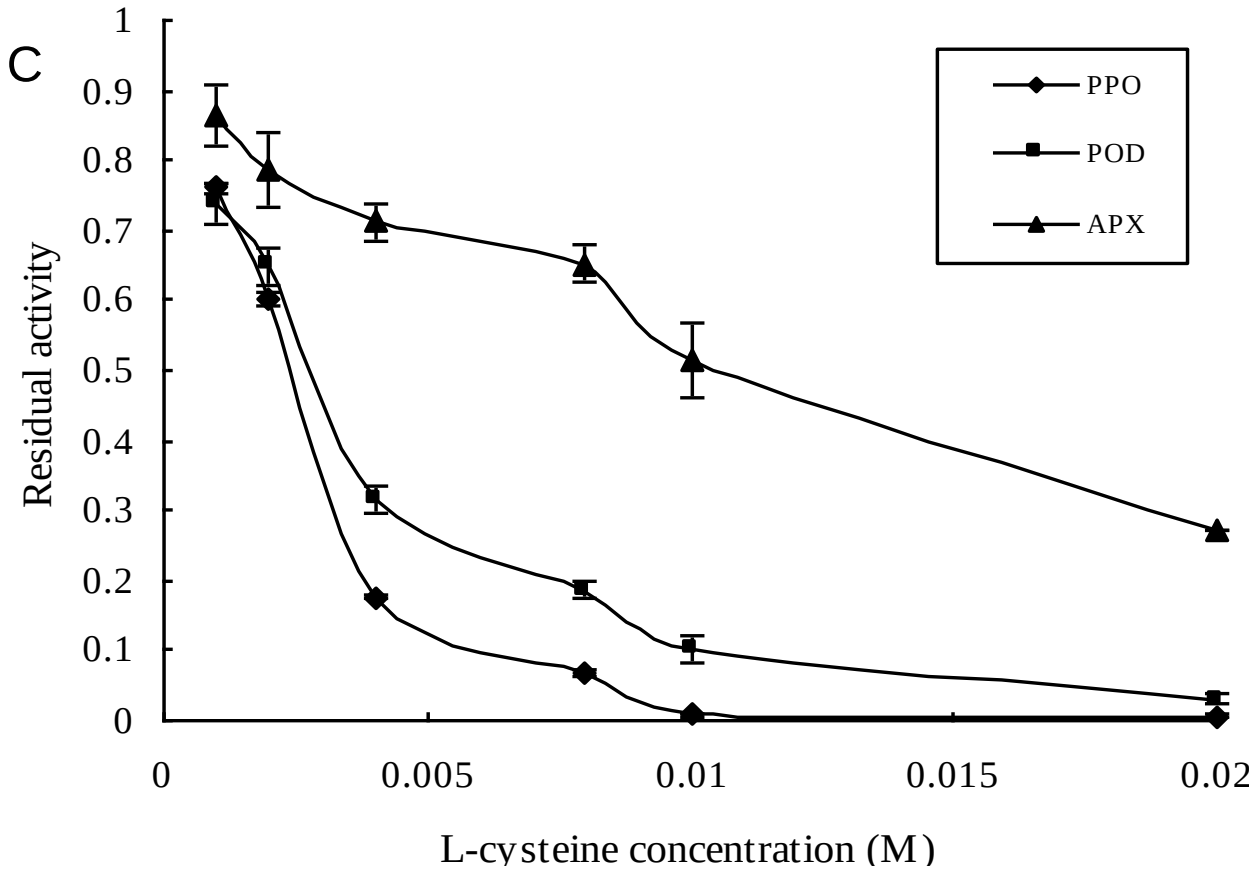


Figure 4. Lineweaver–(a), Burk plots of PPO for catechol, (b), POD for guaiacol, (c) APX for ascorbic acid and (d), H_2O_2.




b
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
Residual activity
0
0.5
1
1.5
2
Citric acid concentration (M)
PPO
POD
APX


C
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Residual activity
0
0.005
0.01
0.015
0.02
L-cysteine concentration (M)
PPO
POD
APX

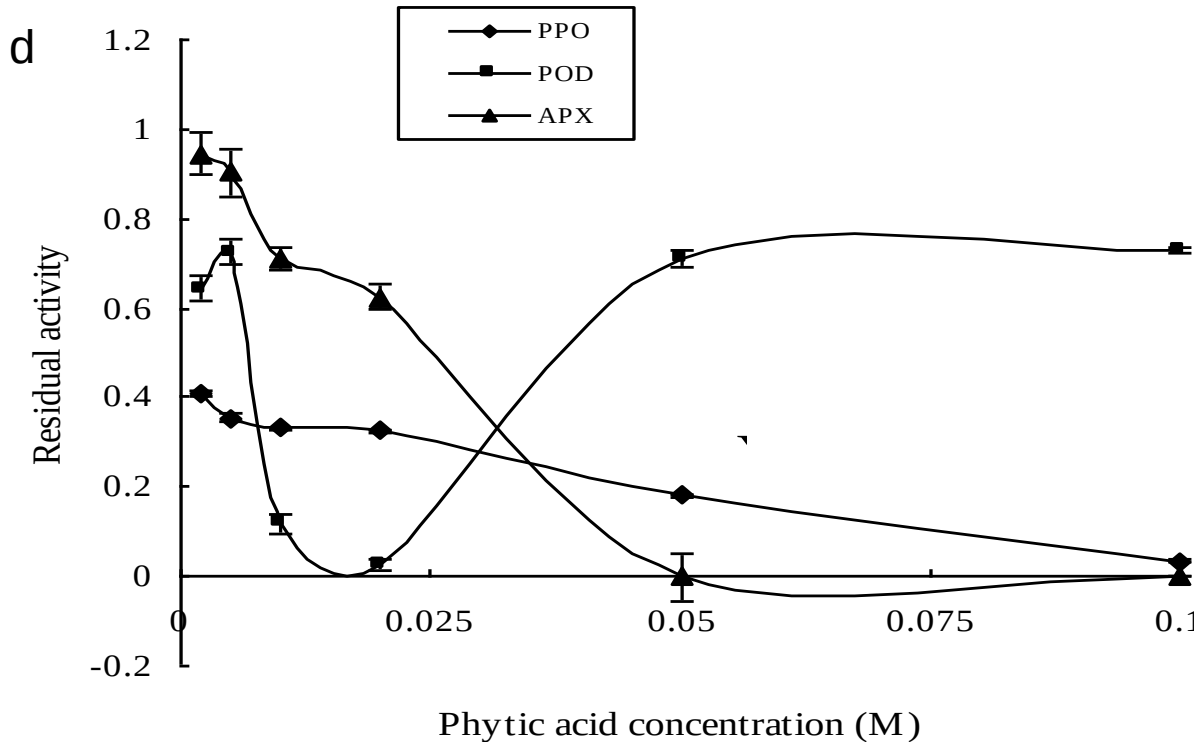


Figure 5. Effects of inhibitors on the activities of PPO, POD and APX. (a) ascorbic acid, (b) citric acid resorcinol, (c) L-cysteine, (d) phytic acid.

PPO (Dogan et al., 2002; Yang et al., 2001), and the effectiveness of each inhibitor varied with the enzyme from different sources. It was shown that the inhibition of PPO from butter lettuce by 10 mM ascorbic acid was 96.6% (Gawlik-Dziki et al., 2008), but the inhibition of PPO from chufa corms by 0.1 M ascorbic acid was 54.9% (Sun et al., 2009).

Conclusion

The enzymatic browning of Yali pear may be caused mainly by PPO and POD at a low temperature, by APX at a high temperature. Appropriate heat treatment and acidic condition could inhibit activities of the three enzymes. The inhibitory effects of the four inhibitors on each enzyme differed, and ascorbic acid, citric acid and L-cysteine were useful inhibitors. Results suggest that the composite inhibitor is more appropriate for processing, and a combined approach could be further tested to preserve Yali pear from browning using the results obtained above.

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