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Purification and Characterization of Polyphenol Oxidase from Rape Flower

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ABSTRACT: The purification and partial enzymology characteristics of polyphenol oxidase (PPO) from rape flower were studied. After preliminary treatments, the crude enzyme solution was in turn purified with ammonium sulfate, dialysis, and Sephadex G-75 gel chromatography. The optimal conditions and stability of PPO were examined at different pH values and temperatures. Subsequently, PPO was also characterized by substrate (catechol) concentrations, inhibitors, kinetic parameters, and molecular weight. Results showed that the optimal pH for PPO activity was 5.5 in the presence of catechol and that PPO was relatively stable at pH 3.5−5.5. PPO was moderately stable at temperatures from 60 to 70 °C, whereas it was easily denatured at 80−90 °C. Ethylenediaminetetraacetic acid, sodium chloride, and calcium chloride had little inhibitive effects on PPO, whereas citric acid, sodium sulfite, and ascorbic acid had strongly inhibitive effects. The Michaelis−Menten constant (K_m) and maximal reaction velocity (V_{max}) of PPO were 0.767 mol/L and 0.519 Ab/min/mL of the crude PPO solution, respectively. PPO was finally purified to homogeneity with a purification factor of 4.41-fold and a recovery of 12.41%. Its molecular weight was 60.4 kDa, indicating that the PPO is a dimer. The data obtained in this research may help to prevent the enzymatic browning of rape flower during its storage and processing.

KEYWORDS: *polyphenol oxidase (PPO), rape flower, catechol, purification, characterization*

INTRODUCTION

Rapeseed is an important oil crop in China and widely planted in the Yangtze River valley and northwestern provinces. In this country, its planting area is >100 million acres and ranks first in the world. It is worth mentioning that rape seed and rape flower are rich in nutrient and functional elements, such as proteins, amino acids, vitamins, minerals, polysaccharides, and flavonoids. Previously, some polysaccharides and two-protein-bound polysaccharides with antioxidant and immunity activities have been extracted from rapeseed.[1,2] Recently, ultrafiltration of polysaccharides from rapeseed has also been studied[3] Having attractive color and plenty of nutrition elements, rape flower is a kind of high-quality seasonal vegetable. Moreover, rape flower has been used as a medical raw material to cure dystocia in China for hundreds of years.

Polyphenol oxidase (PPO) comprises a group of copper-containing enzymes responsible for enzymatic browning occurring in fruits and vegetables. There are some studies about PPO from various fruits and vegetables, such as apple, banana, potato, bean, cabbage, and rapeseeds.[4−9] PPO catalyzes the ortho-hydroxylation of monophenol to *o*-diphenol, and the latter is further oxidized to highly reactive and colored *o*-quinone in the presence of molecular oxygen. Then, *o*-quinone can easily polymerize or react with proteins and amino acids to form complexly red, brown, or black pigments.[10] These pigments have unfavorable effects on the sensory and nutritional qualities of raw materials and products, which depreciate their commodity values. The browning degrees vary with phenolic compounds, reducing substances, oxygen concentration, metal ions, temperature, pH, and kind of PPO. The reactions happen easily after raw materials have suffered from physiological or mechanical injuries, cold storage, or harvest and unfavorably affect the acceptability and palatability of the resulting products. In this regard, enzymatic browning is one of the worst problems during harvesting, handling, processing, and storing various fruits and vegetables. Moreover, it is also a major reason for much quality loss during postharvest.[11,12]

During the blanching and drying processes of rape flower, enzymatic browning easily happens and produces a bad color, inevitably influencing the product quality. However, as far as our literature survey could ascertain, no information is available on the study of PPO from rape flower. This work was designated to isolate, purify, and characterize PPO from rape flower. The results will not only help us to prevent the enzymatic browning of rape flower during its storing and processing but also provide a theoretical basis for in-depth study of PPO from rape flower.

MATERIALS AND METHODS

Materials. Rape flower, 80% of which was in full bloom, was harvested from the suburbs of Hefei city in Anhui province, China, in May. Then, it was stored at −20 °C in a refrigerator (BC/BD-206AZ, Hefei Meiling Corp., China) until use. Catechol, phenylthiourea, sodium hydrogen phosphate, sodium dihydrogen phosphate, sodium hydroxide, sodium chloride, hydrochloric acid, acetic acid, sodium acetate, glycine, sodium sulfite, ascorbic acid, ethylene diamine tetraacetic acid (EDTA), ammonium sulfate, citric acid, polyethylene glycol M-6000 and Sephadex G-75 were purchased from Shanghai Chemical Reagents Company (Shanghai, China). Blue dextran, dextrans (5, 10, 20, 40, 60, 70, and 100 kDa), bovine serum albumin

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(BSA) and tetramethyl ethylene diamine were purchased from Xia-Si biochemical Co. (Beijing, China). All reagents were of analytical grade.

Crude Enzyme Preparation. The extraction procedures of the crude enzyme were carried out as described by Joseph.[13] After rape flower was thawed, its leaves, stems, and impurities were discarded. Then, 100 g of the rape flower was mixed with 1000 mL of distilled water at 0 °C. After homogenization in a paste mill (FDM-100, Zhengjiang Nanfang Corp., China), the solution was incubated at 4 °C for 30 min. Subsequently, the solution was centrifuged with a high-speed refrigerated centrifuge (A1301019, Shanghai Aice Corp., China) at 8000*g* and 4 °C for 30 min. The supernatant was collected as crude enzyme solution and stored at 4 °C until use.

Assay of PPO Activity. PPO activity was determined by monitoring the change in absorbance at 420 nm with a spectrophotometer (721W, Shanghai Xiguang Corp., China). The standard reaction mixture contained 1 mL of the crude enzyme solution and 5 mL of 0.2 mol/L catechol solution in 2 mL of acetic acid−sodium acetate buffer (pH 4.0). The absorbance change was recorded every 30 s for 5 min. Reaction velocity was obtained by calculating the linear slope of the absorbance−time curve. One unit (U) of PPO activity is defined as the amount of the enzyme that increases 0.001 of absorbance at 420 nm per minute per milliliter of PPO solution (0.001ΔAbs min^{-1} mL PPO $solution^{-1}$) due to oxidation of catechol.[10] Specific activity is defined as the change in absorbance per minute per milligram of protein (ΔAbs min^{-1} mg PPO $protein^{-1}$).

Effect of pH on PPO Activity. Catechol was used as substrate in these experiments. The pH values were varied over a pH range of 1.0−8.0 by application of different buffer systems: potassium chloride−hydrochloric acid (pH 1.0−2.0), glycine−hydrochloric acid (pH 2.5−3.5), acetic acid−sodium acetate (pH 4.0−5.5), and disodium phosphate−sodium dihydrogen phosphate (pH 6.0−8.0). Appropriate volumes of the crude PPO solution were added in the above buffers of pH from 1.0 to 8.0 at 0.5 unit intervals, and then PPO activity was measured. Prior to pre-incubation at 40 °C, 2.0 mL of buffers, the pH values of which varied from 1.0 to 8.0 at 0.5 intervals, was put in test tubes before 5 mL of 0.2 mol/L catechol solution. After the addition of 1.0 mL of the crude PPO solution, the resulting solution was further incubated for 10 min. Reaction was terminated by adding 3 drops of phenylthiourea. Then, the resulting solutions were cooled at 25 °C in a water bath (HH-3 Jiangsu Jintan Corp., China) for 5 min, followed by monitoring of the absorbance at 420 nm.

Effect of Temperature on PPO Activity. The optimum temperature was ascertained by measuring residual activity after PPO was incubated at various temperatures varying from 0 to 80 °C at 5 °C intevals, using acetic acid−sodium acetate buffer (pH 4.0). After the substrate was incubated at various the temperatures mentioned above for 2 min, the crude enzyme solution was added. Two milliliters of buffers and 5 mL of 0.2 mol/L catechol were together put into test tubes, and then the solution was pre-incubated at various temperatures for 2 min. After the addition of 1.0 mL of the crude PPO solution, the resulting solution was further incubated. After 10 min, the reaction was terminated by adding 3 drops of phenylthiourea. Then, the resulting solutions were cooled at 25 °C for 5 min, followed by monitoring absorbance at 420 nm.

Temperature Stability of PPO. After 2.0 mL of acetic acid−sodium acetate buffer (pH 4.0) was poured into test tubes and pre-incubated at 60, 70, 80, and 90 °C for 2 min, respectively, 1.0 mL of the crude PPO solution was added. The resulting solutions were incubated at 60, 70, 80, and 90 °C for 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, and 35 min, respectively, before 5.0 mL of 0.2 mol/L catechol solution was quickly poured. The resulting solutions were cooled at 25 °C for 5 min before the absorbance was monitored at 420 nm.

pH Stability of PPO. The crude PPO solution was incubated at 40 °C and pH ranging from 2.0 to 8.0 at 1.0 intervals for 10 min. Then, residual activity was assayed immediately as described.

Effect of Substrate Concentration on PPO Activity and Enzyme Kinetics. After aliquots of 2.0 mL of acetic acid−sodium acetate buffer (pH 4.0) and 5.0 mL of catechol solution at different concentrations were quickly poured into test tubes, the solutions were preheated at 40 °C for 2 min. After the addition of 1.0 mL of the crude PPO solution, the temperature of the solutions remained constant at 40 °C for 10 min. After that, residual activity was measured immediately as described. Reaction rate was defined as the change value in absorbance per minute, and the change of A_{420} per minute was used to indicate reaction rate. The kinetic data were drawn as 1/specific activity ($1/V$) versus 1/substrate concentration ($1/[S]$). Calculated by the experimental data, the linear regression equation was as follows:

$$Y = 1.48X + 1.93 \quad (R^2 = 0.995) \tag{1}$$

The Lineweaver−Burk plot is presented in Figure 6, and the Michaelis−Menten constant (K_m) and maximum reaction velocity (V_{max}) parameters were computed by the obtained slope and intercept, respectively.[14,15]

Effects of Inhibitors on PPO Activity. Ascorbic acid (vitamin C), citric acid, sodium sulfite ($NaHSO_3$), ethylenediaminetetraacetic acid (EDTA), sodium chloride (NaCl), and calcium chloride ($CaCl_2$) were selected as inhibitor candidates of PPO using catechol as the substrate in the experiments. The concentration of all inhibitors was fixed at 4%. Each inhibitor (0, 0.2, 0.4, 0.8, 1.2, 1.6, and 2.0 mL) was added into test tubes, respectively, and the final volume (2.0 mL) was obtained by adding acetic acid−sodium acetate buffer (pH 4.0). After the test tubes were preheated at 40 °C for 2 min, 1.0 mL of the crude PPO solution was added. After a 20 min hold, residual activity was measured immediately as described. The crude PPO solution incubated with the buffer without any inhibitors was used as control and referenced as 100% of enzyme activity.

Precipitation with $(NH_4)_2SO_4$ at 30% Saturation. Sodium hydrogen phosphate−sodium dihydrogen phosphate buffer (0.1 mol/L, pH 6.5) at 0 °C was used as the solvent of ammonium sulfate (30% saturation). After 1000 mL of the ammonium sulfate solution was added to 100 mL of the crude enzyme solution with slow stirring at 0 °C, the resulting solution was kept at 4 °C overnight. Then, the turbid solution was centrifuged at 8000*g* and 0 °C for 30 min before the precipitate was collected. Finally, PPO activity and protein content were determined as described.

Precipitation with $(NH_4)_2SO_4$ at 80% Saturation. Sodium hydrogen phosphate−sodium dihydrogen phosphate buffer (0.1 mol/L, pH 6.5) at 0 °C was used as the solvent of ammonium sulfate (80% saturation). After 100 mL of the ammonium sulfate solution was added to 1.0 g of the precipitated enzyme with slow stirring at 0 °C, the mixture was kept at 4 °C overnight. After centrifugation at 8000*g* and 0 °C for 30 min, the precipitate was collected. The precipitate was dissolved in 50 mL of 0.1 mol/L sodium hydrogen phosphate−0.1 mol/L sodium dihydrogen phosphate buffer (0 °C, pH 6.5). PPO activity and protein content were determined as described.

Dialysis by Dialysis Bag. To desalt and remove some hybrid proteins of small molecules, the enzyme solution was put into a 10 kDa dialysis bag (MD25, Shanghai Ouwei Corp., China) and dialyzed against 0.1 mol/L sodium hydrogen phosphate−0.1 mol/L sodium dihydrogen phosphate buffer at 4 °C for 24 h. The dialysate was replaced every 3 h in the first 12 h and every 4 h in the second 12 h. The resulting solution was concentrated with polyethylene glycol M-6000 at 4 °C for 24 h. PPO activity and protein content were determined as described.

Sephadex G-75 Gel Chromatography and Determination of Molecular Weight of PPO. Two milliliters of the concentrated enzyme solution was submitted to a Sephadex G-75 column (1.6 cm × 60 cm) pre-equilibrated with 0.1 mol/L sodium hydrogen phosphate−0.1 mol/L sodium dihydrogen phosphate buffer (0 °C, pH 6.5). Then, the column was eluted with 0.1 mol/L sodium hydrogen phosphate−0.1 mol/L sodium dihydrogen phosphate buffer (0 °C, pH 6.5) at a flow rate of 0.2 mL/min, and the volume of every collecting tube was 2.4 mL with an automatic collector (SBS-100, Shanghai Luxi Corp., China). Absorbance at 420 nm was detected with a spectrophotometer (HD-21-88, Shanghai Qite Corp., China), and elution peaks were recorded. The purified enzyme was lyophilized.

To prepare the calibration curve, dextran standards of different molecular weights (5, 10, 20, 40, 60, 70, and 100 kDa) were used. Each

was submitted to a Sephadex G-75 column (1.6 cm × 60 cm) and operated at the same conditions. The logarithms of molecular weights (lg M) were plotted against the elution volumes (V_e) of dextran standards. The linear regression equation is as follows:

$$\lg M = -0.0086V_e + 5.11 \quad (R^2 = 0.982) \qquad (2)$$

The elution volume of PPO was plotted in the same graph, and the molecular weight was determined.

Protein Determination. The protein concentration of PPO from rape flower was determined according to the method of Bradford,[16] using bovine serum albumin (BSA) as standard, and the absorbance was measured at 595 nm.

Amino Acid Composition Determination. The amino acid composition of PPO obtained finally was measured according to the modified method described by Sun et al.[1] The obtained amino acids were identified and quantified using a Sycom S-433D automatic 123 amino acid analyzer (Sykam, Eresing, Germany).

Statistical Analysis. Three analyses of each sample were made, and each experiment was repeated in triplicate (n = 3). The standard deviation and mean value were calculated from the data obtained.

RESULTS AND DISCUSSION

Effect of pH on PPO Activity. The plot of relative activity (absorbance at 420 nm) versus pH value of the crude enzyme solution is summarized in Figure 1. It is apparent that the relative activity rose drastically with the increase of pH values. A small peak was observed at pH 4.0. Then, the relative activity decreased slowly before pH 5.0. However, it is noteworthy that the activity was improved again when the pH was >5.0. A big peak appeared at pH 5.5, indicating that PPO exerted its maximum activity at this pH value. Overall, PPO showed high activity in the pH range of 3.5−8.0, and it is further inferred that such a pH range is inappropriate for the storage and processing of rape flower. The two peaks could be explained as follows. In rape flower, there might be two isoenzymes or isomerases of PPO, which have similar molecular weights. As they distribute in different cells or tissues, they evolve different activity. This result is consistent with previous reports that there were PPO isoenzymes in plants, such as Bartlett pears, dog-rose fruit, and avocado fruit.[17−20] The optimum pH of PPO varies with various plant origins. Differences in optimum

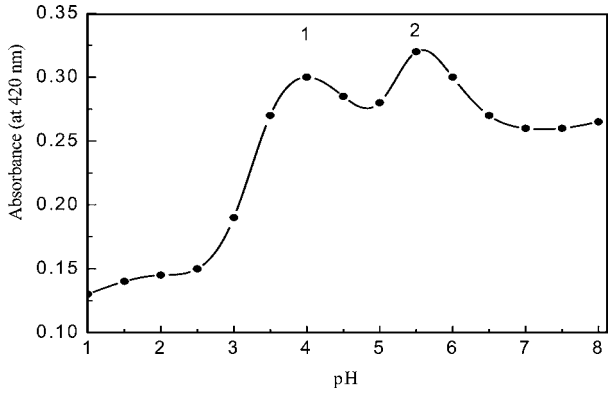

Figure 1. pH activity profile for PPO from rape flower. The buffers used were potassium chloride−hydrochloric acid (pH 1.0−2.0), glycine−hydrochloric acid (pH 2.5−3.5), acetic acid−acetic acid sodium (pH 4.0−5.5), and disodium phosphate−sodium dihydrogen phosphate (pH 6.0−8.0), respectively. The reaction was carried out at 40 °C. Reaction mixtures contained 5 mL of 0.2 mol/L catechol solution, 2.0 mL of the buffers, and 1.0 mL of the crude enzyme extract.

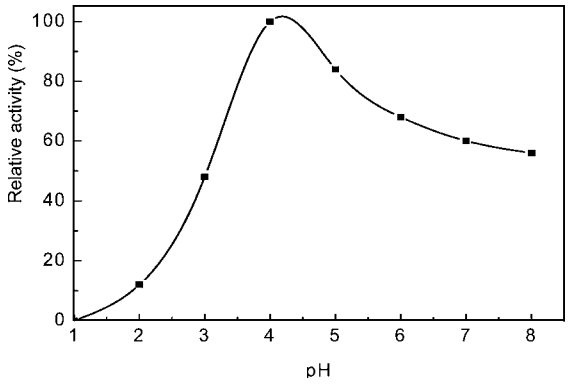

Figure 2. pH stability of PPO from rape flower. Catechol was used as substrate. The pH values of solutions increased from 1 to 8 at intervals of 1 unit. PPO activity was expressed as relative activity (%) compared with the activity determined at pH 4.0.

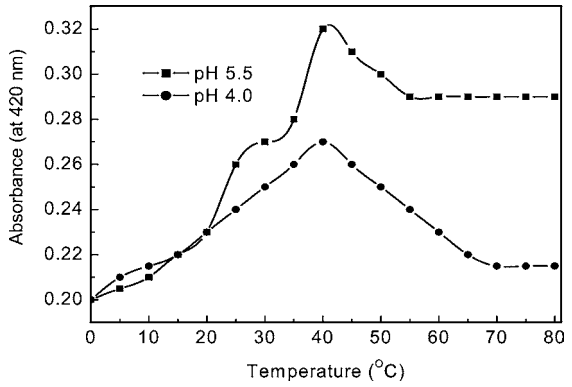

Figure 3. Effect of temperature on PPO activity from rape flower. Substrate used was catechol, which was dissolved in 2.0 mL of acetic acid−acetic acid sodium buffer (pH 4.0). The reaction mixture except the crude enzyme was incubated for 2 min at tested temperatures from 0 to 80 °C at 10 °C intervals. After the crude enzyme was added, the reaction mixture was incubated at the tested temperatures for 1 min. Subsequently, the activity was measured spectrophotometrically at 420 nm as quickly as possible.

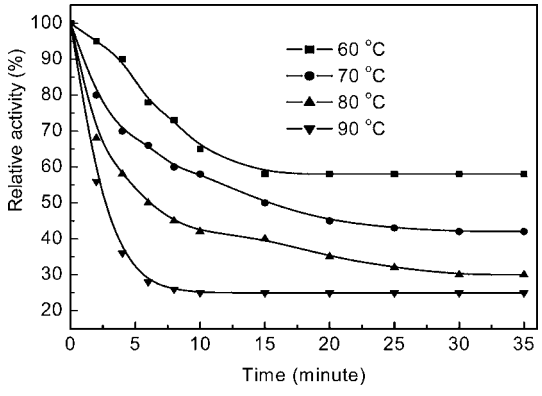

Figure 4. Thermal stability of PPO from rape flower. Catechol was used as substrate, which was dissolved in 2.0 mL of acetic acid−acetic acid sodium buffer (pH 4.0) and preheated in 60, 70, 80, and 90 °C for 2 min, respectively. After the crude enzyme was added, the mixture was incubated at the tested temperatures for certain times from 0 to 35 min at 5 min intervals. The activity was measured spectrophotometrically at 420 nm as quickly as possible.

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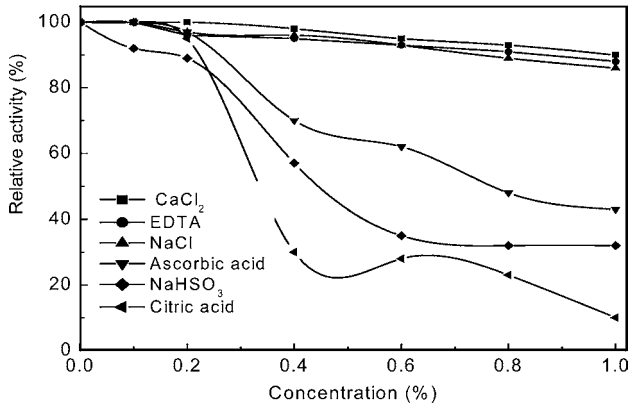

Figure 5. Effects of various inhibitors on PPO activity. Inhibitors were $CaCl_2$, EDTA, NaCl, ascorbic acid, $NaHSO_3$, and citric acid. The concentration of the inhibitors was fixed at 4% by adding acetic acid–sodium acetate buffer (pH 4.0). The solution without any inhibitors was used as control and referenced as 100% of enzyme activity.

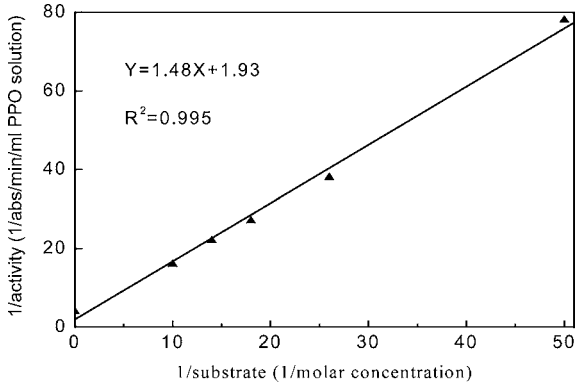

Figure 6. Linerweaver–Burk plot of PPO using catechol as substrate.

pH have also been reported for PPO extracted from strawberry and tea leaf. Furthermore, optimal pH also depends on the nature of phenolic substrates, temperatures, extraction methods, etc.[21−23]

Effect of pH on PPO Stability. To determine PPO stability against valid acidity, the pH values of the crude enzyme solution were adjusted in a range of 1.0–8.0, followed by pre-incubation at 40 °C for 10 min. Figure 2 shows the relative activity of rape flower as a function of initial pH value. It is clear that the profile had a typical bell-shaped curve. The relative activity increased with the increase of pH from 1.0. Later, there appeared a maximum at pH 4.0. After that, the relative activity decreased gradually until it finally reached 60% at pH 8.0. Collectively, PPO was more stable within a pH range of 3.5–5.5, and its relative activity remained about 80%. However, the enzyme was greatly inactive or unstable within a pH range of 1.0–3.5. As pH values decreased from 3.5, which was far from its optimum pH, the relative activity significantly descended from 83.0 to 1.4%. It can be inferred that suitable pH values for the storage of rape flower are below 3.0.

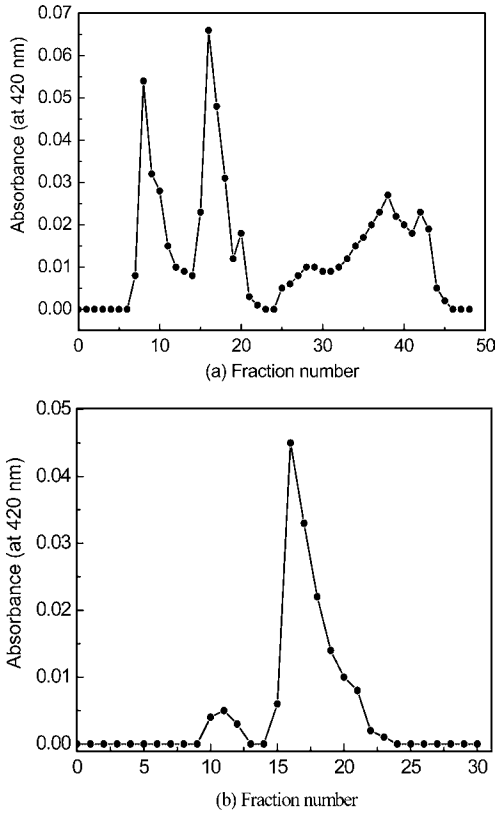

Figure 7. Chromatographic profile of PPO: (a) elution profile of PPO activity from Sephadex G-75 gel column chromatography (I); (b) elution profile of PPO activity from Sephadex G-75 gel column chromatography (II).

Effect of Temperature on PPO Activity. The effect of temperature (0–80 °C) on the relative activity of PPO at pH 4.0 and 5.5 is presented in Figure 3. The relative activity rose with the increase of temperature from the initial and even increased nearly in first order at pH 4.0. Then, maxima appeared at 40 °C, namely, 0.27 and 0.32 at pH 4.0 and 5.5, respectively. In other words, the relative activity was slightly higher at pH 5.5 than at pH 4.0. Later, it decreased despite a further increase in temperature. The observations show that the optimum temperature for PPO from rape flower is at 40 °C, which is not compatible for the storage and processing of rape flower. The optimum temperatures of enzymes are not only different from plant origins but also depend on plant growing conditions. In sum, an appropriate temperature is necessary for enzymes to achieve maximum activity. As can be seen from Figure 3, PPO in general exerted higher activity at pH 5.5 than

Table 1. Purification Table of Polyphenol Oxidase from Rape Flower[a]

purification step	activity (U/mL)	protein (mg/mL)	specific activity (U/mg)	purification factor	recovery (%)
crude extract	2740.7 ± 18.9	6.39 ± 0.02	428.80 ± 5.04	1.00 ± 0.05	100.7 ± 0.25
30% $(NH_4)_2SO_4$	1060.3 ± 7.55	1.42 ± 0.01	746.48 ± 4.16	1.74 ± 0.03	38.69 ± 0.09
80% $(NH_4)_2SO_4$	830.14 ± 5.55	0.91 ± 0.01	912.09 ± 2.91	2.13 ± 0.03	30.29 ± 0.08
dialysis (10 kDa)	540.25 ± 5.47	0.42 ± 0.01	1285.7 ± 9.71	3.00 ± 0.04	19.71 ± 0.09
Sephadex G-75	340.61 ± 4.68	0.18 ± 0.01	1888.9 ± 7.45	4.41 ± 0.04	12.41 ± 0.07

[a]These values were obtained in triplicate.

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Table 2. Amino Acid Composition of Polyphenol Oxidase from Rape Flower

amino acid (mol %)														
Asp	Thr	Ser	Glu	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg
17.34	6.04	9.91	14.39	9.29	9.53	6.43	1.32	4.50	5.61	1.15	2.37	4.47	7.81	3.85

at pH 4.0. However, it was more remarkable that its activity became compromised at temperatures below 10 °C or above 70 °C. There may be two reasons to explain the phenomena. On the one hand, appropriate activation energy is necessary for substrates to complete enzymatic reaction. Low temperature means that substrates have only low activation energy, which is not enough to fully achieve enzymatic reaction. Therefore, PPO exerted low activity at temperatures below 10 °C. On the other hand, heat denaturation of enzymes might occur at high temperature. At temperatures above 70 °C, enzymes may in part or permanently lose their activity. Therefore, PPO displayed lower activity at the tested temperature above 70 °C. The results indicate that the appropriate temperatures to store and process rape flower are below 10 °C and above 70 °C in view of PPO activity.

Effect of Temperature on PPO Stability. The thermal stability of PPO was assayed by incubating the crude PPO in a temperature range of 60−90 °C for 5−35 min, and the results were shown in Figure 4. Obviously, the relative activity decreased within the initial 15 min in a temperature range of 60−90 °C. After treatment at 60 and 70 °C for 15 min, PPO retained only 58.4 and 45.6% of its activity, respectively, whereas at 80 and 90 °C for the same time interval, it retained only 35.2 and 25.7% of its activity, respectively. Later, the relative activity showed nearly no change although the time further increased. The above results indicated that PPO was tolerant in a temperature range of 60−70 °C, whereas it was partly denatured in a temperature range of 80−90 °C. This may due to the unfolding of the tertiary structure of PPO at higher temperatures, so that the relative activity of PPO decreased. It has been reported that PPOs of plum and pear were stable at 60−70 °C and that PPOs of kiwi fruit, mango, Satsuma mandarin, and head lettuce had relatively high thermal stabilities.[24,25] Collectively, the data indicate that high-temperature blanching and low-temperature storage are necessary methods to control the enzymatic browning caused by PPO in rape flower.

Effects of Inhibitors on PPO Activity. The effects of six different inhibitors, namely, EDTA, NaCl, $CaCl_2$, $NaHSO_3$, citric acid, and ascorbic acid, on PPO were examined to determine their potential for inhibition of catechol oxidation by rape flower, and the results are shown in Figure 5. The inhibitive effects of the inhibitors positively correlated with the increase of concentration. Nevertheless, EDTA, NaCl, and $CaCl_2$ had only weak inhibitive effects on PPO activity. As concentrations increased, the relative activity not only had a slight decrease but also was in first-order. On the contrary, $NaHSO_3$, citric acid, and ascorbic acid displayed good inhibitive effects. Especially, $NaHSO_3$ displayed the strongest inhibitive effect among the three inhibitors at concentrations from 0.1 to 0.2%. Surprisingly, after the concentrations exceeded 0.2%, citric acid displayed the best inhibitive effect, followed by $NaHSO_3$ and ascorbic acid. By comparison of these six inhibitors, the order of the inhibition effects is as follows: citric acid > $NaHSO_3$ > ascorbic acid > NaCl > EDTA > $CaCl_2$.

There are several types of mechanisms of PPO activity inhibition. First, some reagents could chelate metallic ions, which act as cofactors of enzymes and reduce certain enzymatic reaction. As a chelator of metallic ions, EDTA has certain specific effects to chelate copper ions and thereby inhibit PPO activity to some extent. Second, oxygen is a prerequisite for PPO to transform monophenol to diphenol. As some substances were dissolved in systems, some oxygen was repelled, and therefore PPO activity was inhibited. In the same way, with the addition of NaCl and $CaCl_2$, oxygen in the solution had appropriate reduction so that PPO activity was inhibited.[26] Furthermore, high salt concentrations might enhance hydrophobic interactions, causing enzyme aggregation and further loss of activity. The phenomenon that high salt concentration suppressed enzymes was also observed by Perera et al., who found that NaCl at concentrations above 1 mol/L had an unfavorable effect on lipolytic activity.[27] Third, some compounds might compete with substrates and favorably combine the binding sites of enzymes so that fewer of the substrates were catalyzed. Similarly, some competitive inhibitors usually prevent the accumulation of quinone and thus inhibit the formation of melanin, which is further transformed to stable colorless products.[8] Fourth, reducing agents, such as $NaHSO_3$, may oxidize some active groups, such as sulfhydryl groups, in enzymes so that these enzymes lose part or all of their activity. Moreover, reducing agents also inhibit the hydroxylation of L-tyrosine into 3,4-dihydroxyphenylalanine or block enzymatic browning through interacting with *o*-quinone and other substances. Because of its high effectiveness and low price, $NaHSO_3$ is broadly applied in vegetable and fruit processing as a color-protecting agent. Fifth, when the concentrations of acidic substances increased, the solution pH values were far from the optimum pH of PPO, which was inactive in an acidic environment. As a kind of organic acid, citric acid not only decreased pH value, which was far from the optimum pH of PPO, but also acted as a chelator to chelate copper ions of the enzyme.[7] For these two reasons, citric acid had strongly inhibitive effects on PPO. Finally, ascorbic acid and its isomers are commonly used as color fixatives in the processing of fruits and vegetables. On the one hand, ascorbic acid could reduce quinone to a phenolic compound and hence prevent brown pigment formation until nearly all ascorbic acid is exhausted.[20] On the other hand, ascorbic acid is also an organic acid and could inhibit PPO activity by raising the system acidity. In short, PPO of different sources may similarly react with inhibitors. Nevertheless, different inhibitors exert different effectiveness on various PPOs so that particular measures are needed to control certain systems.

Effect of Substrate Concentration on PPO Activity and Kinetic Studies. The effect of catechol concentration on PPO activity was further investigated, and the results are presented in Figure 6. When catechol concentration was low, the relative activity increased in a dose-dependent manner. However, after the concentration exceeded 0.14 mol/L, it increased very little with further increase of the concentration. Because catechol is an inactivating reagent or an inhibitor of PPO at high concentrations, its activity decreases at high substrate concentrations in some cases. Fernandez-Lafuente et

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al.[28] reported that when catechol concentrations exceeded 1.0 mmol/L, the activity of catechol 2,3-dioxygenases was inhibited.

K_m and V_{max} values for rape flower PPO with catechol as substrate are 0.767 mol/L and 0.519 Ab/min/mL of PPO solution, respectively. This K_m value is near that (0.6825 mol/L) of cabbage PPO and was greatly higher than that (0.044 mol/L) of mamey PPO, using the same substrate.[7,29]

Purification. To determine the enzymatic characterization of PPO from rape flower, the crude enzyme was further purified, and the results are presented in Table 1. The crude PPO had a specific activity of 428.80 U/mg protein. After purification with 30 and 80% ammonium sulfate in turn, PPO activity increased 1.74- and 2.13-fold, respectively. PPO obtained was further loaded onto a Sephadex G-75 column, and the elution profile is shown in Figure 7a. Notably, six peaks appeared in the elution profile. Fractions 10 and 11 and 15−18 from this column were pooled, respectively. After concentration by dialysis bag, PPO activity was displayed in fractions 15−18, and its purification factor was 3.00. Later, PPO was further loaded onto the Sephadex G-75 column, and the elution profile is shown in Figure 7b. Two peaks appeared in fractions 9−13 and 14−25, respectively. After concentration, PPO activity was displayed in fractions 14−25. PPO showed a 4.41-fold increase in purity and a protein content of 0.18 mg/mL, after purification.

According to the linear regression equation ($\lg M = -0.0086V_e + 5.11$), the apparent molecular mass of PPO from rape flower was 60.4 kDa, which coincided with 60 kDa PPO from broad bean leaves.[30] Other researchers also reported that the molecular weights of PPO ranged from 45 to 67 kDa.[31,32]

Amino Acid Composition. The amino acid composition of PPO obtained finally is listed in Table 2. It is evident that the protein portion of PPO consisted of 15 amino acids, namely, aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, and arginine. In particular, aspartic acid was the most abundant amino acid in PPO from rape flower, and its content was similar to that of cabbage PPO determined by Fujita et al.[8] PPO from rapeseed flower was rich in aspartic acid, serine, glutamic acid, glycine, and alanine, but the contents of methionine, tyrosine, and phenylalanine were very low.

The present study for the first time reports the purification and characterization of PPO from rape flower. PPO exerted its maximum activity at pH 5.5 and was relatively stable at pH 3.5−5.5. Its optimum temperature was 40 °C at either pH 4.0 or 5.5. It was relatively stable at lower temperatures, and most of its activity was even kept at 60−70 °C for 35 min. However, it was easily denatured at higher temperatures, and most of its activity was lost at 80−90 °C for 5 min. EDTA, NaCl, and $CaCl_2$ had slightly inhibitive effects on PPO, whereas citric acid, $NaHSO_3$, and ascorbic acid showed strongly inhibitive effects. In short, storing at low temperatures, blanching at higher temperatures, and choosing suitable inhibitors are effective measures to control enzymatic browning caused by PPO.

K_m and V_{max} values of rape flower PPO with catechol as substrate were 0.767 mol/L and 0.519 Ab/min/mL of PPO solution, respectively. After purification with ammonium sulfate, dialysis with a dialysis bag, and Sephadex G-75 gel chromatography in turn, the activity of the final PPO increased 4.41 times compared with the crude enzyme. The molecular weight of the enzyme was 60.4 kDa by Sephadex G-75 gel chromatography. Overall, the results obtained in this study will substantially aid in controlling the enzymatic browning happening in rape flower, which could be exploited as a new source of functional food and medicine in the future.

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