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Partial purification and characterization of polyphenol oxidase and peroxidase from chestnut kernel

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ABSTRACT

Presently there is very little information about the properties of polyphenol oxidase (PPO) and peroxidase (POD) from chestnut, which are both related with chestnut browning. The chestnut PPO and POD were partially purified by acetone extraction, 30–80 g/100 mL ammonium sulfate fractionation, DE-52 anion-exchange column. PPO and POD activity increased 4.6-fold and 9.52-fold, its yields were 2.03 U/100 U and 4.21 U/100 U, and its specific activity was 1375 and 16,500 U mg^{-1} protein after DE-52. SDS-PAGE results indicated that the molecular mass of PPO and POD was approximately 32.5 and 30.3 kDa, respectively. Its optima pH value for catalyzing catechol and 2-hydroxy phenol was both 7.0. The optimum temperature of PPO and POD for catalyzing catechol and 2-hydroxy phenol was found to be 40 and 50 °C, respectively. PPO showed the highest affinity to catechol among all our selected substrates. The K_m and V_{max} of PPO for substrate catechol were 92 mmol/L and 1.53 △OD/min. The K_m and V_{max} of POD for substrate 2-hydroxy phenol were 49 mmol/L and 0.2373 △OD/min. The investigation on the properties of PPO and POD is greatly important for minimizing the losses caused by fruit browning during chestnut processing.

1. Introduction

Polyphenol oxidase (PPO: EC 1.14.18.1) and peroxidase (POD: EC 1.11.1.7) are widely distributed among higher plants. PPO is the principal enzyme involved in enzymatic browning. PPO is a copper-containing enzyme responsible for hydroxylation of monophenols to *o*-diphenols and their further oxidation to colored and highly reactive *o*-quinones (Martinez & Whitaker, 1995). These *o*-quinones readily polymerize and react with endogenous amino acids and proteins to form complex brown pigments (Matheis & Whitaker, 1984). This leads to organoleptic and nutritional modifications, thus depreciates the food value. PPO has been isolated from various sources, such as strawberry (López-Serrano & Ros Barceló, 1996), green bean (Mdluli, 2005), persimmon (Navarro, Tárrega, Sentandreu, & Sentandreu, 2014), Turkish tea leaf (Altunkaya, 2014) and Jackfruit (Tao, Yao, Qin, & Shen, 2013), and its properties have been extensively studied.

POD, as another oxidoreductase, participates in several metabolic plant processes such as the catabolism of auxins, lignification of the cell wall (Aquino-Bolaños & Mercado-Silva, 2004). It may also be involved in enzymatic browning reaction which catalyzes the oxidation of various electron donors with H_2O_2 and carries a 'b'-type haem as prosthetic group (Elstner & Heupel, 1976).

Chestnut (*Castanea mollissima*), a well-known nut fruit in China, has a long history of reported health effects related to its nutritional composition, especially the absence of gluten and the presence of unsaturated fatty acids (De Vasconcelos, Bennett, Rosa, & Ferreira-Cardoso, 2009). Enzyme browning is easily happened during chestnut storage and processing (Shi, Li, Zhu, & Zhou, 2011), which will affect its nutrition, flavor and color. Many authors (Chisari, Barbagallo, & Spagna, 2008; Gonzlez, de Ancos, & Cano, 2000) studied the properties of crude extract of PPO and POD simultaneous, but no much was done for characterizing purified PPO and POD. PPO and POD of marula fruit was purified, and their properties of PPO and POD was purified and characterized (Mdluli, 2005). PPO of Henry chestnuts has been purified and characterized (Xu, Zheng, Meguro, & Kawachi, 2004). However, information of the properties of purified chestnut PPO and POD is very limited. The purposes of this study were (1) to obtain partially purified PPO and POD of chestnut by acetone extraction, 30–80 g/100 mL ammonium

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sulfate fractionation, DE-52 anion-exchange column; (2) to analysis its chemical properties, such as molecular weight, optimum pH and temperature, substrate specificity.

2. Materials and methods

2.1. Materials

Chestnut variety, Jiangsuluhe, was obtained from a local market in Nanjing, Jiangsu province, China. Chestnut fruits were stored in a cold room at 4 °C for half year, when their POD and PPO enzyme activities are high (Duan, 2006).

DEAE−52 was obtained from Whatman Chemicals Separations, Ltd, Clifton, NJ, USA. The other biochemicals used in this study were of analytical reagent grade. Acetone, ammonium sulfate, disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride were all purchased from Shanghai Chemical Reagent Company, Nanjing, China. Catechol and 2-methoxy phenol were purchased from sigma (Sigma−Aldrich Company, Shanghai, China).

2.2. PPO and POD crude enzyme extraction from chestnut

Chestnut kernel were homogenized with4 times the sample volume of acetone at −20 °C, then the puree was centrifuged at 12,000 g for 20 min and collected precipitate as acetone powder with ratio of 1:10 (w/w). The acetone powder was redissolved in 0.05 mol/L sodium phosphate buffer (pH 7.0), and the supernatant was collected at 12,000 g for 20 min at 4 °C. The enzyme solution was fractionated with solid ammonium sulfate (30−80 g/100 mL), the precipitate was collected by centrifugation at 12,000 g for 20 min at 4 °C, then redissolved and dialyzed in 10 kDa dialysis tubing (Sigma−Aldrich Company, Shanghai, China) at 4 °C against the same buffer. The dialyzed solution were collected and condensed in 10 kDa cutoff dialysis tubing with polyethylene glycol of 20 kDa, then be lyophilized into crude enzyme powder.

2.3. Ion-exchange chromatography on DEAE-52

For further purification, the crude enzyme powder was dissolved in small volume of 0.05 mol/L sodium phosphate buffer (pH 7.0) with ratio of 1:5 (w/w), and loaded it onto a DEAE-52 anion-exchange column, preequilibrated with 0.05 mol/L sodium phosphate buffer (pH 7.0). The column was eluted with the same buffer at the flow rate of 30 mL/h and linear gradient of NaCl concentration from 0.15 to 1.5 mmol/L. Fractions of 4 mL were collected and assayed for PPO and POD activities. The elution process continued until no absorbance at 280 nm was detected.

2.4. Enzyme assay

PPO was assayed with 0.1 mol/L catechol as a substrate by a spectrophotometric procedure (Lowry, Rosebrough, Farr, & Randall, 1951). The assay was performed with 2 mL of 0.05 mol/L sodium phosphate buffer (pH 7.0), 0.7 mL of catechol and 0.3 mL of enzyme solution purified by ion exchange. The increase in absorbance at 410 nm at 37 °C was recorded automatically for 30 s. One unit of enzyme activity was defined as the amount of the enzyme which caused a change of 0.001 in absorbance per minute. Specific activity of PPO was calculated by enzyme activity per milligram of protein.

POD assays were done with 2-hydroxy phenol (0.05 mol/L) and 2 mL/100 mL H_2O_2 in sodium phosphate buffer (Nakano & Asada, 1981). The assay was performed with 2 mL of 0.05 mol/L sodium phosphate buffer (pH 7.0), 1.0 mL of 2-hydroxy phenol, 1.0 mL of H_2O_2 and 0.1 mL of enzyme solution purified by ion exchange. The increase in absorbance at 470 nm at 37 °C was recorded automatically for 30 s. One unit of enzyme was defined as 0.001 change in absorbance per minute. Specific activity of POD was calculated by enzyme activity per milligram of protein.

2.5. Protein determination

Protein content was determined by the dye-binding method (Bradford, 1976) with bovine serum protein as the standard. The assay was performed with 0.1 mL of enzyme solution, 0.9 mL distilled water, and 5 mL dye for 2 min at 595 nm. The standard curve was prepared with various volume of bovine serum protein (0, 0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL, 1 mL), 5 mL dye, then adding distilled water to 6 mL for 2 min at 595 nm.

2.6. SDS-PAGE electrophoresis

Enzyme purity and molecular weight were analyzed by SDS-PAGE in a DYCZ-24D Electrophoresis (Beijing Liuyi Instrument Factory, Beijing, China) with 12 g/100 mL polyacrylamide gel and 4 g/100 mL stacking gel. Proteins were stained by the Coomasie Blue staining technique and molecular weight was estimated by a commercial kit (Sigma−Aldrich Co., Shanghai, China) containing six proteins of known molecular weight (15 kDa, 25 kDa, 35 kDa, 50 kDa, 67 kDa and 94 kDa).

2.7. Effect of pH on PPO and POD activity

The optimum pH value of PPO and POD was determined in a pH range of 3.0−10.0 using 50 mmol/L Na_2HPO_4-citric acid buffer between pH 3.0 and 8.0 at 37 °C whereas 50 mmol/L glycine−NaOH was used in a range of pH 9.0 and 10.0. Assays were performed in triplicate.

2.8. Effect of temperature on PPO and POD activity

The optimum temperature of two kinds of enzyme was determined in the range of 30−80 °C at pH 7.0 by measuring specific activity as described above. Analyses were performed in triplicate under the standard mixing conditions.

2.9. Substrate specificity studies

In order to determine the Michaelis−Menten constant (K_m) and maximum velocity (V_{max}), PPO activity was measured with catechol, 4-methylcatechol and gallic acid as substrates at various concentrations of 0.025, 0.05, 0.1, 0.15 and 0.2 mol/L respectively. POD activity was measured with 2-hydroxy phenol and hydrogen peroxide as substrates. The concentration of 2-hydroxy phenol used were 0.025, 0.05, 0.075, 0.1, 0.15, 0.2 and 0.4 mol/L at constant concentration of hydrogen peroxide 2 mL/100 mL K_m and V_{max} values of the enzyme were calculated from a plot of $1/V$ against $1/[S]$ using the method of Lineweaver and Burk method (Lineweaver & Burk, 1934). Measurements were carried out in triplicate.

2.10. Statistical analysis

Average results of three determinations were analyzed for variation (ANOVA) and statistical significance was analyzed by method of Duncan.

3. Results and discussions

3.1. Purification of PPO and POD

After extraction using acetone, fractionation using 30−80 g/100 mL ammonium sulfate, dialysis, concentration, chestnut PPO