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The purification and characterisation of polyphenol oxidase from green bean (*Phaseolus vulgaris* L.)

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ABSTRACT

Four isoforms of polyphenol oxidases (PPOs) were characterised in purified extracts of coats (PPOIa and PPOIb) and pods (PPOIIa and PPOIIb) of green bean (*Phaseolus vulgaris* L.). The molecular weights of four isoforms have been estimated to be from 57.5 to 39.0 kDa by SDS–PAGE. The PPOII (mixture of PPOIIa and PPOIIb) was used to characterise the PPO of green bean pods. All isoforms activities were stable between pH 6.8 and pH 7.2. PPOIa and PPOII have similar thermal inactivation profiles, and PPOIb has higher thermal stability than that of PPOIa and PPOII. PPOs showed the highest affinity to pyrogallol in all selected substrates. Although activities of PPOs were markedly inhibited by L-ascorbic acid, the activity of PPOI (mixture of PPOIa and PPOIb) was significantly activated by $MnSO_4$ and $CaCl_2$.

1. Introduction

Polyphenol oxidase (PPO: EC 1.10.3.1) is a copper-containing enzyme responsible for the hydroxylation of monophenols to *o*-diphenols and oxidation of *o*-diphenols to *o*-diquinones. PPO is compartmentalised in plastids and their phenolic compounds are located in the vacuoles for most plant tissues. Action of PPO only occurs when this compartmentation is disrupted after tissues are wounded, as observed in diseased tissues or cell disruption caused by processing and storage (Mazzafera & Robinson, 2000). The production of the browning reaction may modify plant proteins and be more toxic to potential phytopathogens (Aydemir, 2004). The reactions also produce undesirable blackening in the products during food processing, or in post-harvest of plant products resulting in a reduction of their sensory properties and nutritional value. For this reason, the PPO have been characterised in several plants, such as butter lettuce (Gawlik-Dziki, Zlotek, & Swieca, 2008), broccoli (Gawlik-Dziki, Szymanowska, & Baraniak, 2007), coffee (Mazzafera & Robinson, 2000), potato (Marri, Frazzoli, Hochkoeppler, & Poggi, 2003), *Ferula* sp. (Erat, Sakiroglu, & Kufrevioglu, 2006), avocado (Gomez-Lopez, 2002), Chinese cabbage (Nagai & Suzuki, 2001), and Marula fruit (Mdluli, 2005).

Green beans (*Phaseolus vulgaris*, L.) are among the most important vegetables produced in China. They are generally harvested seasonally, and the outdoor varieties are excellent in nutrition, colour, texture, and flavour (Martins & Silva, 2004). Consumption of canned green beans has decreased in the last years, but that of fresh bean products has increased continuously (Cano, Monreal, Ancos, & Alique, 1998). Therefore, fresh green beans are more appreciated by consumers due to their sensorial and nutritional characteristics. The quality and shelf life of green beans varies depending on cultivars, maturity, processing, and storage conditions. It is a common phenomenon that browning decreases the storage period and commercial value of green beans. In green beans, the relation between green bean quality and PPO activity is not well known, although it has been described that PPO is one of several reasons causing green bean blackness during storage (Lian et al., 2006). In the literature, there are few studies reported on the purification and characterisation of PPO from green beans. This work covers investigations of purification of PPOs, effects of pH and temperature on PPO activity and stability, effects of inhibitors on PPO activity, and substrate specificity of PPO from green bean coats and pods. The research can lead to understanding the properties of the PPO that catalyses the browning reaction during storage. The results would provide information and effective methods for controlling browning and extending shelf-life.

2. Materials and methods

2.1. Samples and chemicals

Mature green beans of a major cultivar, *P. vulgaris* L., were obtained from a local farm (Heilongjiang Academy of Agricultural

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Science, Harbin, China). The green bean samples were transported to the laboratory within 2 h on the day of harvest and stored at 8 °C until extraction.

Catechol, L-dopa, 4-methylcatechol, pyrogallol, polyvinylploypyrolidone (PVPP), L-ascorbic acid, L-tyrosine, L-cysteine, sodium metabisulphite, Sephadex G-100, and DEAE-cellulose were obtained from Sigma Chemical Co (St. Louis, USA). Molecular Markers (M.W., 14.4–116.0 kD) were purchased from Fermentas Inc. (Glen Burnie, MD, USA). Acrylamide, bis-acrylamide, TEMED, ammonium persulfate and SDS were obtained from Amersham Pharmacia Biotech AB (Björkgatan, Uppsala, Sweden). Other reagents were all of analytical grade.

2.2. Enzyme extraction and partial purification from green bean

One hundred gram fresh green bean pods (or 20 g fresh green bean coats) were homogenised in 300 ml (or 50 ml) of 0.1 M phosphate buffer (pH 6.8, 10% PVPP) for 20 s at 4 °C (HR1724, Philips, Netherlands). The homogenate was filtered through four layers of cheesecloth and centrifuged at 15,000*g* for 20 min at 4 °C (Anke TGL-16G, Anting Scientific Instrument Factory, Shanghai, China). The supernatants were treated with $(NH_4)_2SO_4$ to obtain 60% saturation at 0 °C and then centrifuged at 15,000*g* for 20 min at 4 °C. The crude enzyme extracts were used for further purification.

For further purification of PPO from green bean coats, the precipitate, which was extracted and centrifuged from green bean coats, was dissolved in 0.01 M phosphate buffer (pH 6.8). The crude extract enzyme was extensively dialysed against the same buffer at 4 °C for 24 h with four changes of the buffer during dialysis. The dialysed enzyme solution was fractionated by a Sephadex G-100 column (1.6 cm × 50 cm). The column was equilibrated with 0.01 M phosphate buffer (pH 6.8). The 1.0 ml dialysed enzyme solution was passed through the column and was eluted by same phosphate buffer. The elution rate was adjusted to 30 ml/h. An elution of 5 ml was collected each time. The elution process was continued until obtaining zero absorbance at 280 nm. Protein was qualitatively detected by Protein Nucleic Acid Analyzer (HD-93–1, Kingdom Biochemical Instrument Co., Shanghai, China) at 280 nm. Each isolated PPO sample was then determined for their activity. Thus, values obtained were plotted against the sample numbers. The fractions having PPO activity were collected and purification degrees were determined by measuring specific activity before and after purification. Then the fractions were stored at 0 °C for subsequent analysis.

For the further purification of the PPO extracted from the green bean pods, the extracted and centrifuged precipitate was dissolved in 0.01 M Tris–HCl buffer (pH 7.1). The crude extract enzyme was extensively dialysed against the same buffer at 4 °C for 24 h with four changes of the buffer during dialysis. The dialysed enzyme solution was fractionated by a DEAE-cellulose column (1.6 cm × 10 cm). The column was equilibrated with 0.01 M Tris–HCl buffer (pH 7.1). The 2.0 ml dialysed enzyme solution was passed through the column and was eluted by 0.01 M Tris–HCl buffer (pH 7.1) NaCl at a flow rate of 30 ml/h. Enzyme activity was eluted with a linear gradient of 0–0.5 M NaCl in a total 600 ml of buffer. Fractions of 2.5 ml were collected and assayed for PPO activity and protein content. The fractions having PPO activity were collected and purification degrees were determined by measuring specific activity before and after purification. Then the fractions are stored at 0 °C for subsequent analysis.

2.3. PPO activity assay

PPO activity was determined according to the method of Yamamoto, Yoshitama, and Teramoto (2002) with some modification. The catechol was used as a substrate, and the quinone formation was measured on a spectrophotometer at 410 nm (Spectrum 754PC, Spectrum Instruments Co., Ltd, Shanghai, China). The sample contained 0.5 ml of 0.1 M catechol, 2.0 ml of 0.1 M phosphate buffer (pH 6.8) and 0.5 ml of the enzyme solution. The blank sample contained only 3.0 ml of substrate solution. The sample and blank were kept at ambient temperature for 30 min. PPO activity was assayed in triplicate measurements. One unit of enzyme activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 per min. The initial rate was calculated from the slope of the absorbance–time curve.

2.4. Estimation of molecular weight

The purified PPO was subjected to determine molecular weight based on SDS–polyacrylamide gel electrophoresis (SDS–PAGE) under denaturation conditions by a vertical electrophoresis unit (DYCZ-24D, Liuyi Instrument Factory, Beijing, China). The PPO preparation was followed by heating the PPO in boiling water for 3 min with 2% SDS and β-mercaptoethanol. The SDS–PAGE test employed 12% polyacrylamide gels and the buffer system. Electrophoresis was run at 10–25 mA for 2.0 h at ambient temperature. Gels were stained with Coomassie Brilliant Blue R-250. Molecular markers used were β-galactosidase (*M*r, 116.0 kDa), bovine serum albumin (*M*r, 66.2 kDa), ovalbovine (*M*r, 45.0 kDa), lactate dehydrogenase (*M*r, 35.0 kDa), restriction endonuclease Bsp981 (*M*r, 25.0 kDa), and β-lactoglobulin (*M*r, 18.4 kDa). The Gel-pro Analyzer 4.0 was applied to determine the molecular weight.

2.5. Effect of pH on PPOs activities and stability

The purified PPO (0.5 ml) was mixed with 2.5 ml of 0.1 M citrate buffer (pH 3.0–6.0) and 0.1 M phosphate buffer (pH 6.1–8.0) at the appropriate pH with 0.2 pH unit intervals. The PPO activity, as a function of pH, was determined at different pH values.

To determine pH stability, 0.5 ml purified PPO fractions which were originally isolated from green bean pods and green bean coats were mixed with 2.5 ml of 0.1 M citrate buffer (pH 3.0–6.0) and 0.1 M phosphate buffer (pH 6.1–8.0) at the appropriate pH with 0.2 pH unit intervals. After mixing of the sample and buffer, the pH was re-checked. The mixtures were kept at ambient temperature for 24 h, and then the mixtures were adjusted to pH 7.0. The residual PPO activity was determined.

2.6. Effect of temperature on PPOs activities and stability

A quantity of 2.5 ml substrate solution (0.1 M catechol in 0.1 M, pH 6.8 phosphate buffer) was pre-heated from 0 to 80 °C (at 10 °C intervals) in a water bath for 5 min. Then 0.5 ml PPO fraction was added into the substrate. PPO activity was determined as a function of temperature. PPO activity was calculated in the form of percent residual PPO activity at the optimum temperature.

To determine the thermal stability of PPO (0–95 °C), the test was divided into two temperature ranges. In first temperature range, each 0.5 ml PPO fraction (PPOIa, PPOIb and PPOII) was added into the 2.5 ml substrate solution (0.1 M catechol in 0.1 M, pH 6.8 phosphate buffer). After the mixtures were incubated 30 min at 0–80 °C (at 10 °C intervals), the samples were rapidly cooled in an ice bath for 5 min. Then residual PPO activity was determined at ambient temperature. In second temperature range, 0.5 ml PPOI (mixture of PPOIa, PPOIb) or PPOII (mixture of PPOIIa and PPOIIb) was treated same as above, but the differences were that samples were incubated 5 min at 85–95 °C (at 5 °C intervals). The residual activity was calculated as a percentage of unheated enzymes.

The kinetic data analysis of thermal inactivation can be described by the first-order reaction (Rapeanu, Loey, Smout, & Hendrickx, 2006):