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Purification and structural analysis of membrane-bound polyphenol oxidase from Fuji apple

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ABSTRACT

Membrane-bound polyphenol oxidase (mPPO) in Fuji apple (*Malus domestica* Borkh. cv. Red Fuji) was purified and analyzed with a nanoelectrospray ionization mass spectrometer. The three-dimensional model and binding site of mPPO to 4-methyl catechol were also studied using molecular docking. mPPO was purified 54.41-fold using temperature-induced phase partitioning technique and ion exchange chromatography. mPPO had a molecular weight of 67.3 kDa. Even though a significant level of homology was observed between mPPO and the soluble polyphenol oxidase in the copper binding sequence, there was another region, rich in histidine residues, which differed in 13 amino acids. The three-dimensional structure of mPPO consisted of six α-helices, two short β-strands, and ten random coils. The putative substrate-binding pocket contained six polar or charged amino acids, His191, His221, Trp224, Trp228, Phe227, and Val190. Trp224 and Trp228 formed hydrogen bonds with 4-methyl-catechol.

1. Introduction

Fuji apples (*Malus domestica* Borkh. cv. Red Fuji) have recently become as popular as other traditional apple varieties, e.g., 'Golden Delicious' and 'Red Delicious', due to their distinctive qualities and high economic return (Iglesias, Echeverría, & Lopez, 2012; Varela, Salvador, & Fiszman, 2008). Apples have several health benefits including anti-tumor, antioxidant, and cholesterol-lowering properties (Oszmiański, Wolniak, Wojdyło, & Wawer, 2008). Lots of apples are consumed every year, mostly in the form of apple juice.

Apple juice is commonly processed into a clear juice. However, there has been a growing market trend for natural and cloudy apple juice due to its sensory and nutritional qualities, which resemble those of the fresh product (Komthong, Igura, & Shimoda, 2007; Niu et al., 2010). Discoloration and browning, which commonly affect fruit products, may occur during apple processing via enzymatic browning reactions (Ibrahim et al., 2011). Enzymatic browning is mainly attributed to the action of polyphenoloxidase (PPO), which contribute to the formation of phenolic compounds in the presence of molecular oxygen. Enzymatic browning decreased the commercial quality, organoleptic acceptance and nutritional value of the product (Aka, Courtois, Louarme, Nicolas, & Billaud, 2013).

Traditionally, thermal processes are applied in fruit juice processing to inactivate PPO. However, heat-sensitive nutrients and volatile compounds may be negatively affected by thermal processing (Xu et al., 2011). In recent time, non-thermal methods, such as high hydrostatic pressure (HHP) and dense phase carbon dioxide (DPCD), have been under intense investigation to evaluate their potential as an alternative or complementary process to traditional thermal methods (Duong & Balaban, 2014). Nevertheless, some activation of PPO activity was observed for HHP and DPCD treatment, such as PPO from pears, carrots and apples (Eisenmenger & Reyes-De-Corcuera, 2009). The enhancement in PPO activity was hypothesized to be from the release of membrane-bound enzymes or from separation of a part of the protein molecule and subsequent liberation of a second active site (Eisenmenger & Reyes-De-Corcuera, 2009). But the mechanism has not been extensively explored and is not known.

Plants contain multiple forms of PPO (Mayer, 2006). The enzyme is either located in the thylakoid membrane (membrane-bound) or lumen (soluble form) of chloroplasts (Chazarra, Cabanes, Escribano, & Garcia-Carmona, 1996). The membrane-bound form (mPPO) can be weakly or strongly bound to the thylakoid membrane and is not involved in the synthesis of phenolic compounds, while the soluble form (sPPO) is present in the thylakoid lumen (Mishra, Gautam, & Sharma, 2012). Even though the role of mPPO in plant tissue is not well understood, it has been hypothesized that mPPO can be easily activated, thereby contributing to browning and rotting (Orenes-Piñero, García-Carmona, &

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Sánchez-Ferrer, 2006). However, few studies have focused on the effect or structure of mPPO in Fuji apples. Furthermore, there is no report on the binding sites of mPPO in Fuji apples.

Because of the considerable economic and nutritional losses induced by enzymatic browning, it is crucial to have a better understanding of the molecular properties of mPPO. In this study, we purified mPPO from Fuji apples. Additionally, we generated three-dimensional models to determine the potential binding sites of mPPO and to have a better understanding of the structure of mPPO in Fuji apples. Experiments involving amino acid mutations might reveal mechanistic insights and shed light on potential strategies for the design of PPO inhibitors.

2. Materials and methods

2.1. Materials

Red Fuji apples were purchased from a local market in Beijing, China and stored at 4 °C. Triton X-100, polyvinylpyrolidone (PVPP), and ammonium sulfate were purchased from Solarbio (Beijing, China). Tris-base, phenylmethylsulfonyl fluoride (PMSF), L-cysteine, citric acid, and phenolic compounds were obtained from Sigma–Aldrich (St. Louis, Mo., USA).

2.2. Enzyme extraction

In this experiment, mPPO, isolated from 100 g of apples, was homogenized at maximum speed for 1 min in an mini-mixer 230 (Midea Inc., Beijing, China) with 100 ml cold sodium phosphate buffer (0.05 M, pH 6.80), containing 30 mM ascorbic acid and protease inhibitors [2 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM ethylenediaminetetraacetic acid (EDTA)]. The homogenate was passed through six cheesecloth layers and centrifuged at 10,000 rpm for 20 min at 4 °C.

The resulting pellet (containing mPPO) was solubilized with 0.25% (v/v) Triton X-100 in Tris-buffer (0.05 M, pH 6.80) under constant stirring for 1 min. The homogenate was sonicated for 10 min and centrifuged at 12,000 rpm for 15 min. The resulting supernatant was subjected to temperature-induced phase partitioning (kept at 4 °C for 30 min and heated to 35 °C for 15 min). The treated extract was centrifuged at 5000 rpm for 10 min at 25 °C. The resulting supernatant represented the crude mPPO.

2.3. Partial purification of mPPO

mPPO extract was subjected to 60–80% ammonium sulfate precipitation, followed by centrifugation at 11,000 rpm for 15 min. The precipitate was re-suspended in a minimum volume of sodium phosphate buffer (0.05 M, pH 6.80) and dialyzed through an 8-kDa cut-off membrane in 5 L phosphate buffer at 4 °C with three buffer changes. The dialyzed mPPO fraction was used in subsequent purification experiments.

2.4. Purification of mPPO by DEAE sepharose fast flow anion-exchange chromatography

The mPPO was purified according to the method of Liu, Zhao, Gan, and Ni (2015). The DEAE sepharose fast flow column material (25 ml) was washed with distilled water (250 ml) and sodium phosphate buffer (pH 6.80, 0.05 M). The material was poured into the column (1.0 cm × 10 cm) in one continuous motion, using a glass rod held against the wall of the column to minimize the formation of air bubbles. The column was equilibrated with 500 ml sodium phosphate buffer (pH 6.80, 0.05 M). The dialyzed mPPO extract was passed through a 0.45-μm syringe filter and loaded into the column. The elution was carried out using five gradients of NaCl (0, 0.1, 0.2, 0.4, 0.5 M) in 180 ml of sodium phosphate buffer (pH 6.80, 0.05 M) at a flow rate of 0.3 ml min^{-1}; the elution was monitored at 280 nm. In this experiment, 3-ml fractions were tested for enzyme activity; fractions were pooled for the measurement of maximum enzyme activity.

2.5. Enzyme assays

The enzymatic activity of purified mPPO was assessed by a colorimetric method. In this experiment, 2.5 ml of 0.2 M catechol dissolved in 0.05 M sodium phosphate buffer (pH 6.80) was mixed with 0.5 ml of enzyme solution. The activity was determined in triplicate at 20 °C in a UV 1800 spectrophotometer (Shimadzu, Japan) at 410 nm. One unit of enzyme activity was defined as a 0.001-unit change in absorbance per min per ml.

2.6. Protein content

Protein content in the mPPO extract was determined by the Bradford method at 595 nm (Bradford, 1976), using bovine serum albumin (BSA, Sigma Chemical, St. Louis, USA) as the standard.

2.7. Denaturing SDS–PAGE

Purified mPPO was subjected to SDS–PAGE in a Mini Protean III Electrophoresis Cell (Bio-Rad), with 12% resolving and 4% stacking gels. Purified mPPO (25 μl) was loaded in each lane and the slab gels (1.5-mm thickness) were run at a constant current of 110 V. Proteins was stained with Coomassie Blue R-250. The molecular weight was estimated by comparison to molecular weight markers (solarbio, Beijing, China): phosphorylase B (99.0 kDa), BSA (70.0 kDa), actin (43.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (24.0 kDa), and lysozyme (15.0 kDa).

2.8. Native-PAGE

Activity staining of PPO was performed according to the method Laemmli on an 8% non-denaturing polyacrylamide gel (Laemmli, 1970). Loading buffer without SDS and thiol-reducing agent was used. PPO (20 μl) was loaded in each lane. A constant power supply of 80 V was used. All steps were performed at 4 °C.

PPO bands were detected by immersing the gel in a solution containing sodium phosphate buffer (0.5 M, pH 6.80), catechol (0.3 M), and *o*-phenylenediamine (0.06% dissolved in 0.01 M oxalic acid) at a ratio of 1:3:1 (v:v:v). Color development occurred within 15 min.

2.9. nESI-LC–MS/MS analysis

Trypsin-digested peptides were separated in a C18 column and analyzed with a nanoelectrospray ionization mass spectrometer (nESI-LC–MS/MS) according to the method of Chen, Zhang, and Cheung (2012) with some modification. Chromatographic separation was achieved using a Q-Exactive (Thermo, USA) LC–MS/MS system equipped with an analytical column (3.5 μm × 150 mm × 75 μm; ZORBAX 300SB-C18, Agilent, USA). The injection volume was 7 μl. The peptides were separated with a gradient consisting of solvent A (100% water with 0.1% formic acid) and solvent B (20% water and 80% acetonitrile with 0.08% formic acid) at a flow rate of 300 nl/min: 0–5 min (8% B), 5–40 min (35% B), 40–46 min (90% B), 46–55 min (90% B), 55–56 min (4% B), and 56–60 min (4% B). The nanospray voltage was 2.5 kV and the mass spectra was measured over a range of m/z 400–1500 Da.

The peptides were arranged at 1^+, 2^+, and 3^+. A protein hit with a confidence level >95% ($p < 0.05$) was considered to be an identified