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Aggregation and conformational change of mushroom (*Agaricus bisporus*) polyphenoloxidase subjected to thermal treatment

Lei Zhou, Wei Liu *, Liqiang Zou, Zhiqiang Xiong, Xiuting Hu, Jun Chen

State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang 330047, China

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ABSTRACT

This study investigated changes in the activity, conformation and microstructure of mushroom polyphenoloxidase (PPO) subjected to thermal treatment. The inactivation of PPO can be achieved by high temperature-short time or mild temperature-long time treatment. Circular dichroism and fluorescence spectra suggested that heating process induced the rearrangement of secondary structure and the disruption of tertiary structure. Red shifts of fluorescence spectra showed positive correlations with the inactivation rate of PPO. There were significant differences in the conformation and molecular microstructure among PPO samples with the same relative activity, which were obtained by treating PPO at 45, 55 and 65 °C for different times. In summary, PPO molecules were deformed at mild temperature, while higher temperature induced the formation of large aggregates. PPO with the same relative activity might exist in different forms.

1. Introduction

Polyphenoloxidase (PPO, E.C 1.14.18.1) is a binuclear copper-containing enzyme, which is widely distributed in animals, plants, bacteria and fungi (Mayer, 2006). It is reported that crystal structure of mushroom (*Agaricus bisporus*) PPO comprises two H subunits and two L subunits (Ismaya et al., 2011). The H subunit contains a binuclear copper-binding site in the deoxy-state and three histidine residues coordinate each copper ion (Ismaya et al., 2011). PPO is able to insert oxygen in a position *ortho*-to an existing hydroxyl group in an aromatic ring (Mayer, 2006). Therefore, PPO can catalyze the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones. Further, *o*-quinones polymerize to brown pigments (Espín, Jolivet, & Wichers, 1998; Virador et al., 2010). PPO plays an important role in the browning of damaged fruits and vegetables. Besides the color change, enzymatic browning induced by PPO deteriorates the nutritional quality of food. Therefore, it is necessary to inactivate PPO and prevent enzymatic browning in food processing.

Over the years, many methods have been applied to inactivate PPO, including addition of organic acid (Zhou et al., 2016), high pressure (Yi et al., 2012), ultrasound (Cheng, Zhang, & Adhikari, 2013), pulsed electric fields (Zhong et al., 2007), γ irradiation (Sun & Song, 2003) and thermal treatment (Gouzi, Depagne, & Coradin, 2012). Among these methods, thermal treatment is considered as the most effective, economical and widely used technique to inactivate PPO and control enzymatic browning (Awuah, Ramaswamy, & Economides, 2007; Baltacioglu, Bayindirli, Severcan, & Severcan, 2015). In many studies, PPO was investigated in the temperature range of 50–80 °C, and only activity assay and kinetic approaches were used to represent the inactivation process (de Aguiar Cipriano, Ekici, Barnes, Gomes, & Talcott, 2015; Goyeneche, Di Scala, & Roura, 2013; Terefe, Delon, Buckow, & Versteeg, 2015). PPO does not belong to the extremely heat-stable enzymes, and it is sufficient to inactivate PPO by exposing products to high temperatures for a short time (Vamos-Vigyazo, 1981). To date, a limited number of literatures have reported the conformational change of PPO during thermal treatment. Tse, Kermasha, and Ismail (1997) found that the secondary structure of PPO was predominately α-helix in nature. Above 40 °C, secondary structure of PPO began to change and protein-protein aggregate formation appeared during thermal treatment (Tse et al., 1997). Similar and detailed results were obtained by using FTIR spectroscopy in a recent report. The α-helix and β-sheet of PPO decreased as temperature increased, while aggregated β-sheet, turns and random coil increased (Baltacioglu et al., 2015). Besides, in the study of Ionita, Aprodu, Stanciuc, Rapeanu, and Bahrim (2014), phase diagrams were used to analysis the existence of structurally distinct species, and aggregates were found by fluorescence spectroscopy experiments. Molecular dynamics simulations indicated the important structural

* Corresponding author.
E-mail address: liuwei@ncu.edu.cn (W. Liu).

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rearrangements of PPO subjected to thermal treatment (Ionita et al., 2014). Nevertheless, how can we obtain the PPO samples with the same relative activity by different thermal treatment? And are there any differences in conformation among PPO samples with the same relative activity? In addition, the effect of thermal treatment on the molecular microstructure of PPO is not clear yet.

The aim of the study was to explore the changes in the activity, conformation and molecular microstructure of PPO subjected to thermal treatment. In order to characterise the deformation and aggregation of PPO subjected to thermal treatment, atomic force microscopy (AFM) was used to observe the changes in molecular microstructure of PPO. These contents not only provide useful information for preventing browning by thermal treatment but also contribute to understand the structure-activity relationship of PPO.

2. Materials and methods

2.1. Materials

Mushroom (*Agaricus bisporus*) polyphenoloxidase (T3824-50 Ku, 5771 u/mg) was purchased from Sigma Chemical Co. (St. Louis, MO). Reaction substrate L-DOPA (L-3,4-dihydroxyphenylalanine, 99% purity) was purchased from Aladdin Chemicals Co. (Shanghai, China). All other chemicals were of analytical grade, solutions were prepared in double-distilled water.

2.2. Thermal treatment of PPO

Thermal treatment experiments were divided into two parts. For heating process, PPO solution was incubated in a test tube at 25 °C and then heated from 25 to 80 °C at a heating rate of 4 °C/min. At predetermined temperature intervals (30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80 °C), 3 mL samples were pipetted into test tubes that were immediately transferred to an ice bar to stop thermal inactivation instantaneously (Gouzi et al., 2012). For the thermostability of PPO, PPO solutions were incubated at 45, 55 and 65 °C for different holding times (0–240 min). After thermal treatment, enzyme solutions were rapidly immersed in an ice bar (Liu, Cao, Yang, & Qi, 2015a).

2.3. Activity assay

According to our previous study, PPO activity was determined by a UV–vis spectrophotometer (MAPADA, Shanghai, China) (Liu et al., 2009b; Yi et al., 2012). The reaction mixture included 0.2 mL of 0.01 mol/L L-DOPA and 2.7 mL of phosphate buffer (50 mM, pH 6.8). Reaction mixture was incubated in a test tube at 37 °C. Then 0.1 mL of the PPO solution (0.1 mg/mL) was added to the reaction mixture to initiate the enzyme reaction. The absorbance of the reaction mixture was monitored immediately at 475 nm for 1 min and specific activity of PPO was calculated from the slope of a linear segment:

Specific activity = A_{475nm}/1 min/0.1 mL of enzyme solution.

$$\text{Relative activity} = \frac{\text{Activity of treated PPO}}{\text{Activity of untreated PPO}} \times 100\%.$$

2.4. Circular dichroism (CD) analysis

CD measurement was conducted according to our previous studies (Liu et al., 2013; Zhou et al., 2016). MOS-450 spectropolarimeter (French Bio-Logic SAS, Claix, France) was used to record CD spectra with a quartz cuvette of 1 mm optical path length at room temperature (25 ± 1 °C). CD spectra were scanned in the far UV range (250−190 nm) at 50 nm/min. The step resolution and band width were 1 and 0.5 nm, respectively. Concentration of PPO used in CD analysis was kept at 0.35 mg/mL. Dichro Web Online SELCON 3 algorithms with the reference set No. 4 (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) was used to estimate the secondary structures elements (Wang, Zhang, Yan, & Gong, 2014). The CD data was expressed in terms of mean residual ellipticity (θ), in mdeg cm^2/dmol, using a Mean Residual Weight (MRW) of 113.7. The results of four replicates were averaged and Origin 8.0 was used to smooth the spectra.

2.5. Fluorescence emission spectra analysis

Fluorescence emission spectra measurement was conducted with a FS F-4500 Spectrophotometer (Hitachi, Tokyo, Japan). Samples were scanned at room temperature (25 ± 1 °C). Emission spectra of PPO solutions were measured in the wavelength range of 290–420 nm with the maximum excitation wavelength of PPO (λ_{ex} = 280 nm). Excitation and emission slits were 5 nm (Liu, Geng, Zhao, Chen, & Kong, 2015b; Liu et al., 2009a). Each spectrum was corrected by subtracting the baseline spectrum of phosphate buffer (50 mM, pH 6.8).

2.6. Atomic force microscopy (AFM) analysis

The microstructure observations were performed with an AFM (Agilent 5500, Agilent Technologies, Santa Clara, CA, USA), according to the description of Yu, Zeng, and Lu (2013) and Zou et al. (2014) with some modifications. AFM images were carried out in tapping mode at room temperature (25 ± 1 °C). PPO was sufficiently dissolved in phosphate buffer (pH 6.8, 50 mM). Afterwards, 10 μL PPO solutions (65 μg/mL) were uniformly dropped on a freshly cleaved flat mica surface and subsequently dried in a desiccator. The scan size was 3 μm and scan rate was 1.0 Hz. Images were processed and analyzed with Pico Image Basic 6.2 and Pico-View 1.14 softwares.

2.7. Statistical analysis

All experiments were performed in at least triplicate. The values are expressed as means ± standard deviation (SD). The analyses of variance (ANOVA) were performed for all experimental runs to determine significance at 95% confidence.

3. Results and discussion

3.1. Inactivation of PPO by thermal treatment

The relative activity of PPO during the heating process from 25 to 80 °C at 4 °C/min was shown in Fig. 1A. PPO retained most of the activity when temperature increased from 25 to 40 °C. From 40 to 80 °C, relative activity of PPO dramatically decreased with increasing temperature. It was almost completely inactivated when temperature reached 75 °C. Results of thermostability showed that the relative activity of PPO decreased with increasing treating time at 45, 55 and 65 °C (Fig. 1B). The enzyme activity decreased slowly at 45 °C. After incubation at 45 °C for 20, 110 and 240 min, the relative activity of PPO were 84.8%, 52.4% and 26.2%, respectively. Activity of PPO gradually decreased at 55 °C. It remained 52.7% and 25.7% after 10 and 15 min incubation, respectively. At 65 °C, PPO was extremely unstable and a rapid inactivation occurred. After 90 s incubation, the relative activity of PPO was 48.5%. Moreover, it only remained 22.5% after 150 s incubation. Gouzi et al. (2012) examined the thermal inactivation of PPO from *Agaricus bisporus* between 50 and 73 °C in relation to exposure time, and