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Effect of Ultrahigh Hydrostatic Pressure on the Activity and Structure of Mushroom (*Agaricus bisporus*) Polyphenoloxidase

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ABSTRACT: High hydrostatic pressure (HHP, treatment pressure ≤700 MPa) is approved to be the most successful commercial nonthermal processing due to its minimal modifications in nutritional and sensory quality. However, for some pressure stable enzymes such as PPO, this unique technology can hardly inactivate them at treatment pressure below of 700 MPa. This study investigated the effects of ultrahigh hydrostatic pressure (UHHP, treatment pressure >700 MPa) on the activity of *Agaricus bisporus* mushroom polyphenoloxidase (PPO) both in the phosphate buffer and in the mushroom puree, and on the structure of the enzyme by means of circular dichroism (CD), fluorescence emission spectra, and sulphydryl group detection. The results showed that UHHP treatment at pressure from 800 to 1600 MPa caused significant inactivation on the PPO both in the phosphate buffer and in the mushroom puree. UHHP treatment at 1400 and 1600 MPa for 1 min reduced the enzyme activity by 90.4% and 99.2% in the buffer;, however, higher enzyme activity remained in the puree after UHHP treatment at the same condition. CD and fluorescence spectra analysis showed that the secondary and tertiary structures of UHHP treated mushroom PPO were changed. The sulphydryl group (SH) detection revealed that the SH content on the surface of UHHP treated mushroom PPO was increased. It has been suggested that the inactivation of mushroom PPO by UHHP treatment at pressure higher than 1000 MPa was due to the synergistic effect of the pressure and the heat arising from pressurization, in which heat plays a major role.

KEYWORDS: *ultrahigh hydrostatic pressure, polyphenoloxidase, adiabatic compression heating, inactivation, structure*

INTRODUCTION

Polyphenoloxidase (PPO, EC1.14.18.1) is ubiquitous in plants, animals, fungi, and bacteria, and it is responsible for the browning of damaged fruits and vegetables. PPO from all of the species has six histidine residues that ligats the two copper ions of the active site.[1] Recently, Ismaya et al.[2] reported the crystal structure of *Agaricus bisporus* mushroom polyphenoloxidase, which comprises two H subunits of ∼392 residues and two L subunits of ∼150 residues. The H subunit contains a binuclear copper-binding site in the deoxy-state, in which three histidine residues coordinate each copper ion. In the presence of molecular oxygen and PPO, natural phenolic compounds are oxidized to the corresponding *o*-quinones, which subsequently polymerize nonenzymatically to brown pigments.[3] The enzymatic browning induces deterioration of sensory and nutritional quality and affects appearance and organoleptic properties. Therefore, inactivation of PPO is desirable for the preservation of foods.[4] Several methods have been used to inhibit enzymatic browning in plant foods, for example, the exclusion of oxygen or the addition of antioxidants such as the widely used ascorbic acid or sulfur dioxide.[5] However, thermal processing is still the most efficient and economical method for the inactivation of PPO. Unfortunately, thermal processing has limits such as the loss of sensory and nutritional qualities of food products;[6,7] thus, there is a need to optimize conventional processing techniques currently applied in food industries and to develop novel processing techniques such as high hydrostatic pressure (HPP) processing.

HHP processing is an interesting alternative to traditional food processing and preservation methods due to its limited effects on covalent bonds resulting in minimal modifications in nutritional and sensory qualities. Since HHP processing can inactivate microorganisms and enzymes, in the past few years, numerous studies have been reported, and it has been considered as a promising methodology for food preservation.[8−10] Meanwhile, as the most successful commercial nonthermal processing, this technology will receive more interest in the food industry in the next few years.

Generally, the pressure levels of HHP treatment used to process food are lower than 700 MPa because at pressure above 680 MPa, there are no vessels available for commercial applications, and this is due to the fact that water is changed to solid phase (Ice V) at ambient temperature when the pressure exceeds 700 MPa.[11] However, for some pressure stable enzymes such as PPO, this unique technology can hardly inactivate them at pressure levels lower than 700 MPa,[12−16] thus resulting in enzymatic browning during storage. The completely inactivation of PPO is very important in food processing. Therefore, using higher pressures (800−1600 MPa) to inactivate it was considered to better understand the contribution of pressure on the inactivation of PPO. For this objective, the effects of extreme pressure on inactivation and molecular structure change of mushroom PPO were investigated, in turn, to provide some guidance on enzyme inactivation at commercial HHP treatment conditions (≤700 MPa). In this work, the treatment of HHP at pressure levels higher than 700 MPa was defined as ultrahigh

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hydrostatic pressure (UHHP), which used oil or oil/water mixture as the pressure transmission medium due to the phase change phenomenon of water.

PPO has been known to be highly stable under extreme pressure; thus, it is an ideal material to investigate the effect of high pressure treatment on enzyme inactivation. Several works have been published on the effects of HHP treatment (≤700 MPa) on PPO from different sources.[12−16] Buckow et al.[14] studied the inactivation effects of HHP at different pressure−temperature domains on apple PPO and found that HHP treatment at the pressure below 700 MPa at mild temperature (<60 °C) was not enough to inactivate PPO completely. Terefe et al.[13] found that the maximum inactivation of strawberry PPO after treatment at the most intense processing condition (690 MPa, 90 °C) was about 23%. Gomes et al.[5] reported that after HHP treatment at 600 MPa for 10 min, the activity of mushroom PPO only decreased by about 50%. However, little information was available regarding the inactivation and changing of molecular properties of PPO at pressure levels higher than 700 MPa. Weemaes et al.[17] reported that plum PPO was not inactivated by UHHP treatment at 900 MPa and 25 °C. Garcia-Palazon et al.[12] reported that an inactivation of red raspberry PPO by 29% was observed after UHHP treatment at 800 MPa and 18−22 °C for 15 min, and an only slight inactivation of pear PPO was observed in the same treatment conditions. Sun et al.[18] found that after exposure to 800 MPa for 10 min at temperature around 35 °C, the activity of mushroom PPO reduced by 28%. Rapeanu et al.[19] found that the activity of grape PPO reduced by 22% after UHHP treatment at 800 MPa and 25 °C for 15 min. Gomes et al.[5] also reported that UHHP treatment at 800 MPa for 1 min could not inactivate mushroom PPO completely. On the basis of these results, UHHP treatment at pressure lower than 1000 MPa at mild temperature had limited inactivation effects on mushroom PPO, and the minimal pressure to inactivate mushroom PPO completely was still unknown. Therefore, the effect of UHHP treatment at higher pressure levels on the inactivation of mushroom PPO is worth investigating. In addition, little information was available on the inactivation effects of UHHP on PPO in a real food matrix, especially in comparison to the inactivation effects between PPO in the buffer and in the food matrix. The objective of this work was to study the effects of UHHP treatment on the activity of mushroom PPO both in the phosphate buffer and in the mushroom puree at pressure from 800 to 1600 MPa and further elucidate the alteration of some molecular properties induced by UHHP, such as the secondary and tertiary structures.

MATERIALS AND METHODS

Materials. Mushroom (*Agaricus bisporus*) PPO was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and used without further purification.[18] Fresh mushrooms (*Agaricus bisporus*) were obtained from a local grocery and used within 12 h for the experiment. Catechol was purchased from Beijing Chemicals Co. (Beijing, China). All other chemicals used in the investigation were of analytical grade.

UHHP Process System. Pressure treatments were conducted using a UHHP experimental system designed by China Agricultural University (Beijing, China) and Baotou Kefa Co., Ltd. (Inner Mongolia, China). The system was composed of a hydraulic intensifier, pressure vessel, pumping system, and control panel. All parts of the system exposed to high pressure were made of stainless steel. The pressure vessel was 330 mm in outer diameter, 28 mm in inner diameter, and 400 mm in height and had a volume of approximately 250 mL, which was designed to withstand a pressure of 2000 MPa. A copper tube jacket in thermal contact with the outer surface of the vessel wall was connected to the circulator to allow temperature control. The maximum working pressure of the UHHP unit was 1600 MPa. Di-2-ethylhexyl sebacate was used as the pressure transmission medium in the UHHP unit. A hydraulic intensifier was located at the top of the device. At the downward movement of the plunger rod, high pressure was achieved directly by the compression of the pressure transmission medium. The pressure increase and release rates were about 30 MPa/s and 30−60 MPa/s, respectively. The pressures and treatment time were displayed on a control panel and were continuously recorded by a computer during the pressurization cycle.

UHHP Treatment. The mushroom PPO was dissolved in 50 mM phosphate buffer (pH 6.5) to a concentration of 1.0 mg/mL. To investigate the effect of UHHP on the activity of PPO, the enzyme solution was subjected to different pressure levels (800, 1000, 1200, 1400, and 1600 MPa) at 20 °C for 1 min and an untreated sample running through the same protocol was used as a control. Then, 3.0 mL of 1.0 mg/mL mushroom PPO solution was filled into a polyethylene bag and vacuum-sealed. To avoid the influence of the heat that generated during the heat sealing process, the polyethylene bag (15 cm in length and 3 cm in width) was submerged by ice water throughout the whole sealing procedure. A previous study showed that there was no significant decrease in the activity of mushroom PPO after heat sealing due to the cooling method. Then, the sealed bag was placed and enclosed in the UHHP vessel and pressurized to the required pressure level, and the pressure was held for 1 min. Each UHHP treatment was performed in triplicate. The treatment time reported in this study did not include the pressure increase and release time. Then, depressurization was performed by raising the plunger rod. After UHHP treatment, the PPO solution was taken out from the vessel and immediately cooled in an ice bath. The activity measurement of the enzyme was performed after 0.5 to 1 h storage. Preliminary results showed that no reactivation occurred during this time.

To investigate the effects of UHHP treatment on mushroom PPO in a real food matrix, chilled fresh mushrooms were cut into small pieces (~10 mm^3) and homogenized at 4 °C for 3 min with double their volume of distilled water. The concentration of PPO in the diluted mushroom puree was lower than the concentration used in the phosphate buffer; therefore, to eliminate the influence of the concentration, 8.5 mg of purified mushroom PPO was dissolved in 10 mL of puree to an activity of 1100 ± 50 U/mL, which was the same as the activity in the buffer. Then the mushroom puree was vacuum packed in polyethylene bags, and the cooling method above was also adopted during this procedure. Pressure treatments were performed the same as the treatment condition of purified enzymes above.

Heat Treatment. When the initiation temperature of the UHHP device vessel and the pressure transmission medium was 20 °C, the temperature of di-2-ethylhexyl sebacate would reach about 65, 75, 78, 80, and 82 °C when pressurized to 800, 1000, 1200, 1400, 1600 MPa, respectively.[20] It has been indicated that the real temperature of di-2-ethylhexyl sebacate in the vessel would be lower than the theoretic value due to the heat transfer during the pressure build up and dwelling period. In this work, according to the pressure−temperature curve of di-2-ethylhexyl sebacate[20] and taking into account of the heat-absorbing effect of the container during the pressurization as well as the heat insulation effect of the polyethylene bag, the temperature of the enzyme samples would reach close to 60, 65, 70, 75, and 80 °C at 800, 1000, 1200, 1400, and 1600 MPa, respectively.

Therefore, to investigate the influence of adiabatic heating effect of the pressure transmission medium on mushroom PPO during pressurization, heat treatment at 60, 65, 70, 75, and 80 °C for 1 min was performed. A 3.0 mL of 1.0 mg/mL mushroom PPO solution was filled into a polyethylene bag and vacuum-sealed, then samples were heated for preset times in a water bath with a thermostat. Temperature increasing time was not included. Samples were immediately cooled in ice water upon withdrawal from the water bath.

Enzyme Assay. The activity of mushroom PPO measurement was carried out using an UV-1800 spectrophotometer (Shimadzu Co., Japan).[21] Then, 0.05 mL of 1.0 mg/mL mushroom PPO solution was mixed with 2.95 mL of substrate solution (0.1 M catechol in phosphate

buffer (50 mM, pH 6.5)). For the blank, 0.05 mL of PPO was heated at 95 °C for 2 min to be inactivated completely and added to 2.95 mL of substrate solution. The absorbance of the mixture was immediately monitored at 420 nm for 5 min at a temperature of 25 ± 1 °C. The specific activity was calculated from the slope of a linear segment, expressed as $A_{420\ nm}$/min/0.1 mL of undiluted sample. In this study, the residual activity of PPO was obtained with the following formula:

$$\text{relative activity} = \frac{\text{activity of HHP} - \text{treated PPO}}{\text{activity of untreated PPO}} \times 100\% \quad (1)$$

For the determination of the activity of PPO in mushroom puree, 5.0 mL of treated puree was centrifuged at 14 000*g* and 4 °C for 30 min. The supernatant was used as the crude enzyme extract for the assay of PPO, and the assay method was as the same as the method above.

Circular Dichroism (CD) Analysis. CD spectra were recorded by a JASCO-J-720 CD spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan), using a quartz cuvette of 0.2 cm optical path length at temperature 25 ± 1 °C.[18] CD spectra were scanned in the far UV range (260−190 nm) at 50 nm/min and a bandwidth of 1 nm. Then, 0.2 mL of 1.0 mg/mL mushroom PPO was scanned within 24 h after each treatment. All CD spectra measured were corrected with baseline by phosphate buffer (50 mM, pH 6.5). The CD data were expressed in terms of mean residual ellipticity (θ) in deg cm^{-2} dmol^{-1}, using a mean residual weight (MRW) of 113.7, which was calculated by MRW = $M/(N - 1)$, where M is the molecular mass of the polypeptide chain (in Da), and N is the number of amino acids in the chain.[22] The secondary structure elements of PPO were computed from the data using the online SELCON3 algorithms with the reference set No. 4.[23] The results of three replicates were averaged, and the spectrum was smoothed using Origin 8.0 through the Fast Fourier Transform (FFT) noise reduction routine that allows reduction of most noisy spectra without distorting their peak shapes.

Fluorescence Spectroscopy Analysis. Fluorescence spectra were measured with a Cary Eclipse Fluorescence Spectrophotometer (Varian, Inc., USA), using a quartz cuvette of 0.2 cm optical path length.[21] Then, 0.2 mL of 1.0 mg/mL mushroom PPO was scanned at a temperature of 25 ± 1 °C. The excitation and emission slits were both 10 nm. The emission spectra (λ_{em} from 600 to 295 nm) of mushroom PPO were obtained by scan at its maximum excitation wavelength (λ_{ex} = 280 nm). All samples were scanned within 24 h after UHHP treatment.

Determination of Sulphydryl Groups. Changes in sulphydryl group (SH) content on the surface of mushroom PPO was determined by the method of Ellman and modified.[24] Then, 0.5 mL of 1.0 mg/mL mushroom PPO solution was added to 2.5 mL of sodium phosphate buffer (50 mM, pH 6.5) containing 25 μL of 10 mM 5,5′-dithiobis-2-nitrobenzonic (DTNB) acid solution, and the absorbance of the mixture was measured at 412 nm after incubation at 25 °C for 20 min. Values of surface SH content were obtained by dividing the value of the absorbance by the molar extinction coefficient of 13 600 M^{-1}cm^{-1}.[24] The results were expressed in terms of moles of SH per gram of mushroom PPO.

Statistical Analysis. All experiments were performed in triplicate. Analyses of variance (ANOVA) was carried out by using the software Microcal Origin 8.0 (Microcal Software, Inc., Northampton, USA). The ANOVA test was performed for all experimental runs to determine significance at 95% confidence.

RESULTS AND DISCUSSION

Effect of UHHP Treatment on Mushroom PPO Activity. Effects of UHHP treatment on the activity of mushroom PPO in phosphate buffer were shown in Figure 1. The treatment of PPO at 800 and 1000 MPa for 1 min reduced the enzyme activity by 15.1% and 20.6%, which indicates that UHHP treatment at pressure levels lower than 1000 MPa for 1 min have a little influence on the activity of mushroom PPO.

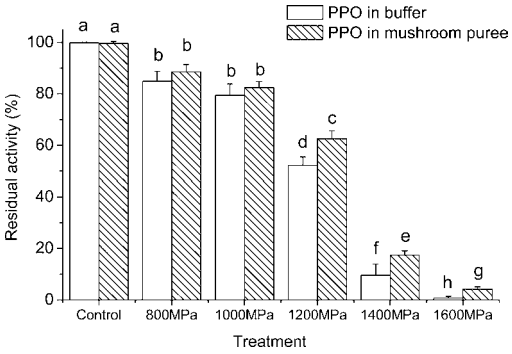

Figure 1. Residual activity of UHHP treated mushroom PPO. Samples were treated at 800, 1000, 1200, 1400, and 1600 MPa for 1 min, respectively. The initial temperature of the sample and the pressure transmission medium were 20 °C. The reaction system was 0.05 mL of a 1.0 mL enzyme solution and 2.95 mL of a 50 mM phosphate buffer at pH 6.5, which contains 0.1 M catechol.

However, PPO activity was reduced by 90.4% and 99.2% after UHHP treatment at pressure levels of 1400 and 1600 MPa for 1 min, respectively, which meant treatment at 1600 MPa for 1 min could not inactivate mushroom PPO completely. Meanwhile, when the treatment pressure increased to 1400 MPa from 1200 MPa, the enzyme activity of mushroom PPO dropped sharply, indicating that the pressure between 1200 and 1400 MPa was probably an inflection for larger inactivation of PPO.

Figure 1 also shows the results of inactivation effects of UHHP treatment on PPO in mushroom puree. The results showed that the enzyme activities in the mushroom puree were decreased to 88.5%, 82.4, 62.5%, 17.4%, and 4.2% after treatment at 800, 1000, 1200, 1400, 1600 MPa, respectively, indicating that UHHP treatment has strong inactivation effects on PPO in the mushroom puree. The inactivation effects were insignificant between purified PPO in the phosphate buffer and crude enzyme in the mushroom puree when treated at 800 and 1000 MPa; however, higher enzyme activity remained in the puree after UHHP treatment at 1200, 1400, and 1600 MPa than in the phosphate buffer. This was probably due to the protection effects of other components in the puree or the release of membrane-bound enzymes[5] and may relate to a transition from the latent to the active state of the enzyme.[25]

Though several studies have been performed on the inactivation of PPO by UHHP, the results were ambiguous. Weemaes et al.[17] reported that plum PPO was not inactivated by UHHP treatment at a pressure of 900 MPa and 25 °C. Garcia-Palazon et al.[12] reported that an inactivation of red raspberry PPO by 29% was observed after UHHP treatment at 800 MPa and 18−22 °C for 15 min. Sun et al.[18] also found that after exposure to 800 MPa for 10 min at temperature around 35 °C, the activity of mushroom PPO reduced by 28%. Gomes et al.[5] reported that as treatment pressure exceeded 400 MPa, the activity of mushroom PPO decreased rapidly with increasing of the pressure level, and after 10 min UHHP treatment at 800 MPa at ambient temperature, complete inactivation of mushroom PPO was achieved. Four reasons were suggested for this discrepancy: (i) the difference of PPO source. The amino acid composition and its sequence affect the enzyme molecular microstructure,[2,3] thus influencing their pressure stability. In addition, the degree of subunit polymerization also influences their pressure stability.[4] For instance, the stimulation effect induced by pressure was only observed for monomeric enzymes. (ii) Different pressure transmission media have different compression heating characteristics, thus influencing the sample temperature during treatment.

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(iii) The pressure increase and decompression rate of the device may influence the inactivation effects of enzyme based on the fact that this rate affected the inactivation of the microorganism.[26] In addition, the vessel volume of the UHHP device was very small in most cases; this rate could prolong/shorten heat transfer time throughout the vessel, pressure transmission medium, and sample, thus altering the treatment temperature. (iv) Some procedures before pressure treatment may lead to inactivation of the enzyme to some extent. For example, the heat that generated during the vacuum sealing process may inactivate the PPO before UHHP treatment. The results of our previous study showed that this heat could even inactivate PPO completely if the sealing procedure was not carefully manipulated.

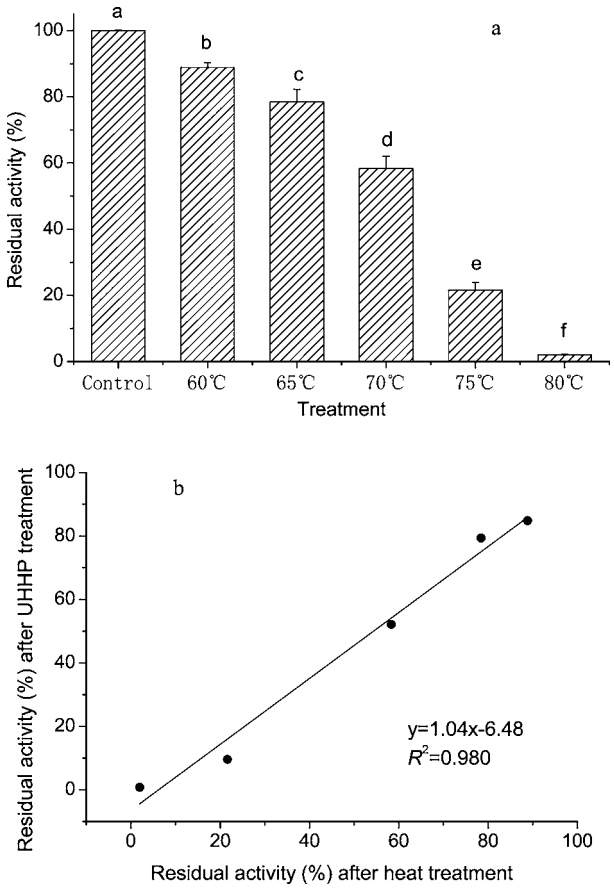

Figure 2. (a) Residual activity of heat treated mushroom PPO. Samples were treated at 60, 65, 70, 75, and 80 °C for 1 min. The reaction system was 0.05 mL of a 1.0 mL enzyme solution and 2.95 mL of a 50 mM phosphate buffer at pH 6.5, which contains 0.1 M catechol. (b) Correlation analysis between effects of UHHP treatment and heat treatment on the activity of mushroom PPO.

Effect of Heat Treatment on Mushroom PPO Activity. As shown in Figure 2a, heat treatment at 60, 65, 70, 75, and 80 °C for 1 min reduced the activity of mushroom PPO by 11.1%, 21.5%, 41.7%, 78.4%, and 97.7%, respectively. Meanwhile, these results showed that the trends of inactivation effect of heat treatment on PPO by heating at 60, 65, 70, 75, and 80 °C were similar to the effect of UHHP treatment at 800, 1000, 1200, 1400, 1600 MPa, which reduced the activity of PPO by 15.1%, 20.6%, 47.8%, 90.4%, and 99.2%, respectively. Moreover, the correlation analysis (Figure 2b) showed that the effects of heat treatment were highly correlated (R^2 = 0.980) to the effects of UHHP treatment on the inactivation of PPO, indicating that the inactivation of PPO by UHHP could be relative to the compression heating.

As the increasing rate of temperature, which was induced by adiabatic compression heating, decreased dramatically with the rising of treatment pressure,[20] the temperature of enzyme samples could reach close to 60, 65, 70, 75, and 80 °C at 800, 1000, 1200, 1400, and 1600 MPa, respectively. Since the temperature of the pressure transmission medium will exceed 65 °C at 1000 MPa due to the adiabatic pressure heating during pressurization and this heat alone has a significant inactivation effect on the activity of mushroom PPO,[27] this implies that the adiabatic compression heating was an important faactor for the inactivation of PPO by UHHP treatment at pressure levels higher than 1000 MPa. Some previous reports also showed that rising treatment temperature could increase the inactivation effects of PPO. Weemaes et al.[17] reported that plum PPO was not inactivated by UHHP treatment at 900 MPa and 25 °C, but inactivation was detectable when treatment temperature increased to 50 °C. In the case of pear PPO, treatment temperature increased from 25 to 35 °C resulted in a 3-fold reduction of the inactivation rate constant.

On the basis of the results above, there was no significant difference between the residual activity of PPO treated by UHHP at 800−1000 MPa (84.9% and 79.4%) and the heat treatment at 60−65 °C (88.9% and 78.5%), indicating that pressure had little influence on the activity of PPO at lower pressure (1000 MPa) and that heat was the main factor on inactivation of PPO. However, the residual activity of PPO treated by UHHP at 1200−1400 MPa (52.2% and 9.6%) was significantly higher than heat treatment at 70−75 °C (58.3% and 21.6%), indicating that the inactivation was not just caused by next generated heat but by a synergic effect contributed by both factors during treatment at pressure higher than 1200 MPa. The results also showed that there was no significant difference between the residual activity of PPO treated by UHHP at 1600 MPa (0.8%) and the heat treatment at 80 °C (2.3%). This insignificance was because when treated at extreme pressure or heat, both of activities of PPO were almost inactivated completely. Therefore, it is speculated that the contribution of the pressure on PPO inactivation was limited when treated at pressure lower than 1000 MPa. As the treatment pressure rises, the contribution of the pressure on PPO inactivation increases.

Effect of UHHP on the Secondary Structure of Mushroom PPO. Common secondary structures in protein solution contain α-helices, β-sheets, and β-turns. They have characteristic far-UV CD spectra, which directly characterize the change of protein secondary conformation.[28] Two negative peaks at 208 and 222 nm are characteristic of the α-helix secondary conformation of proteins, and 214 nm is characteristic of β-sheet secondary conformation of proteins.[28] As shown in Figure 3a, the UHHP treatment at various pressure levels modified the CD spectra of mushroom PPO. The CD spectrum of UHHP treated (800 MPa) mushroom PPO showed a slight alteration, whereas the spectra gave obvious modifications following UHHP treatment at pressure higher than 1000 MPa. Mostly, the negative ellipticity values in the range of 200 and 225 nm were decreased after UHHP treatment, indicating the decrease of the ordered structure. Estimation of the secondary structure exhibited that UHHP treatment caused a decrease in α-helices, an increase in β-structure, and a fluctuation in the unordered fraction of PPO secondary structure (Table 1). The results also showed that the untreated mushroom PPO had

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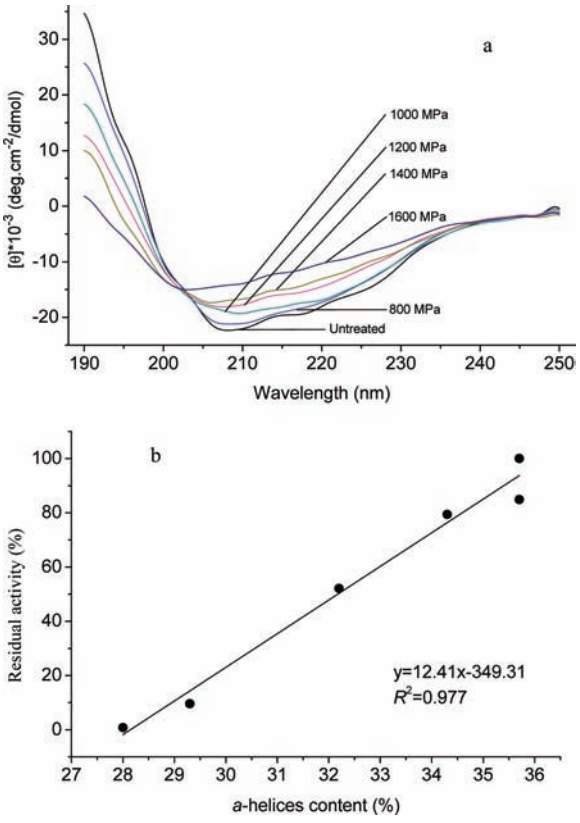

Figure 3. (a) Far-UV CD spectra of UHHP treated mushroom PPO. Samples were treated at 800, 1000, 1200, 1400, and 1600 MPa for 1 min. 0.2 mL of 1.0 mg/mL mushroom PPO was scanned at a temperature of 25 ± 1 °C. CD spectra were scanned in the far UV range (260−190 nm) at 50 nm/min and a bandwidth of 1 nm. (b) Correlation analysis between the α-helices content and the residual activity of mushroom PPO.

Table 1. Secondary Structure Content of UHHP Treated Mushroom PPO[a]

treatment	α-helices (%)	β-sheet (%)	β-turn (%)	random coil (%)
untreated	35.7 ± 0.1	6.8 ± 0.6	18.7 ± 0.5	38.8 ± 0.2
800 MPa	35.7 ± 0.5	8.6 ± 0.5	18.5 ± 0.3	37.2 ± 0.8
1000 MPa	34.3 ± 1.1	9.4 ± 1.0	19.6 ± 0.4	36.7 ± 0.5
1200 MPa	32.2 ± 0.1	10.0 ± 0.3	19.4 ± 0.4	38.4 ± 0.2
1400 MPa	29.3 ± 0.4	12.3 ± 0.5	20.0 ± 0.8	37.8 ± 0.7
1600 MPa	28.0 ± 0.4	14.7 ± 1.2	20.0 ± 0.2	37.3 ± 0.7

[a]Data are presented as the mean ± SD, n = 3.

35.7% α-helices, 6.8% β-sheet, 18.7% β-turn, and 38.8% random coil. After UHHP treatment at 1600 MPa, the α-helices content of mushroom PPO decreased to 28.0% with concurrent increase of the β-sheet content to 14.7%.

The UHHP-induced disruption in the secondary structure of enzymes was also reported in previous studies. Tedford et al.[29] claimed that significant deviations in the CD spectra from that of native lysozyme were found to occur at 600 MPa and 40 °C, resulting in 66% loss in the α-helices content. Mushroom PPO had a similar pattern of decrease of the ordered structure. Sun et al.[30] found that UHHP treatment at 800 MPa causes noticeable decrease in the composition of α-helices of mushroom PPO. As shown in Figure 3b, the decrease of PPO α-helix content correlated to the decrease of its activity after UHHP treatment at various pressure levels (R^2 = 0.979), which indicated that the content of α-helix structure was of vital importance to the catalytic activity of PPO. These results were in agreement with the fact that the secondary structure of mushroom PPO was primarily α-helix, and the core of the enzyme molecule was formed by a four-helix bundle and the two active site copper ions, which were coordinated by three histidine residues contributed from these four helices of the α-bundle.[1] Therefore, it was speculated that the disruption of α-helix structures induced by UHHP treatment led to malfunction of the enzyme active site, thus inducing partial or complete denaturation of mushroom PPO.

Effect of UHHP on the Tertiary Structure of Mushroom PPO. The fluorescence spectrum was determined chiefly by the polarity of the environment of the tryptophan and tyrosine residues and by their specific interactions.[31] The quantum yield of fluorescence decreases when the chromophores interact with quenching agents, either a solvent or in the protein itself.[15] The changes in intrinsic tryptophan fluorescence emission correspond to changes in the tertiary structure of the protein.[21] The fluorescence emission spectra of UHHP treated mushroom PPO at λ_{ex} = 280 nm is shown in Figure 4. The relative

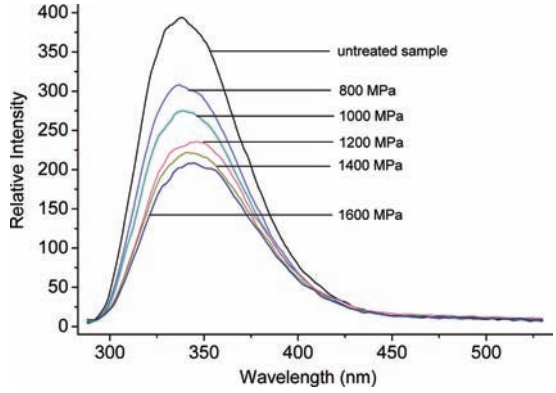

Figure 4. Fluorescence emission spectra of UHHP treated mushroom PPO. Samples were treated at 800, 1000, 1200, 1400, and 1600 MPa for 1 min. 0.2 mL of 1.0 mg/mL mushroom PPO was scanned at a temperature of 25 ± 1 °C. The emission spectra (λ_{em} from 600 to 295 nm) of mushroom PPO were obtained by scanning at its maximum excitation wavelength (λ_{ex} = 280 nm). The excitation and emission slits were both 10 nm.

fluorescence emission value of UHHP-treated PPO exhibited an obvious decrease, and the higher treatment pressure level could result in a greater reduction in the fluorescence intensity. The fluorescence intensity of UHHP treated mushroom PPO at a pressure of 800 and 1600 MPa was decreased by 22.4% and 47.2%, respectively. Those results were in accordance with the findings of Sun et al.[18] who reported that the fluorescence intensity of PPO at 338 nm decreased by 27.4% after exposure to UHHP at 800 MPa at a temperature around 35 °C. The maximum fluorescence emission wavelength of untreated PPO was observed at 337 nm;[30] however, this emission peak shifted to 344 and 346 nm after UHHP treatment at 1400 and 1600 MPa, respectively.

The decrease in the fluorescence intensity indicated that UHHP treatment induced unfolding of the mushroom PPO molecules and the exposure of amino acid residues such as tryptophan residues to the polar solvent environment; thus, the fluorescence was quenched by the environment. Therefore, the well-organized tertiary structure was lost during UHHP treatment.

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The importance of noncovalently bound water in protein structure, function, and stability was well recognized.[32] Modification of electrostatic and hydrophobic interactions, which were the major forces maintaining the tertiary structure, was accompanied by large hydration changes, which were assumed to be primary sources of volume change associated with pressure denaturation of enzymes.[33] In the condition of extreme pressure, the rupture of electrostatic interactions was accompanied by the exposure of charged groups, which has the tendency to reorient water molecules and lead to a loss of the densely packed water structure. The disruption of hydrophobic bonds leads to the exposure of nonpolar amino acid residues to the aqueous solution. Meanwhile, the densely packed water structure was disturbed, and a loosely structured hydrophobic hydration layer was formed. Thus, the pressure-denatured proteins might resemble molten globule structure when the hydrodynamic radii of protein are 10% to 20% greater than those of native conformations.[34] Also, heating was known to amplify hydrophobic interactions and unfolding of protein molecules, which meant that hydration of nonpolar molecules become less favorable with an increase in temperature.[35] Therefore, it was speculated that as an initial step of thermal inactivation occurred at a treatment pressure above 1000 MPa, the enzyme lost a number of essential water molecules, and this loss may give rise to rearrangements in the enzyme. Meanwhile, high pressure fortified this process by further disorder the electrostatic and hydrophobic interactions, thus leading to the denaturation of the mushroom PPO.

In conclusion, UHHP treatment could result in the exposure of amino acid residues to the polar solvent environment and disorder the natural balance of hydrophobic interactions of the enzyme, thus changing the well-organized tertiary structure. However, the exposure of charged groups could also result in reorientation of the surrounding water molecules of the enzyme and lead to the disrupture of the enzyme's natural hydration shell.

Effect of UHHP on the Surface SH Group Content of Mushroom PPO. Sulphydryl groups (SH) and thioether (S−S) bonds significantly influenced the functional properties of food enzymes.[36] Pressure induced denaturation of proteins was a complex phenomenon where hydrophobic bonds and salt bridges between subunits may be disrupted.[37] Kajiyama et al.[38] reported that surface SH content of soybean protein was increased after pressurization at 500 MPa for 30 min, indicating conformational change of the protein molecule.

The surface SH content of mushroom PPO after pressurization is shown in Figure 5a. The changes of PPO surface SH content after UHHP treatment at a pressure below 1000 MPa were insignificant from the native enzyme. As the treatment pressure increased to 1200 MPa, the surface SH group content of mushroom PPO increased significantly. The surface SH content of untreated mushroom PPO was 2.48×10^{-5} M/mg, and the maximum surface SH content of 7.89×10^{-5} M/mg was observed after UHHP treatment at 1600 MPa.

Virador et al. reported that the subunits of mushroom PPO were linked by a number of S−S bonds and that these thioether linkages were vital to maintaining its native conformation and catalytic activity.[3] As shown in Figure 5b, the increase of mushroom PPO surface SH content showed a negative correlation (R^2 = 0.979) with the decrease of its activity after UHHP treatment, which indicated that the rupture of the thioether linkage might induce unfolding of the enzyme, thus leading to the loss of enzyme activity.

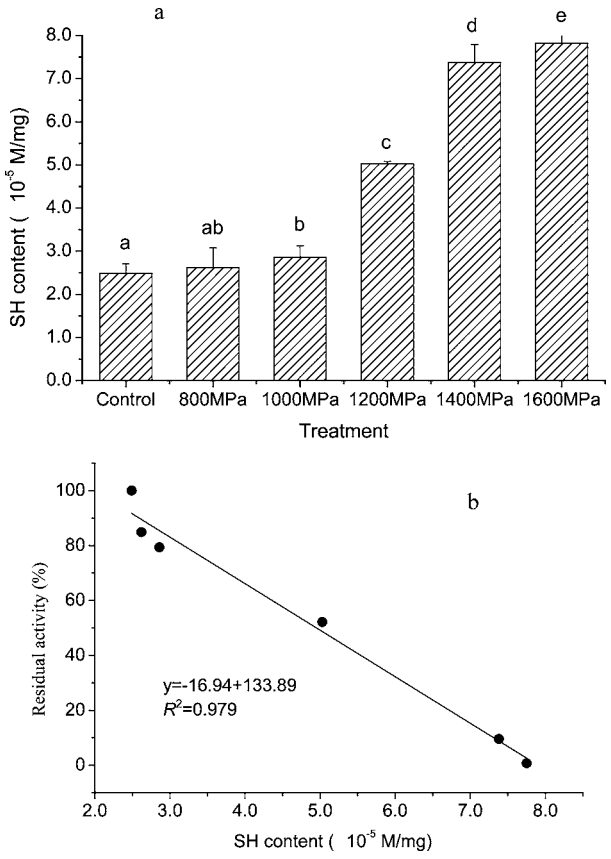

Figure 5. SH content on the surface of UHHP treated mushroom PPO. Samples were treated at 800, 1000, 1200, 1400, and 1600 MPa for 1 min. The reaction system was 0.5 mL of a 1.0 mL enzyme solution and 2.5 mL of sodium phosphate buffer (50 mM, pH 6.5) containing 25 μL of 10 mM 5,5′-dithiobis-2-nitrobenzonic (DTNB). (b) Correlation analysis between the surface SH content and the residual activity of UHHP treated mushroom PPO.

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ABBREVIATIONS USED

UHHP, ultrahigh hydrostatic pressure (pressure >700 MPa); HHP, high hydrostatic pressure (pressure ≤700 MPa); PPO, polyphenoloxidase; UV, ultraviolet; DTNB, 5,5′-dithiobis-2-nitrobenzonic; CD, circular dichroism; rmsd, root-mean-square deviation; SH, sulphydryl group; FFT, fast Fourier transform; MRW, mean residual weight

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