Effects of flat sweep frequency and pulsed ultrasound on the activity, conformation and microstructure of mushroom polyphenol oxidase

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ABSTRACT

The effects of thermal processing (TP) and flat sweep frequency and pulsed ultrasound (FSFPU) treatment with different frequency modes on the activity, conformation and physicochemical properties of mushroom polyphenol oxidase (PPO) were investigated. The results showed that the relative enzymatic activity of PPO gradually decreased with increasing temperature and duration, and thermosonication decreased the PPO activity to a greater extent compared with thermal processing. FSFPU treatment with dual-frequency of 22/40 kHz mode showed the most significant effect. Circular dichroism (CD) showed that the content of α-helix and β-turn dropped, while that of β-sheet and random coil raised after FSFPU treatment. The intensity of endogenous fluorescence decreased, indicating that PPO protein unfolded and the tertiary structure was destroyed. The amount of free sulphydryl, protein aggregation index, and turbidity all rose. Moreover, FSFPU treatment led to the aggregation of protein from the analysis of atomic force microscope (AFM). Conclusively, FSFPU can be used as an effective method to inhibit the activity of endogenous enzymes in food.

1. Introduction

Polyphenol oxidase (PPO) is an oxidase containing binuclear copper atoms at its active site; one copper atom is connected to histidine while another one is to cysteine [1]. It is widely distributed in microorganisms, animals, and plants. Among them, the plant PPO can promote browning reaction in presence of oxygen, where PPO catalyzes the hydroxylation of monophenol to produce catechol [2]. Catechol is further catalyzed and oxidized to produce o-benzoquinone, which is further polymerized into black or brown substances [3]. In addition, the fruit color degradation is caused by PPO, an essential endogenous enzyme. It not only causes fruit juice to brown and the appearance of the product to deteriorate, but it also affects the texture and aroma [4]. As a result, reducing enzymatic browning has long been a priority in the food industry.

Traditional methods of inhibiting enzyme activity include the use of heat treatment and chemical reagents. Among them, heat treatment is the most commonly and widely used technology in industrial production but this method is likely to cause nutrient loss and flavor change [5]. The effects of chemical reagents, such as organic acids and plant extracts, are transient and reversible and chemical residues have potential threats to human health. In recent years, there have been alternative modern methods to replace traditional methods, such as pulsed light, ultra-high pressure, high-pressure carbon dioxide, and plasma [6]. In particular, ultrasound, as a non-thermal technology, is considered to be a promising treatment to improve food quality [7].

Ultrasound has been widely used in food and biological fields. When ultrasonic wave propagates in liquid medium, strong physical effects lead to continuous compression and expansion of liquid, high local temperature and pressure, accompanied by the production of hydrogen and hydroxyl radicals from water decomposition will chemically damage proteins, causing the loss of enzyme activity. Many studies have shown the positive effect of ultrasound on enzymes. For example, with ultrasonic pretreatment in coconut water, Rojas et al. [8] noticed a higher thermal sensitivity of POD, reducing the traditional thermal processing time, which ultimately reduced the damage to food quality. However, the effect of using ultrasound treatment alone to inactivate enzyme is limited. Many people have studied the combination of ultrasound and other treatment methods to inactivate enzymes, including...
heat, lactic acid, and ultraviolet.

Flat sweep frequency and pulsed ultrasound (FSFPU) means that the ultrasonic frequency fluctuates up and down with time in the central frequency range, thus the propagation in the medium has stronger vibration and higher acceleration [9]. Acoustically, sweep frequency ultrasound can provide a suitable environment that is more favorable to enhancing the cavitation effect. Long-time processing causes temperature rise and nutritional loss, while pulsed ultrasound has a greater energy conversion efficiency [10]. In addition, it is worth noting that the ultrasonic frequency is an important index, affecting the sonochemical reaction, which is directly proportional to the cavitation’s intensity [11]. Different ultrasonic frequency working modes with different actual power consumption also have different effect on product quality. Furthermore, dual-frequency ultrasound can cause a greater cavitation effect than single-frequency ultrasound, which may be due to the dual-frequency synergism and the formation of new microbubble clusters with different characteristics at two different ultrasonic wavelengths. The investigations of dual-frequency ultrasound have been successfully conducted in protein [12], starch [13], juice [14], and drying [11,15]. Various researches have demonstrated that ultrasonic treatment can inactivate the endogenous enzymes of fruits and vegetables, but only a few have looked at how ultrasound in different frequency modes affects the structure and characteristics of the enzyme. As a result, the goal of this research was to examine the influences of FSFPU on activity, structure, physicochemical properties and molecular microstructure of PPO. The PPO inactivation process was fitted using first order, fractional conversion, and Weibull distribution models. The secondary and tertiary structures of PPO were investigated using circular dichroism (CD) and fluorescence spectra. The physicochemical changes of PPO were reflected by free sulphydryl concentration and protein aggregation, and the alterations in molecular microstructure were examined using an atomic force microscope (AFM).

2. Materials and methods

2.1. Raw materials and chemical reagents

Mushroom polyphenol oxidase (PPO) (500 U/mg) was acquired from Xinhai Biotechnology Co., Ltd. (Shanghai, China) and utilized without further preparation. The molecular weight of polyphenol oxidase was 128 kDa. All chemicals were bought from Sinopharm Chemical Reagent Company (Zhenjiang, Jiangsu, China).

2.2. Polyphenol oxidase (PPO) preparation and activity assay

PPO (10 mL) was prepared in 0.05 M phosphate buffer (pH 6.8) with the concentration of 0.05, 0.10, 0.15 and 0.20 mg/mL (initial enzyme activity of 5-, 10-, 15- and 20-U), respectively. The effect of flat sweep frequency and pulsed ultrasound treatment on the PPO activity in strawberry juice has been investigated in our previous study [14]. Here, the different concentrations of mushroom PPO solutions were prepared to obtain a concentration closer to the enzyme activity in strawberry juice to investigate the exact mechanism of PPO inactivation by ultrasound.

The activity of PPO was quantified using a UV spectrophotometer, as described by Mustapha Abdullaleet et al. [16] with minor modifications. 0.003 M L-DOPA substrate solution was prepared with above mentioned phosphate buffer. The reaction system consisted of 2.8 mL substrate solution and 0.2 mL enzyme solution, while in the blank group, phosphate buffer was added instead of enzyme solution. The absorbance (wavelength 475 nm) change at the first three min time period was recorded. The quantity of enzyme that causes a change in absorbance unit of 0.001 in one minute is known as enzyme activity. The results are expressed as relative residual enzyme activity (Eq. (1)): 

\[ RA = \frac{A_t}{A_0} \times 100\% \]  

where, \( RA \) represents the residual enzyme activity, \( A_0 \) is the untreated enzyme activity, and \( A_t \) is the residual activity at the treatment time \( t \).

2.3. Thermal and flat sweep frequency and pulsed ultrasound (FSFPU) processing

The thermal processing (TP) was carried out at three temperature levels of 40, 50 and 60 °C. Different concentrations of 5, 10, 15, and 20 U enzyme solutions (each 10 mL) in 25 mL jacketed beakers were heated in a water bath, which was sampled and cooled in an ice bath at a selected time. All the experiments were repeated for 3 times.

The ultrasonic generator, ultrasonic transducer (i.e., ultrasonic vibrating plate), and ultrasonic tank (300 × 360 × 114 mm) were all connected to a computer controller in the FSFPU device. Sweep frequency is the frequency of an ultrasonic wave that moves up and down within a predetermined range around the core frequency during a specific sweep interval. The frequency fluctuation range is expressed as \( \pm \beta \) (where, \( \alpha \) is the center frequency, and \( \beta \) is the frequency fluctuation range). The center frequencies (\( \alpha \)) in this experiment were 22 and 40 kHz (single-frequency 22 and 40 kHz, dual-frequency 22/40 kHz), while \( \beta \) was 2 kHz. Moreover, the ultrasonic power, power density, pulsation ratio, and the scanning frequency cycle were 600 W, 75 W/L, 10:5 (10 s working time and 5 s stop time), and 18 ms, respectively. The water flow was controlled by a peristaltic pump, which was connected with a water bath to keep up with the temperature of water. 10 mL of PPO enzyme solution was placed into a 25 mL jacketed beaker, which was fixed with a 5 cm-high iron frame to fix the sample position in the center of the ultrasonic plate. Ultrasonic treatment was carried out under different ultrasonic frequencies (single-frequency of 22 or 40 kHz, and dual-frequency of 22/40 kHz) combined with thermal treatment (40, 50, 60 °C).

2.4. Kinetic models of PPO inactivation

Inactivation of enzymes is typically characterized using the first order, fractional conversion and Weibull distribution models [17,18,19]. The first order model is represented by following equation:

\[ \ln \left( \frac{A_t}{A_0} \right) = -k_1t \]  

where, \( A_0 \) is the initial activity, \( A_t \) is the residual activity at time \( t \) (min), and \( k_1 \) is the inactivation rate constant (min \(^{-1}\)).

Equation (2) can be converted into fractional form as follows (Fractional conversion model):

\[ \frac{A_t}{A_0} = A_r + (A_0 - A_r)\exp(-k_1t) \]  

where, \( A_r \) is the final residual PPO activity, \( A_0 \) and \( A_t \) are PPO activity at initial time and at time \( t \), respectively.

In terms of the Weibull distribution model, equation (2) can be expressed as:

\[ \frac{A_t}{A_0} = \exp(-b\cdot t^\gamma) \]  

where, \( b \) is the proportion parameter, \( n \) is the shape parameter, \( A_0 \) and \( A_t \) are the PPO activity at the initial time and at time \( t \), respectively.

The coefficient of determination (\( R^2 \)) and root mean square error (RMSE) were determined to assess the model’s accuracy.
where, $R_{Ap}$ and $R_{As}$ represent the predicted value and experimental value, respectively; $n$ is the number of observations.

2.5. PPO structure analysis

2.5.1. Circular dichroism (CD) spectral determination

At room temperature, the secondary structure of the sample was determined by a circular dichroic spectrometer (Model Jasco J-815, Jasco Corporation, Tokyo, Japan). As described by Murata et al. [20], 3 mL of diluted sample (0.025 mg/mL) was taken into a quartz cuvette and the scanning range was 190–250 nm with a 0.5 nm scanning step and a 1 nm bandwidth, respectively. The scanning speed was 50 nm/min. The determination was repeated for three times with PBS as a blank. The percentage of secondary structure was calculated by CDNN software.

2.5.2. Intrinsic fluorescence spectral determination

The endogenous fluorescence emission spectrum of PPO was measured by a Card-F980 fluorescence spectrometer (Lengguang, Shanghai, China) to describe the changes of the tertiary structure of the enzyme [21]. The experiment was carried out utilizing a quartz cuvette with a 10 mm optical path. The scanning speed was 300 nm/min. The excitation wavelength was 280 and 295 nm, and the excitation and emission slit width was 10 nm. The experiment was done for 3 times with a blank control of PBS buffer. The intensity of the fluorescence was measured in an arbitrary unit (A.U.).

2.6. PPO physicomechanical properties analysis

2.6.1. Determination of the free sulfhydryl group content in PPO

The free sulfhydryl group contents in samples were measured using a slightly modified technique developed by Wu et al. [22]. Adding 2 mL Tris-Gly buffer and 0.2 mL Ellman reagent in 1 mL of PPO sample solution treated with TP and FSFPU, respectively. The free sulfhydryl (-SH) content was calculated using the equation below:

$$-SH(\text{mol/g}) = \frac{73.53 \times A_{412}}{C} \times \frac{D}{(10^2 / (1.36 \times 10^3))}$$

where, 73.53 is the molar absorption coefficient, which is calculated from $10^2 / (1.36 \times 10^3)$; $A_{412}$ is the absorbance at 412 nm; $D$ is the sample dilution ratio; and $C$ is the sample concentration (mg/mL).

2.6.2. Determination of the degree of PPO aggregation

2.6.2.1. Aggregation index. At room temperature, the absorption of PPO solution (1 mg/mL) was measured using an ultraviolet-visible spectrophotometer (T6 New Century, Puxi, Beijing, China) at 280 nm and 340 nm [23]. As a control, PBS was employed. The aggregation index was calculated as follows:

$$A1 = \frac{A_{280}}{A_{340} - A_{280}} \times 100$$

where, $A_{280}$ and $A_{340}$ are the absorbance values at 280, 340 nm wavelength, respectively.

2.6.2.2. Turbidity measurement. An ultraviolet visible spectrophotometer (T6 New Century, Puxi, Beijing, China) was used to test the absorbance of PPO solution at 420 nm and the turbidity was expressed as its absorbance value. Distilled water was used as a blank to correct the turbidity measurement [24].

2.7. PPO molecular microstructure analysis

Images of PPO molecular microstructure and morphology were captured at the molecular level by atomic force microscopy (AFM, Bruker Corporation, Karlsruhe, Germany) [12]. 5 μL sample (0.005 mg/mL) was successively dropped on the surface of clean mica sheet and air dried in a fume hood. Bruker ScanAsyst needle was used to obtain a size of 10 μm × 10 μm AFM image in peak force QMN mode. The surface roughness and height distribution of the sample were further analyzed by using NanoScope Analysis 1.8 offline software. The mean roughness ($R_{a}$) and root mean square roughness ($R_{q}$) were determined.

$$R_{q} = \frac{1}{N} \sum_{i=1}^{N} (Z_{i} - \bar{Z})$$

$$R_{a} = \sqrt{\frac{1}{N} \sum_{i=1}^{N} (Z_{i} - \bar{Z})^2}$$

where, $Z_{i}$ is the height corresponding to the ith value, $\bar{Z}$ is the average height of all points within the measurement range and $N$ is the number of samples.

2.8. Statistical analysis

All of the experiments were repeated in triplicate. The data were examined by analysis of variance and Duncan test with social science statistical program (SPSS26). The difference was statistically significant when $p < 0.05$. The deactivation mechanical parameters were analyzed by linear regression with Origin software version 2021.

3. Results and discussion

3.1. Effect of FSFPU on PPO enzyme activity

The effect of thermal processing on polyphenol oxidase activity at different concentrations (5, 10, 15, and 20 U) and temperatures (40, 50, and 60 °C) is shown in Fig. 1. As can be seen from the figure, PPO activity dropped significantly ($p < 0.05$) with increasing temperature at the same concentration. At the same temperature, the lower the enzyme concentration, the easier the inactivation of PPO and the shorter the time required for thermal processing. When the enzyme concentration was high, PPO enzyme activity showed a trend of first increasing and then decreasing, and it still could not decrease by 10% even after 120 min of thermal processing at 40 and 50 °C. For example, when the concentration was 15 U and thermal processing time was 5 min at 40 °C, the PPO activity was maximum (124.35%, Fig. 1C). At the concentration of 20 U, PPO enzyme activity increased by 140.07% and 120.54% after thermal processing at 40 °C and 50 °C for 5 min, respectively (Fig. 1D). Previous studies have also shown that low temperature had a reverse and limited inhibitory effect on PPO activity [25] and PPO maintained completely active when thermal processing was as high as 40 °C [26]. Similar results were found in other studies as well, when PPO in melon juice was treated by ultrasound, PPO activity increased as the ultrasonic intensity increased from 100 to 400 W and subsequently decreased at 500 W [27]. PPO may exhibit a latent form, which can be activated by ultrasonic treatment at low intensities and short treatment durations. Conversely, PPO may be inactivated with increasing ultrasonic intensity or treatment duration.

The residual enzyme activity of PPO after FSFPU treatment at 22 kHz, 40 kHz, and 22/40 kHz at different temperatures is shown in Fig. 2. Under different treatment temperatures (40, 50, and 60 °C), the relative enzyme activity of PPO dropped steadily as treatment time increased. When the PPO was treated with 22 kHz ultrasonic frequency, the activity of PPO changed relatively slower and decreased to 48.68% within 40 min at 40 °C. At 50 and 60 °C, the variation of PPO was dramatically increased and the minimum residual enzyme activity reached to 9.04% (50 °C, 40 min) and 3.26% (60 °C, 20 min). The residual enzyme activities after thermal processing at 40, 50 and 60 °C for 40 min were 70.00%, 23.23% and 2.26%, respectively (Fig. 2). Therefore, compared to thermal processing, thermosonication considerably ($p < 0.05$).
enhanced the PPO inactivation. When the ultrasonic temperature was 40 °C, the residual enzyme activities under ultrasonic frequencies of 22 kHz, 40 kHz, and 22/40 kHz for 40 min were 48.68%, 40.22% and 37.50%, respectively. A similar trend was observed for ultrasonic treatment at 50 °C and 60 °C. When the effect of thermosonication treatment on mushroom PPO was investigated, similar findings were achieved [28]. Therefore, ultrasonic frequency, temperature, and time had significant ($p < 0.05$) effects on the inactivation of PPO. Because the enzyme activity of thermal processing, 22 kHz, 40 kHz and 22/40 kHz was 29.35%, 21.81%, 10.23% and 1.47%, respectively, when treated at 50°C for 30 min. The enzyme activity was at different levels and the variability was more pronounced. The further structural characterization was conducted under the concentration of 10 U at 50 °C for 30 min.

It can be seen that dual-frequency ultrasound 22/40 kHz greatly outperformed single-frequency ultrasound in enzyme inactivation, which might be attributed to the stronger cavitation impact of dual-frequency ultrasound. Yin et al. [29] discovered that dual-frequency ultrasound would produce disturbance in mediums, addressing the drawbacks of single-frequency ultrasound’s unequal volume energy consumption and directional sensitivity. Kerboua et al. [30] investigated the effect of dual-frequency ultrasound on bubbles and found that coupling the wave to a lower frequency enhanced the collapse duration compared to a single frequency. Avaru et al. [31] found a synergistic effect of dual-frequency operation in increasing sonochemical yields, enabling higher sonochemical yields at an equivalent level of total power dissipation. Consequently, under this dual-frequency synergistic effect, the cavitation effect is stronger and the mechanical damage of PPO is bigger, leading to the improvement of the enzyme inactivation effect.

### 3.2. Kinetics of PPO inactivation

The values fitted by PPO model are displayed in Table 1. The coefficient of determination ($R^2$) and root mean square error (RMSE) are acquainted with measure the fitting level of the model; the nearer the $R^2$ worth to 1, and the lower the RMSE value, the better the fitting degree of the model [32]. The results showed that the first-order dynamic model was not ideal, which can be seen from the high RMSE. The $R^2$ of the fractional conversion model was low. In contrast, the Weibull distribution model fitted the PPO inactivation process satisfactorily, due to the high correlation coefficient ($R^2 = 0.996$) and a low prediction error (RMSE = 0.011). Zhang et al. [23] also found that the inactivation of PPO by pulsed light follows the Weibull distribution model. Weibull model has also been applied to the inhibition of enzyme activity by other non-thermal processing technologies, such as plasma, high-pressure carbon dioxide, and ozone [33].

### 3.3. Effect of FSFPU on secondary structure of PPO

Circular dichroism can be utilized to explore secondary structure and swiftly monitor changes in protein structure, such as α-helix, β-sheet, β-turn, and random coil. Untreated PPO showed two negative peaks at 208 nm and 222 nm, as well as a positive peak at 193 nm, indicating an obvious circular dichromatic characteristic with α-helical structure. The negative peak at 220–230 nm is typical for β-sheet structure [34]. Fig. 3A shows the effects of TP and FSFPU on the CD spectrum of PPO. Compared with the control group, the positive peak at 193 nm gradually decreased after thermal and ultrasound treatment at different frequencies and the peak at 193 nm continued to red shift to 195 nm. This is related to the loss of α-helix content and the interaction between protein molecules may be destroyed by thermal and ultrasonic treatment,
although some spectral changes are not significant [35]. When treated with dual-frequency ultrasound (22/40 kHz), the intensity and shape of the peak changed significantly and the shape and intensity of the CD peak also changed, indicating that the secondary structures of different forms in PPO molecules were changed.

In order to describe this transformation in detail, the percentage of each secondary structure was calculated quantitatively by CDNN software. As shown in Fig. 3B, after thermal processing at 50 °C (residual enzyme activity 29.35%) α-helix content dropped from 30.3 to 23.0%, while β-sheet content rose from 20.2 to 21.1%, accompanied by a slight decrease in β-turn. On the contrary, the random coil showed an upward trend. FSFPU showed the same trend, α-helix content dropped to 16.9% and β-sheet content dropped to 25.9% at 22/40 kHz (residual enzyme activity 5.84%). This was similar with the research consequences of Zhou et al. [36]. These outcomes showed that the reduction of α-helix content was related to the decrease in PPO activity. PPO is mainly composed of α-helix conformation and the catalytic center is surrounded by four α-helical beam [37]. The TP and FSFPU treatment caused the loss

| Ultrasonic frequency (kHz) | Temperature (°C) | Time for inactivation of 90% PPO (min) | First-order kinetic model parameters | Fractional conversion kinetic model parameters | Weibull kinetic model parameters |
|---------------------------|-----------------|--------------------------------------|---------------------------------------|-----------------------------------------------|----------------------------------|
|                           |                 |                                      | R² | RMSE | R² | RMSE | R² | RMSE | R² | RMSE |
| 22 ± 1                    | 40              | 123.73                               | 0.019 | 0.993 | 0.036 | 0.045 | 0.973 | 0.004 | 0.025 | 0.917 | 0.996 | 0.011 |
|                           | 50              | 40.25                                | 0.057 | 0.988 | 0.026 | 0.049 | 0.987 | 0.009 | 0.080 | 0.884 | 0.991 | 0.012 |
|                           | 60              | 15.08                                | 0.153 | 0.997 | 0.087 | 0.165 | 0.969 | 0.008 | 0.176 | 0.933 | 0.996 | 0.010 |
| 40 ± 1                    | 40              | 102.38                               | 0.022 | 0.996 | 0.053 | 0.047 | 0.975 | 0.017 | 0.029 | 0.925 | 0.998 | 0.012 |
|                           | 50              | 31.81                                | 0.072 | 0.993 | 0.060 | 0.078 | 0.990 | 0.027 | 0.079 | 0.967 | 0.991 | 0.012 |
|                           | 60              | 12.65                                | 0.182 | 0.997 | 0.104 | 0.193 | 0.997 | 0.019 | 0.241 | 0.863 | 0.998 | 0.008 |
| 22/40 ± 1                 | 40              | 94.21                                | 0.024 | 0.999 | 0.061 | 0.045 | 0.977 | 0.025 | 0.028 | 0.958 | 0.999 | 0.012 |
|                           | 50              | 23.94                                | 0.096 | 0.992 | 0.084 | 0.100 | 0.995 | 0.049 | 0.120 | 0.912 | 0.996 | 0.008 |
|                           | 60              | 10.83                                | 0.213 | 0.995 | 0.118 | 0.225 | 0.996 | 0.033 | 0.328 | 0.779 | 0.999 | 0.01 |
| Average value             |                 |                                      | 0.994 | 0.071 | 0.987 | 0.021 | 0.996 | 0.011 |

Fig. 2. Residual PPO activities (doted lines) and predicted enzyme activities (solid lines) at different times under thermal processing (TP) and flat sweeping frequency and pulsed ultrasound (FSFPU): (A) First-order kinetic model, (B) Fractional conversion kinetic model, (C) Weibull kinetic model.
of α-helix conformation, leading to the inactivation of PPO.

### 3.4. Effect of FSFPU on tertiary structure of PPO

Fluorescence spectroscopy is a commonly used method to characterize the tertiary structure of proteins because the inherent fluorescence of aromatic amino acid residues in proteins is sensitive to the polarity change of microenvironment [12,38]. When the excitation wavelength was 280 nm, the hydrophobic Trp residues and hydrophilic Tyr residues were the predominant sources of endogenous fluorescence. Only hydrophobic Trp residues were found when the excitation wavelength was 295 nm [39].

As shown in Fig. 3C, when the excitation wavelength was 280 nm, the peak emission wavelength of the control group was 341 nm, and the fluorescence intensity of PPO fell dramatically ($p < 0.05$) after TP treatment. After FSFPU treatment, the fluorescence intensity of PPO decreased in turn and the maximum absorption peak shifted to 345 nm. PPO unfolded slightly at 50°C, meanwhile ultrasonic treatment accelerated the exposure of amino acid residues, which explained the rapid inactivation of PPO during thermal ultrasound. The red shift means that more fluorescent groups are presented to the protein surface and the structure of PPO is induced to unfold, resulting in the transfer of fluorescent groups to a more polar environment. This caused a reduction of fluorescence intensity and the destruction of enzyme's tertiary structure [40]. When the excitation wavelength was 295 nm (Fig. 3C), the maximum emission wavelength of PPO was in the range of 340–349 nm, indicating that FSFPU gradually exposed Trp anchored on the surface of protein molecules to water. Compared with the control group (340 nm), the $\lambda_{\text{max}}$ of 22/40 kHz dual-frequency mode was the largest (349 nm), followed by 40 kHz (344.5 nm), 22 kHz (344.5 nm), and TP (343 nm). This indicated that different ultrasonic frequencies had a significant effect on the tertiary structure changes of PPO molecules.

Appropriate mode conditions can promote the expansion of protein structure, increase Trp polarity, and decrease endogenous fluorescence intensity. These findings were consistent with that of Zhou et al. [34], when ultrasound combined with lactic acid treated mushroom PPO. It was also found that a sharp decline in fluorescence intensity as well as a red change in the maximum peak wavelength. However, some studies have found that ultrasound can lead to the increase of fluorescence intensity. In citrus juice, ultrasonic treatment at 300 W resulted in the blue shift and the increase of fluorescence intensity [35].

### 3.5. Effect of FSFPU on the physical and chemical properties of PPO

The free sulfhydryl (–SH) is an important active group affecting the changes of protein properties. It can be oxidized to form disulfide bond and maintain the three-dimensional protein structure. It can be seen from Fig. 3D that after treatment with TP and FSFPU, the free sulfhydryl content of PPO increased significantly ($p < 0.05$). The greater free sulfhydryl content suggested that the inner -SH was exposed to the molecular surface due to the expansion of natural protein caused by ultrasound [41]. Zhang et al. [23] reported that the pulsed strong light could induce partial unfolding of natural PPO, break disulfide bond to form new -SH, and further promote partial unfolding. FSFPU treatment also had similar effects. Due to the cavitation phenomena of turbulence, high pressure and shear force, the buried sulfhydryl groups may be exposed and some proteins unfold [42]. Therefore, -SH content appears to be linked to conformational changes and protein expansion.

The degree of protein aggregation in PPO solution can be expressed by polymerization index and turbidity, which can reflect the particle size and insoluble particle quantity in the solution. After thermal and ultrasonic treatment, both the polymerization index and turbidity increased (Fig. 3D), indicating that the protein was constantly
aggregating. Protein aggregation is closely related to temperature. Thermal processing leads to the exposure of hydrophobic groups and induces protein aggregation; the higher the temperature, the bigger the size of protein polymer. The relatively long time of ultrasonic treatment also increases the turbidity due to the formation of soluble polymers. This is probably due to the effect of turbulent force and microfluidic flow, which increases the speed of collision and protein particle aggregation [43]. Liu et al. [44] reported that long sonication time promoted the interaction between protein molecules, leading to protein aggregation and larger molecule formation.

3.6. Effect of FSFPU on the surface topography of PPO

To investigate the molecular effects of thermal and ultrasound on PPO, AFM was used to observe the particle morphology, size and height of PPO molecules [23]. As shown in Fig. 4A, the original distribution of PPO in the control group was uniform; most proteins had similar heights without apparent changes and the protein coating on the crystal cluster’s surface was uniform (Fig. 4A1). After thermal processing, the structure of the protein was slightly deformed and expanded, showing a relatively long dispersed strip and irregular shape. Moreover, the height also increased, indicating that the protein still retained a highly ordered state (Fig. 4A2). After single-frequency ultrasonic treatment at 22 kHz (Fig. 4A3) and 40 kHz (Fig. 4A4), PPO molecules began to accumulate continuously, forming large and deformed particles. PPO molecules treated with dual-frequency 22/40 kHz (Fig. 4A5) were further polymerized to form larger aggregates, which exceeded the measurement range, as evidenced by the plain white region [45]. This corresponded to the results of the above protein aggregation degree (Fig. 3). It revealed that due to the polymerization of proteins, the active center of the enzyme was covered up, thus preventing the binding of the matrix and ultimately resulting in the loss of the catalytic function of the enzyme molecule and the reduction of PPO activity [6].

Fig. 4B is the height distribution histogram of PPO correlating to the AFM image. Untreated PPO (control) was highly concentrated at 4–7 nm (Fig. 4B1). After heat treatment, the height increased to 10–12 nm, and the percentage of protein with this height was also increased slightly. Fig. 4B3-5 showed that large polymeric protein particles were formed due to cavitation and mechanical effects and the height was further increased. The average roughness ($R_a$) and root mean square roughness ($R_q$) are used as indicators of surface roughness to reflect the degree of aggregation [46]. As shown in Table 2, the untreated PPO had the largest surface roughness with values of $R_a$ and $R_q$ of 1.317 $\pm$ 0.07 and 2.029 $\pm$ 0.04, respectively. After TP and FSFPU treatment, both $R_a$ and $R_q$ values decreased. This indicated that after proteins aggregated into macromolecular groups in the form of dimers or tetramers, the surface roughness was reduced, thus reducing the space of biomolecules bound to them, resulting in PPO inactivation [47].

| Treatments | $R_a$ (nm) | $R_q$ (nm) |
|------------|------------|------------|
| Control    | 1.317 $\pm$ 0.07$^a$ | 2.029 $\pm$ 0.04$^a$ |
| Thermal    | 0.735 $\pm$ 0.04$^b$ | 1.046 $\pm$ 0.02$^b$ |
| 22 kHz     | 0.683 $\pm$ 0.01$^e$ | 0.831 $\pm$ 0.01$^e$ |
| 40 kHz     | 0.536 $\pm$ 0.03$^d$ | 0.712 $\pm$ 0.02$^d$ |
| 22/40 kHz  | 0.339 $\pm$ 0.02$^f$ | 0.683 $\pm$ 0.01$^f$ |

Table 2: Effect of FSFPU and TP treatment on surface roughness of PPO (Mean $\pm$ S.D.).

4. Conclusions

In this research, we thoroughly analyzed the effects of thermal processing (TP) and flat sweep frequency and pulsed ultrasound (FSFPU) in multiple frequency modes on enzyme activity, structure, physicochemical properties, and molecular microstructure of mushroom PPO. The results showed that TP and FSFPU treatment reduced PPO activity and the FSFPU treatment with dual-frequency mode of 22/40 kHz had the most noticeable effect and its inactivation was successfully fitted with the Weibull distribution model. In addition, after TP and FSFPU treatment, the $\alpha$-helix and $\beta$-turn of PPO enzyme decreased, resulting in the occurrence of unfold in secondary structure. Moreover, the tertiary structure was destroyed after treatment from the analysis of fluorescence spectroscopy. The microstructure of PPO enzyme was changed with the reduction of surface roughness and aggregation of the protein. This caused the active center of the enzyme covered, which ultimately led to the loss of the catalytic function of the PPO enzyme molecule and
reduction of its activity. In summary, FSFPU had a positive impact on the work reported in this paper.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CRediT authorship contribution statement

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