The activation mechanism of peroxidase by ultrasound

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1. Introduction

Peroxidase (EC 1.11.1.7) is an oxidase containing ferriporphyrin that catalyzed the oxidation of monophenols and triphenols in terms of functions \cite{1}, contributing to the formation of o-quione and brown or black pigments by self-polymerization in plants. In recent decades, peroxidase has been well studied and widely applied in the fields of life science, medicine, environmental preservation and biosensors. On the one hand, peroxidase is a critical factor that caused browning of fruit and vegetables in processing and storage and responsible for the economic loss. On the other hand, peroxidase with high activity could effectively inhibit microorganism and guaranteed the safety and hygiene of food \cite{2}.

Over the past decades, the effects of various inhibitors and physical-treatment methods on peroxidase activity have been identified and put into practice \cite{3,4}. While, the influence of novel approaches, such as ultrasonic treatment, microwave radiation, high hydrostatic pressure and dense phase carbon dioxide \cite{5}, to peroxidase activity is still ongoing.

Ultrasonic wave is a kind of sound wave with a frequency higher than 20 kHz, which is named for its lower frequency which is higher than the upper limit of hearing of human. It has great directionality, strong penetrating ability, and is easy to obtain concentrated sound energy. It can be used to for distance measurement, speed measurement, cleaning, welding gravel, sterilization, medicine, military and food industry \cite{6}. The effect of using ultrasound or in combination with other chemicals with reducibility on peroxidase activity and browning in food is of great concern \cite{7}. The current study showed that peroxidase and polyphenol oxidase activities from fresh-cut apple and vegetables were inhibited by simultaneous ultrasound treatment and reductants. Therefore, ultrasound treatment many have tremendous prospects in promoting food quality by controlling discoloration of fruit and vegetables to fulfil the desire of consumers. To the best of our knowledge, ultrasound has certain limitations to inhibit browning of fruit and vegetables. Studies have shown that the activity of peroxidase is activated under low-power ultrasound, and gradually decreased under high-power ultrasound required high power consumption. However, the potential activation mechanism is unknown.

The objective of the present study was to investigated the activated mechanism of ultrasound on peroxidase activity based on spectroscopy including ultraviolet–visible spectrometry, fluorescent spectrometry, Fourier transform infrared spectra (FT-IR), atomic force microscopy (AFM) and circular dichroism (CD) with a view to providing basis and application guidance for the application of ultrasound in resisting browning.

2. Materials and methods

2.1. Materials and ultrasonic treatment

Hydrogen peroxide, guaiacol, chlorogenic acid, phosphate buffer, horseradish peroxidase were all purchased from Sigma (St. Louis, MO, USA).
USA). Hydrogen peroxide, chlorogenic acid and guaiacol were diluted with ultra-purified water to 1%, 4%, 4%, respectively. Peroxidase solution (1.0 × 10⁻⁵ mol L⁻¹) was prepared in phosphate buffer (0.05 mol L⁻¹, pH 6.5).

3 mL of ultrasound treatment of peroxidase solution was put into the centrifugal tube and then fixed in the center of the ultrasonic apparatus (KQ500DE, Herchuang Co., Kunshan, China) for 10 min. The bath is a rectangular container (23 cm × 14 cm × 10 cm), annealed at the bottom of the 40 kHz transducer. After measurement, the actual power of ultrasound is 100 ± 0.5 W. By adding ice packs intermittently, check with a thermometer and keep at 0 to 4 °C temperature to control the temperature inside the solution.

2.2. Enzyme activity

Peroxidase activity determination system consisted of 2.775 mL of phosphate buffer (0.05 mol L⁻¹, pH 6.5), 100 μL of 4% m/v guaiacol or chlorogenic acid, 100 μL of 1% v/v H₂O₂ and 25 μL of peroxidase solution. The change of OD₄₇₀nm was recorded by UV-2550 spectrometer (Shimadzu, Japan) was used to record the change of OD₄₇₀nm within 120 s. One enzyme unit (U) was defined as increase 0.01 △OD₄₇₀nm min⁻¹, and results were expressed as U m L⁻¹. Peroxidase solution without ultrasound treatment was regarded as control.

2.3. UV-visible spectra scanning

3 mL of peroxidase solution (2.0 × 10⁻⁶ mol L⁻¹) with and without ultrasound treatment were scanned on a UV-2550 spectrometer (SHIMADZU, Japan) with a wide range from 300 to 700 nm, respectively.

2.4. Fluorescence spectra

Peroxidase solution with ultrasound treatment (0–100 W) for 20 min were scanned the emission spectra on F-2500 (HITACHI, Japan) from 250 to 500 nm, the excitation wavelength were 280 nm, the excitation and emission width were 2.5 nm.

The synchronous fluorescence spectra of peroxidase solution with and without ultrasound treatment were recorded by excitation and emission wavelength interval (Δλ) at 15 and 60 nm.

2.5. Infrared spectroscopic analyses

The peroxidase solution (2 × 10⁻⁶ mol L⁻¹) with and without ultrasonic treatment were lyophilized and dried. 3 mg sample and 300 mg potassium bromide were ground in an agate mortar until evenly pressed [8]. The sample and control were tested on Nicolet 6700 FTIR instrument (ThermoElectric Corporation of America) equipped with DTGS detector, and the resolution is 4 cm⁻¹, scanning 32 times for spectral
accumulation. FTIR spectra were recorded within the wavenumber range of 1800–1400 cm⁻¹/nm.

2.6. Surface structure

The surface measurement of horseradish peroxidase was carried with AFM (Dimension ICON10800, BRUKER OPTICS company, USA) at room temperature. The horseradish peroxidase solutions with and without ultrasound treatment were tiled on the surface of the lat and brand-new mica surface in advance, and allowed to air dry for several hours. Samples were scanned in tapping mode, and the rate was 0.65 Hz.

2.7. CD spectra

CD spectra of peroxidase with and without ultrasound treatment were conducted on a CD spectrometer (MOS-50010400, Bio-Logic, Claiix, France) under constant nitrogen flush at room temperature. The CD spectrometer recorded the CD spectra change from 190 to 250 nm. The online program SELCON3 was used to calculate the contents of different secondary structures of peroxidase with and without ultrasound treatment CD spectra data (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) [9].

3. Results and discussion

3.1. The effect of ultrasound on the peroxidase

It has been recognized that the expression level and activity of peroxidase are directly related to the browning of fruit and vegetables. Therefore, it is particularly important to find technical methods to control the enzyme activities. In this study, the effect of ultrasound to the activity of peroxidase was investigated. The influence of ultrasonic power on enzyme activity and the results were shown in Fig. 1A, the peroxidase activity was prior to the control due to ultrasonic treatment. The enzyme activity increased gradually after ultrasonic treatment and reached its maximum values at 60 W and 90 W. In addition, the absorption values of the control group were 1.05 ± 0.05 and 0.43 ± 0.03 at 120 s with guaiaicol and chlorogenic acid as substrates, respectively (Fig. 1B); while the sample groups were 1.59 ± 0.04 and 0.65 ± 0.03, respectively (Fig. 1C), indicating that ultrasound could effectively improve the affinity between the peroxidase and the substrate and accelerate the substrate reaction. Actually, in recent decades, it has been observed ultrasound could promote the enzyme activity of alcohol dehydrogenase, alkaline phosphatase, protease, lipase, pectinmethylesterase and laccase [10–13] due to It has been reported that the increase in enzyme activity is caused by ultrasound has several main functions, such as enhancement. micromixing mass transfer, enzyme release because of the stimulation of cell division and biochemical reactions to enhance the production of specific enzymes in cellular tissue [14].

3.2. UV spectra

The ultraviolet spectra results of peroxidase with and without ultrasound were shown in Fig. 1D. The ultraviolet absorbance of controls existed a maximum values at 403 nm, while the sample with ultrasound had a slight blue shift and maximum absorbance at 400 nm, which implied that ultrasound exposed the hydrophobic groups and chromophores embedded in the protein, resulting in increased absorbance and redshift of the wavelength.

3.3. Fluorescence spectra analysis

The way in which protein molecules stretch or fold, the number of aromatic amino acids and chromophores, which led to differences in
protein fluorescence intensity, could be inferred from the state of protein molecular structure based on changes in peak and shift in protein fluorescence emission spectra. The Fig. 2A showed that the fluorescence intensity of peroxidase increased with the increasing ultrasonic power at 311 nm. When the power was 90 W, the fluorescence intensity of peroxidase increased by 142%.

Synchronous fluorescence spectroscopy is an effective method to detect the changes of tyrosine and tryptophan in protein molecules under the influence of external factors. Compared with the controls, Compared with the control group, the fluorescence intensity of tyrosine residues at 286 nm in the treatment group gradually increased with the increase of ultrasonic power, but the corresponding fluorescence peak wavelength did not shift (Fig. 2B). Similarly, the fluorescence intensity of tyrosine and tryptophan residues gradually increased with the increase of ultrasonic power, which was mainly attributed to the fact that ultrasound could induce peroxidase structure unfolding and denaturation by destroying the hydrophobic interactions of the molecules, contributing to exposure of hydrophobic residues and thus fluorescence intensity increased [15], and the corresponding fluorescence peak wavelength showed a weak shift from 326 nm to 323 nm (Fig. 2C), indicating that the ultrasonic treatment improves the polarity of tryptophan residues [16], The red shift of the fluorescence peak of tryptophan residue contributed to the conformation change of peroxidase, indicating that a certain degree of ultrasound treatment can cause the movement of the residues inside the molecule, the exposure of chromophore and conformation of the enzyme.

3.4. CD spectra

The change of peak shape of protein circular dichromatogram could reflect the change of its secondary structure. Normally, the α-helix of the natural protein has a positive band around 192 nm, and negative peak around 222 nm and 208 nm; the β-sheet has a positive band at 185–200 nm and is at 216 nm [17]. There is a positive band around β-turn around 206 nm, and the change of circular dichroic band position and absorption intensity can reflect the nature of protein or peptide bonds.

When peroxidase was treated by 80 W ultrasound, CD spectrum results showed that the wavelength corresponding to the characteristic peak of peroxidase secondary structure did not shift (Fig. 3A), but the content of secondary structure changed slightly, β-sheet, random coil and β-antiparallel increased from 5.37% to 9.42%, from 29.56% to 33.07% and from 3.7% to 5.65%, while the α-helix and β-turn decreased from 35.32% to 30.94% and from 26.05% to 20.92%, respectively, leading to a uncompacted conformation due to the decrease of α-helix. The reason for the conformation change might be that ultrasonic cavitation and mechanical shearing disrupt the hydrogen bond and hydrophobic force network that maintained the secondary structure of the protein, and promotes peroxidase structure stretching and rearrangement [18], which benefited the entrance of substrates to active sites and
promote the catalytic reaction.

3.5. FT-IR

The protein is characterized with amide I band (1700–1600 cm\(^{-1}\), mainly C–O stretch) and amide II band (1600–1500 cm\(^{-1}\), mainly C–N stretch coupled with a N–H bending mode) in Fourier transform infrared spectra, which were used to calculated the change of the secondary structure of protein [19].

Fourier transformed infrared spectrum was convolved, and then double integral analysis was performed. The position of the amide I band altered from 1654 to 1655 cm\(^{-1}\), and the amide II band moved from 1549 to 1550 cm\(^{-1}\) with ultrasonic treatment (Fig. 3B), indicating that ultrasound may affected the C–O and C–N groups in the protein structure, leading to the conformational change. β-sheet, random coil and β-antiparallel increased from 5.57% to 8.23%, from 30.21% to 31.28% and from 4.23% to 6.31%, while the α-helix and β-turn decreased from 34.87% to 31.87% and from 25.12% to 22.31%, respectively, indicating that ultrasound could induce changes in the secondary structure of peroxidase (Fig. 3C and D), which will lead to the exposure of the active center, which is conducive to the rapid binding of the substrate to the enzyme. In addition, sonochemically induced radical attack or shear forces can increase the enzyme activity [20].

3.6. AFM

Atomic Force Microscope (AFM), an analytical instrument that can be used to study the surface structure of solid materials including insulators. The structure of the protein determined its morphology, it’s necessary to observe the microstructure of peroxide with and without ultrasonic treatment. The Fig. 4A showed that the free peroxidase, with average 5.47 nm in roughness and average 17.1 nm in height at the resolution of 1 µm (Fig. 4B). While the sample with ultrasonic treatment 0.84 nm and 2.9 nm in height (Fig. 5B). Compared with free peroxidase, the availability of peroxidase molecular group with ultrasonic treatment was generally increased within the solution (Fig. 5A), which promoted the combination between peroxidase and substrates and enhanced the activity [21].

It has been reported that amino acid residues interact to determine the function and natural conformation of this protein. When other proteins are available for proteins, the attraction between other protein residues may lead to the formation of intermolecular clusters or
In this experiment, the effect of ultrasound on peroxidase was investigated based on spectroscopy. It was found that ultrasound could enhance the polarity of peroxidase tryptophan residues and cause the conformational change of peroxidase, increasing the content of β-sheet, random coil, and β-antiparallel, and decreasing the content of α-helix and β-turn. The conformational change made the catalytic center more receptive to substrate catalysis, and thus showed better catalytic efficiency for monophenol-triphenol compounds. This study will further explore its potential mechanism and application, hoping to find more ways to inhibit peroxidase by ultrasonic treatment. In order to reduce browning of fruits and vegetables and the loss in food processing and storage, it will also contribute to broadening the application of ultrasonic treatment in the food industry.

4. Conclusions

In experiment, the effect of ultrasound on peroxidase was investigated based on spectroscopy. It was found that ultrasound could enhance the polarity of peroxidase tryptophan residues and cause the conformational change of peroxidase, increasing the content of β-sheet, random coil, and β-antiparallel, and decreasing the content of α-helix and β-turn. The conformational change made the catalytic center more receptive to substrate catalysis, and thus showed better catalytic efficiency for monophenol-triphenol compounds. This study will further explore its potential mechanism and application, hoping to find more ways to inhibit peroxidase by ultrasonic treatment. In order to reduce browning of fruits and vegetables and the loss in food processing and storage, it will also contribute to broadening the application of ultrasonic treatment in the food industry.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultrasonch.2020.105362.

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