

Research Article

Effects of Different Working Modes of Ultrasound on Structural Characteristics of Zein and ACE Inhibitory Activity of Hydrolysates

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Ultrasound was used as a new technology to pretreat protein prior to proteolysis to improve enzymolysis efficiency. The effects of different working modes of ultrasound on the angiotensin I-converting enzyme (ACE) inhibitory activity of zein hydrolysates and the structural characteristics of zein were investigated. The solubility, surface hydrophobicity (H_0), ultraviolet-visible (UV-Vis) spectra, intrinsic fluorescence spectra, and circular dichroism (CD) spectra of zein pretreated with ultrasound were determined. All ultrasound pretreatments significantly improved the ACE inhibitory activity of zein hydrolysates ($p < 0.05$). The highest ACE inhibitory activity, representing an increase of 99.21% over the control, was obtained with dual sweeping frequency ultrasound of 33 ± 2 and 68 ± 2 kHz. The effects of single sweeping frequency and dual fixed frequency ultrasound were stronger than those of single fixed frequency ultrasound for improving the ACE inhibitory activity of zein. Structural changes in zein were induced by ultrasound, as confirmed by changes in the solubility, H_0 , UV-Vis spectra, intrinsic fluorescence spectra, and CD spectra of zein, and these were consistent with the corresponding ACE inhibitory activities of zein hydrolysates. Thus, ultrasound working mode and frequency have significant effects on the structure of zein and the ACE inhibitory activity of zein hydrolysates.

1. Introduction

Corn gluten meal, a main byproduct from the corn wet-milling process, contains 60–71% (w/w) protein. The major protein fractions in corn gluten meal are zein and glutelin, accounting for 68% and 28%, respectively, of the total protein weight [1]. At present, corn gluten meal is mainly used as a foodstuff or disposed of, largely due to the water insolubility of zein and the severe imbalance of amino acids that compose it [2]. Zein belongs to the class of prolamins with a molecular weight of apparently 24 kDa and 27 kDa (α -zein) and 17 kDa (β -zein) and consists of mostly hydrophobic nonpolar amino acids that are buried inside the molecule [3]. It has been reported that the bioavailability of its proteins could be improved significantly via enzymatic modification [4]. However, the water insolubility of zein and its extensive intermolecular interactions create a barrier, making it difficult for the enzyme to attack the protein bonds and leading

to long processing times and low enzyme utilization rates and protein conversion rates. Therefore, innovative technologies to overcome these shortcomings are in high demand.

Low-frequency (20–100 kHz) ultrasound treatment, which has been widely used in the extraction of target compounds, production of bioactive peptides, and hydrolysis of proteins [5], has attracted increasing attention in the food processing industry. Owing to its thermal, cavitation, and mechanical effects, ultrasound can increase mass transfer, enhance the extrusion of media molecules, and change media density, function, and structure. Especially for acoustic cavitation, the collapse of cavitation bubbles can produce violent physical forces (e.g., microjets, shear forces, and shock waves) and free radicals [6]. Ultrasound is considered the fundamental process that causes the most sonochemical reactions in liquids [7]. Acoustic mode and ultrasonic frequency are two major factors that affect the production and intensity of liquid cavitation and influence

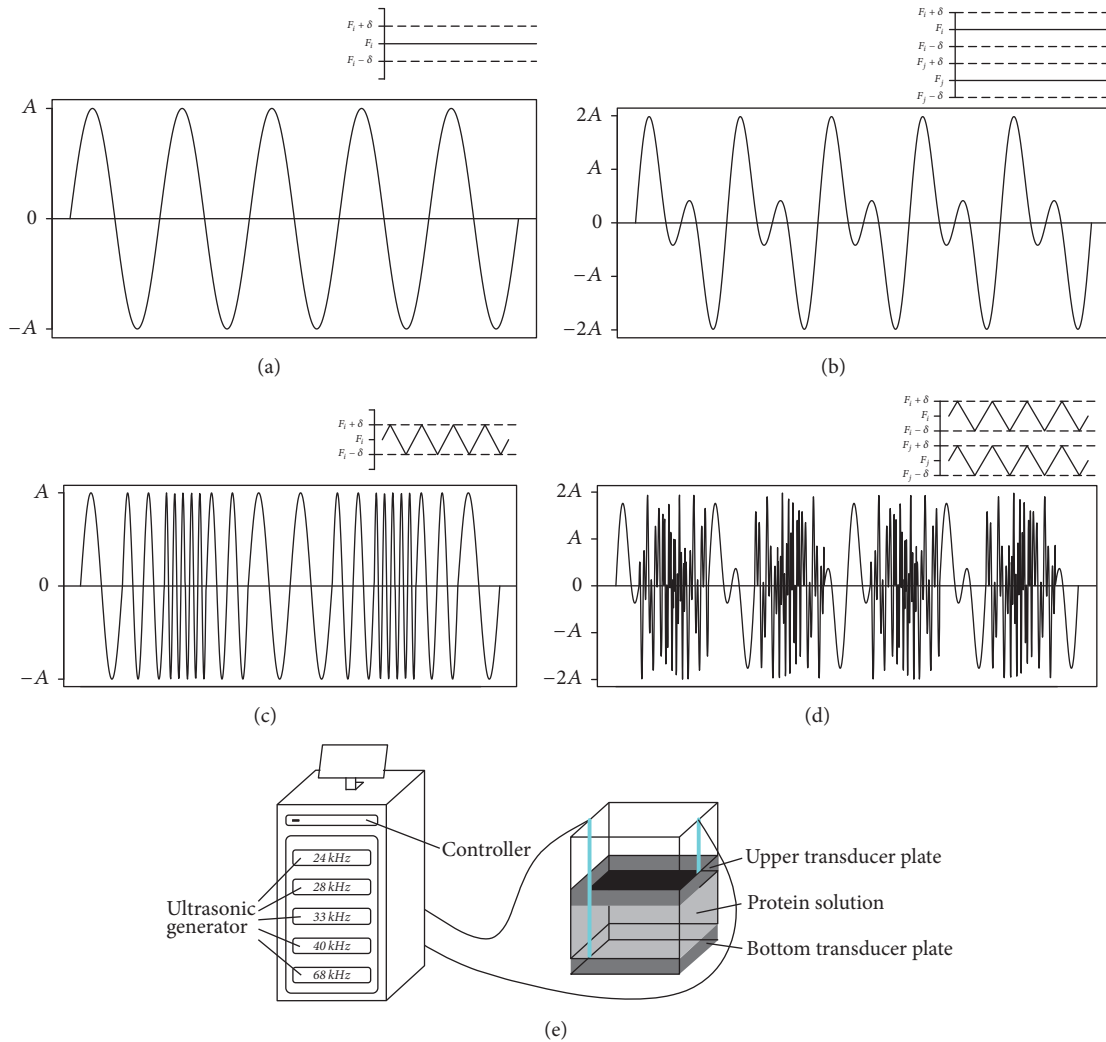


FIGURE 1: Ultrasonic patterns of single fixed frequency ultrasound (a), dual fixed frequency ultrasound (b), single sweeping frequency ultrasound (c), and dual sweeping frequency ultrasound (d). Equipment used for fixed and sweeping frequency ultrasound (e).

the ultrasound treatment effect. Qu et al. [8] found that a single sweeping frequency ultrasound pretreatment at 24 ± 2 kHz can increase the enzymolytic efficiency and angiotensin-converting enzyme (ACE) inhibitory activity of wheat germ protein hydrolysates. Jin et al. [9, 10] reported that dual sweeping frequency ultrasound pretreatment at $28 \pm 2/68 \pm 2$ kHz can change the surface microstructure and chemical structure of proteins. Jia et al. [11] demonstrated that ultrasound with a single fixed frequency of 24 kHz resulted in increases of 21.0–40.7% in the ACE inhibitory activity of wheat germ protein hydrolysates. Ultrasound pretreatment can significantly change the structure of proteins and improve the functionality of protein hydrolysates. There have also been several studies focused on pretreatments with simple ultrasound modes of one or several frequencies on different raw proteins. However, there has been no systematic research conducted on the effect of different ultrasound frequencies and working mode pretreatments on raw materials.

Our team has researched the application of different working modes of ultrasound technology in food physical

processing for many years. The equipment for fixed frequency and sweeping frequency ultrasonic baths has been well developed, consisting of upper and lower plates (Figure 1(e)), each of which can deliver its own frequency separately. In general, there are four working modes of ultrasound with different frequencies induced by this equipment: single fixed frequency (f_i kHz) ultrasound (Figure 1(a)), dual fixed frequency (f_i and f_j kHz) ultrasound (Figure 1(b)), single sweeping frequency ($f_i \pm \delta$ kHz) ultrasound (Figure 1(c)), and dual sweeping frequency ($f_i \pm \delta$ and $f_j \pm \delta$ kHz) ultrasound (Figure 1(d)). On the basis of an earlier report, we previously investigated the effect of single-frequency ultrasound treatment on enzymatic preparations of ACE inhibitory peptides from zein [12]. However, there is limited information regarding the effect of dual-frequency ultrasonic treatment on zein. Therefore, the objective of this research was to (1) compare the effects of pretreatment with the four working modes of ultrasound at two frequencies on the degree of hydrolysis (DH) of zein and the ACE inhibitory activity of

zein hydrolysates and (2) compare resulting changes in the microstructure of zein.

2. Materials and Methods

2.1. Materials. Corn gluten meal with 56–60% protein content was obtained from Fenda Starch Co. Ltd. (Jiangsu, China). Zein with 90.23% protein purity was extracted according to the methods of a previous study [13]. Alcalase with an activity of 132,507 U/ml was purchased from Novozymes Biotechnology Co. Ltd. (Shanghai, China). ACE was extracted from pig lungs, and its activity was assessed using Hippuryl-His-Leu (Sigma-Aldrich Trading Co. Ltd., Shanghai, China) as a substrate. All other reagents were of analytical grade.

2.2. Ultrasound Pretreatment of Zein. An aliquot of zein suspension (200 mL, 2 g zein) was sealed in a high-pressure-resistant bag and treated in an ultrasound bath (internal dimensions 362 mm × 294 mm × 502 mm; Shangjia Biotechnology Co., Wuxi, Jiangsu, China) with 5.4 L of water. The two frequencies tested were 33 and 68 kHz. The four working modes of operation included single fixed frequency (33 kHz or 68 kHz) ultrasound, dual fixed frequency (33 and 68 kHz) ultrasound, single sweeping frequency (33 ± 2 kHz or 68 ± 2 kHz) ultrasound, and dual sweeping frequency (33 ± 2 & 68 ± 2 kHz) ultrasound. The other pretreatment parameters were as follows: sweeping cycle, 500 s; power, 600 W; pulsed on/off time, 10 s/3 s; and sonication time, 40 min. Control samples were prepared using a magnetic stirrer rather than ultrasound treatment, with other conditions remaining unchanged.

To study the effect of ultrasound on the structural characteristics of zein, precipitation was performed by centrifugation at 2,500g for 10 min and lyophilization for further analysis.

2.3. Enzymatic Hydrolysis of Zein. Before enzymatic hydrolysis, the zein suspension was adjusted to pH 8.0 and incubated in a water bath at 50°C for 10 min. Then, Alcalase ($E/S = 3500$ U/g) was added to start the reaction, and the pH was maintained at 8.0 by continuously adding 1 M NaOH. After enzymatic hydrolysis for 40 min, the mixture was adjusted to pH 7.0 and boiled for 10 min to inactivate the enzyme. Trichloroacetic acid (6% v/v) was added to the mixture at a ratio of 1:1 to precipitate the protein. Finally, the hydrolysates were cooled and centrifuged at 5000g for 15 min, and the supernatant was acquired for further analysis.

2.4. Determination of Enzymatic Hydrolysis of Zein

2.4.1. Degree of Hydrolysis. The DH of zein was determined using the pH-state method [14] and was calculated using

$$\text{DH (\%)} = \frac{h}{h_{\text{tot}}} = \frac{N_b \times B \times 100}{\alpha \times M_p \times h_{\text{tot}}}, \quad (1)$$

where B is the NaOH volume consumed (mL), N_b is the normality of the NaOH (mmol/mL), M_p is the protein weight (g), α is the average dissociation degree of $\alpha\text{-NH}_2$ in substrate

(0.985 for zein), and h_{tot} is the total number of peptide bonds in the protein substrate (7.35 mmol/g for zein).

2.4.2. Measurement of ACE Inhibitory Activity. The ACE inhibitory activity of the zein hydrolysates was measured using a previously reported method with slight modifications [15]. Briefly, 45 μL of substrate borate buffer (1.0 mM FAPGG, 80 mM HEPES, 0.1 M borate buffer, and 300 mM NaCl; pH 8.3) was mixed with 40 μL of sample in a microtiter plate well. The reaction was initiated by adding 20 μL of ACE (0.1 U/mL). After reacting for 30 min at 37°C, the absorbance of the reaction mixture was immediately monitored at 340 nm. The ACE inhibitory activity was calculated as follows:

$$I = \frac{(A_C - A_S)}{(A_C - A_B)} \times 100, \quad (2)$$

where I is the ACE inhibitory activity (%), A_C is the absorbance of the control group without protein hydrolysates, A_B is the absorbance of the blank group without ACE, and A_S is the absorbance of the sample group with ACE and protein hydrolysates.

2.5. Determination of Physicochemical Properties and Structure of Zein

2.5.1. Solubility Measurement. Bovine serum albumin was chosen as a standard, and the crude protein content of the supernatant after ultrasound treatment was determined according to the Lowry method [16]. Solubility was expressed as the percentage of protein in the supernatant compared with the total amount of primary protein.

2.5.2. Surface Hydrophobicity (H_0) Measurement. The surface hydrophobicity of zein dispersions was determined using the method described by Kato and Nakai [11, 17] with slight modifications using 1-anilino-8-naphthalene-sulfonate as a fluorescent probe (ANS). The ultrasound-pretreated and control samples were dissolved in 0.01 M phosphate buffer saline (PBS) to 0.5 mg/mL. Then, 50 μL of ANS was added to the 5 mL of zein supernatant and mixed at 25°C. Relative fluorescence intensity was monitored immediately using a Cary Eclipse spectrophotometer at an excitation wavelength of 700 nm (slit 5.0 nm), emission wavelength of 360–750 nm, and scanning speed of 5 nm/s. The surface hydrophobicity was expressed as the relative fluorescence intensity at a protein concentration of 0.5 mg/mL.

2.5.3. Ultraviolet-Visible (UV-Vis) Spectroscopy. Freeze-dried protein samples were dissolved in 60% (v/v) ethanol to 0.2 mg/mL. They were then scanned, and ultraviolet-visible spectra were recorded in the wavelength range of 200–400 nm using a Varian Cary 100 UV-Vis spectrophotometer (Varian Inc., Palo Alto, USA) at 25°C with a 60 nm/min scan rate and a 2.0 nm bandwidth, in a quartz cell with a path length of 1.0 cm.

2.5.4. Measurement of Intrinsic Fluorescence Spectrum. The intrinsic fluorescence emission spectrum of zein (0.2 mg/mL

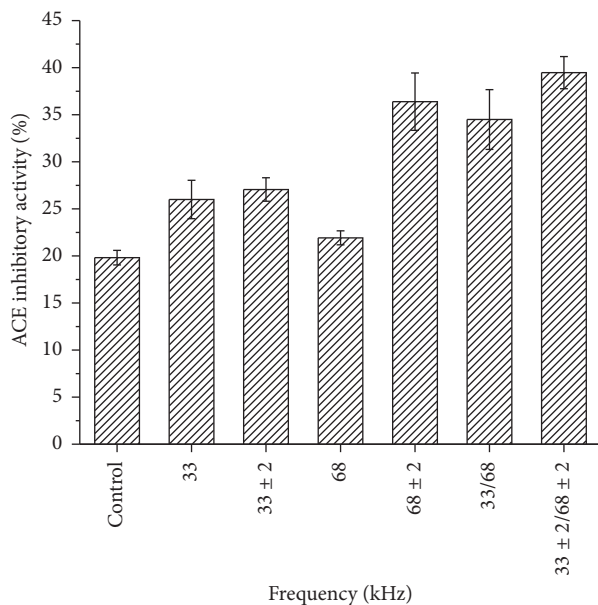


FIGURE 2: Effects of pretreatment with ultrasound bath with four different working modes and two frequencies on the ACE inhibitory activity of zein hydrolysates.

in 60% ethanol) was measured at 25°C using a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, USA) equipped with a cell with a path length of 1 cm, using an excitation wavelength of 350 nm (slit = 5 nm), emission wavelength of 360–750 nm (slit = 5 nm), and scanning speed of 20 nm/s.

2.5.5. Circular Dichroism (CD) Spectroscopy. The CD spectra of zein (0.2 mg/mL in 60% ethanol) were assessed at a wavelength range of 190–250 nm with a Jasco J-715 CD spectropolarimeter (Jasco Corp., Tokyo, Japan) using a quartz cuvette with a 10-mm optical path length under nitrogen flux at a temperature of 25°C, scanning rate of 50 nm/min, and bandwidth of 1.0 nm. The content of the protein secondary structure was calculated from the far-UV CD spectra using the DICHROWEB procedure [18–22].

2.6. Statistical Analysis. All experiments were performed at least in duplicate, and the results are presented as the mean ± standard deviation. Differences among treatments were determined by analysis of variance (ANOVA), and $p < 0.05$ was considered significant. All graphs were generated and calculations were performed using OriginPro8.5 and SPSS 18.0.

3. Results and Discussion

3.1. Effect of Ultrasound Pretreatment on ACE Inhibitory Activity of Zein Hydrolysates. The ACE inhibitory activity of zein hydrolysates pretreated with ultrasound was higher than that of the control, to varying degrees, and is presented in Figure 2. At a single fixed frequency of 68 kHz, the ACE inhibitory activity was only increased by 10.62% over that of the control, representing a smaller increase than that with a single fixed frequency of 33 kHz (31.18% increase). This finding that a

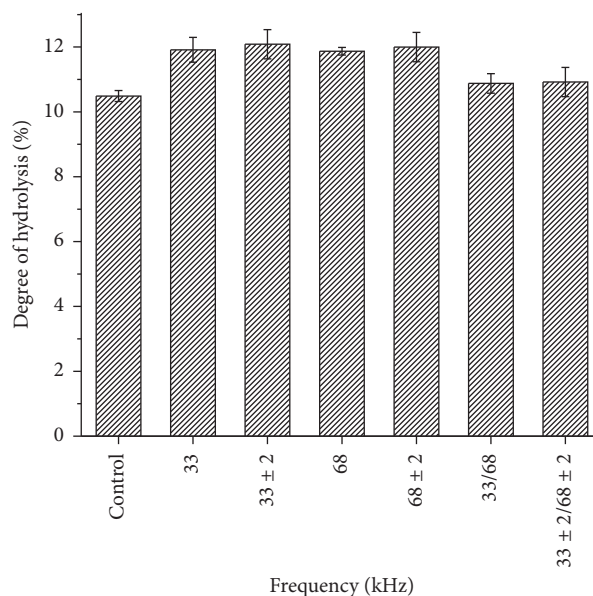


FIGURE 3: Effects of pretreatment with ultrasound bath with four different working modes and two frequencies on the degree of hydrolysis of zein.

lower frequency induced a higher ACE inhibitory activity is consistent with a report on defatted wheat germ proteins [1], which attributed this decrease to controlling the attenuation of ultrasound at high frequencies, resulting in lower energy loss [23]. In the case of ultrasound pretreatment with a single sweeping frequency of 68 ± 2 kHz, we found that the ACE inhibitory activity was higher than that for the ultrasound treatment with a dual fixed frequency of 33 and 68 kHz. This might be due to the resonance frequency of ultrasound. Notably, with a dual sweeping frequency of 33 ± 2 and 68 ± 2 kHz, the ACE inhibitory activity was significantly increased, reaching a maximum (99.21% increase). Compared with the dual fixed frequency, the dual sweeping frequency was more conducive to increasing the ACE inhibitory activity of zein hydrolysates. Arzeni et al. [24] reported that dual-frequency ultrasound can significantly increase the cavitation yield compared with that by two individual ultrasonic irradiation types. This higher cavitation yield is due to increased transfer of the input electrical energy and an increased resonance effect, which contribute to bubble growth and yield the maximum bubble size [25]. Furthermore, the dual sweeping frequency ultrasound contributed to unfolding of the molecular structure of zein and hydrolysis of zein-releasing peptides with high ACE inhibitory activity.

3.2. Effect of Ultrasound Pretreatment on Degree of Hydrolysis (DH) of Zein. Figure 3 shows the DH values for control and ultrasound-assisted enzymolysis of zein. The 33, 33 ± 2, 68, and 68 ± 2 kHz ultrasound pretreatments significantly improved the DH of zein by 13.13–15.22%, which may reflect changes in the molecular conformation of the protein. As zein has a three-dimensional spatial structure and predominantly hydrophobic nonpolar amino acid composition, Alcalase

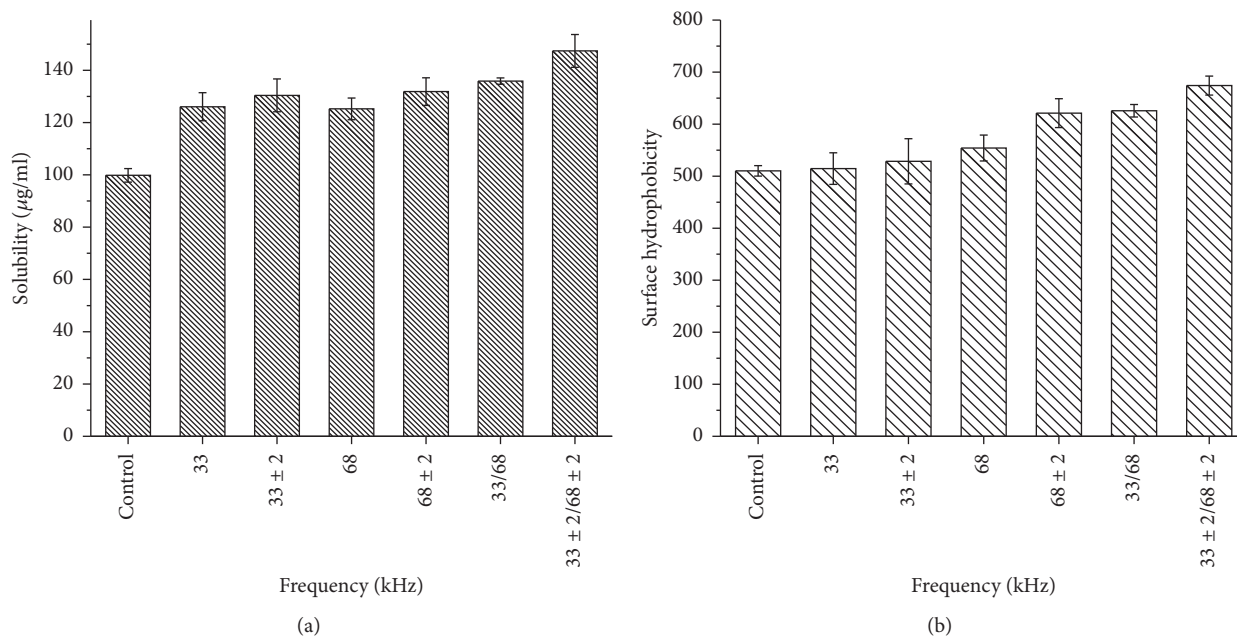


FIGURE 4: Effects of pretreatment with ultrasound bath with four different working modes and two frequencies on the solubility (a) and surface hydrophobicity (b) of zein.

did not hydrolyze zein effectively [3]. After treatment with ultrasound, however, the protein macromolecules became more loosely arranged, with peptide bonds previously buried inside the protein being exposed [9, 26]. This increases the probability of the enzyme coming into contact with the active site of the protein. In contrast, pretreatment using dual-frequency ultrasound had little effect on the DH of zein, although the ACE inhibitory activity of the zein hydrolysates was considerably enhanced under the same conditions. This phenomenon is consistent with that reported by Sun et al. [27] and Zhou et al. [1], who found that some ultrasound pretreatments have little effect on protein DH but significantly increase the ACE inhibitory activity of protein hydrolysates. This suggests that there is no direct relationship between increases in ACE inhibitory activity and the DH of zein.

3.3. Effect of Ultrasound Pretreatment on Structural Characteristics of Zein

3.3.1. Effect of Ultrasound Pretreatment on Solubility of Zein. Solubility is the most effective way to measure the degree of protein denaturation and aggregation in solution, and it is a good indicator of protein function [28]. After ultrasound treatment, the solubility of zein was significantly increased by 20.81–32.28% compared with that of the control (Figure 4(a)). These results indicate that ultrasound enhances the unfolding of protein molecules, exposure of hydrophilic amino acids, and interaction between proteins and water molecules. This result is consistent with similar findings for ultrasound-pretreated proteins, such as black bean protein isolate [29], soy protein isolate [30], and milk protein concentrate [31]. The different working modes of ultrasound greatly changed the solubility, with the highest solubility observed in the

sample that was treated with dual sweeping frequency ultrasound at 33 ± 2 and 68 ± 2 kHz. Thus, treatment using dual sweeping frequency ultrasound is more efficient at increasing the solubility of zein than the other working modes of ultrasound.

3.3.2. Effects of Ultrasound Pretreatment on the Hydrophobicity (H_0) of Zein. H_0 of a protein is one of the structural features used to evaluate changes in protein conformation that affect functional properties and stability [17]. Figure 4(b) shows that ultrasound treatment increased H_0 of zein to varying degrees. Comparing the H_0 values of zein when sonicated at the two frequencies, single-frequency ultrasound treatments of 33 kHz and 33 ± 2 kHz resulted in only small increases in H_0 , indicating that H_0 increased with increasing ultrasonic frequency (33 kHz versus 68 kHz). For zein treated with ultrasound of different working modes (68 kHz versus 68 ± 2 kHz), the effect on H_0 was stronger with sweeping ultrasound treatment than with fixed frequency ultrasound. In addition, the largest increase in H_0 was observed with treatment with dual sweeping frequency ultrasound at 33 ± 2 and 68 ± 2 kHz. The results for the ACE inhibitory activity of the zein hydrolysates and the solubility of zein pretreated by dual sweeping frequency ultrasound at 33 ± 2 and 68 ± 2 kHz are therefore consistent with the results for H_0 . These findings suggest that the differences in the H_0 of zein were due to differences in the working mode and frequency of the ultrasound, which may change the structure of the zein macromolecule and result in more or fewer hydrophobic groups on the inside of the protein molecule being exposed.

3.3.3. Effects of Ultrasound Pretreatment on the UV-Vis Spectrum of Zein. Figure 5 shows the typical UV-Vis spectra

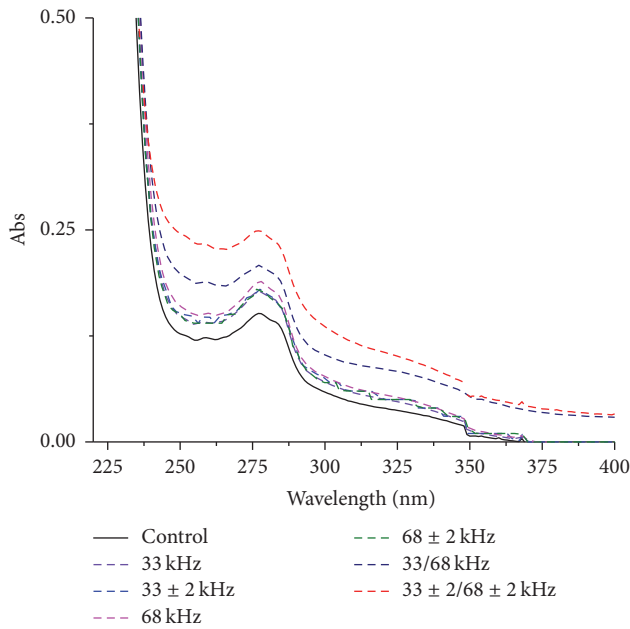


FIGURE 5: Effects of pretreatment with ultrasound bath with four different working modes and two frequencies on the ultraviolet-visible spectra of zein.

of zein after ultrasonic treatment using the four working modes and the two frequencies. Tyrosine, tryptophan, and phenylalanine have characteristic absorptions of 275 nm, 279 nm, and 257 nm, respectively [32]. These hydrophobic amino acids usually prefer to be buried in hydrophobic protein cores. As shown by the spectra, the absorbance intensity of zein macromolecules was increased by ultrasound treatment, indicating that ultrasonic treatment contributed to the exposure of hydrophobic amino acids. This finding is similar to that of Jin et al. [9] in corn gluten meal. The effect of dual sweeping frequency ultrasound pretreatment on the absorbance of zein was much stronger than that of the other ultrasound treatments, possibly because the combination of frequencies for sweeping frequency ultrasound has a resonance effect.

3.3.4. Effect of Ultrasound Pretreatment on Intrinsic Fluorescence Spectrum of Zein. Because the intrinsic fluorescence of aromatic amino acid (tryptophan, tyrosine, and phenylalanine) residues is sensitive to the polarity of the microenvironment during transformation, intrinsic fluorescence has been shown to be an efficient method of determining the tertiary structure transition for soluble proteins. Zein in aqueous ethanol was excited at 350 nm and scanned with an emission wavelength of 360–750 nm. As shown in Figure 6, the emission fluorescence intensity reached maximum values at wavelengths of 420 and 700 nm. Compared with that of the control, the relative proportions of the fluorescence peak areas (at 420 nm) of zein after pretreatment with ultrasound increased, agreeing with findings of Li et al. Moreover, the effect of dual sweeping frequency ultrasound on the absorbance intensity was significantly stronger than that

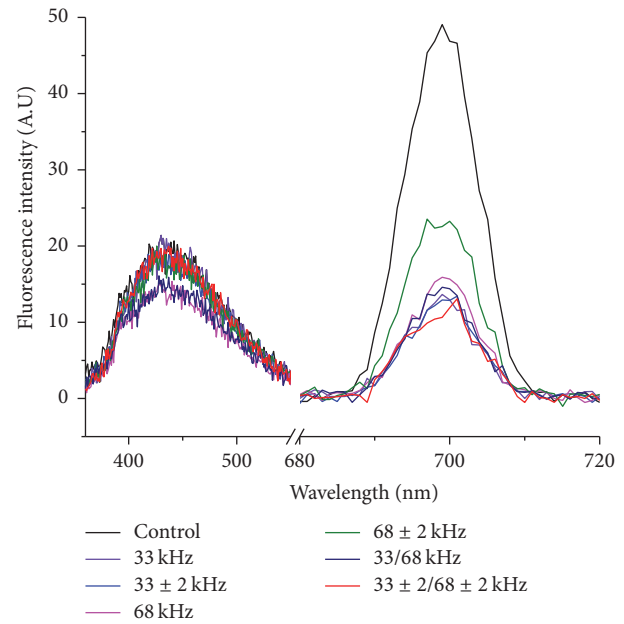


FIGURE 6: Effects of pretreatment with ultrasound bath with four different working modes and two frequencies on the intrinsic fluorescence spectra of zein.

of the other ultrasound pretreatments. The above results indicate that ultrasound pretreatment can alter the protein conformation and disrupt protein bonds, leading to increased exposure of buried hydrophobic groups.

3.3.5. Effects of Ultrasound Pretreatment on the Circular Dichroism (CD) of Zein. To obtain further information on the changes in protein structure induced by ultrasound, the effects of ultrasound pretreatment at different frequencies and working modes on the secondary and tertiary structures of zein were investigated. α -Helix, β -sheet, β -turn, and random coil structures, each, give rise to a CD spectrum of characteristic shape and magnitude. The CD spectra of zein are shown in Figure 7. Ultrasound pretreatment clearly increased the intensity of the positive peak, whereas the opposite was true for the negative peak. These changes reflect an increase in the disorder of the protein molecules. To quantify the changes in zein structure, the DICHROWEB procedure was used to calculate the α -helix, β -sheet, β -turn, and random coil contents (Table 1). There was a slight increase in the α -helix content of zein treated by ultrasound. However, Li et al. reported contradictory findings showing that sonication decreased the α -helix content of rice protein. In contrast, Jayani et al. found that ultrasound resulted in a 10% increase in the α -helix content of whey protein, which is in agreement with the present results. Therefore, the difference between the present results and those of Li et al. obtained for rice protein might reflect differences in the native protein and ultrasound conditions. Compared with the control, ultrasound treatment induced considerable increases in the β -sheet, β -turn, and random coil contents of zein, which is consistent with findings reported by Jin et al. [9]. This phenomenon may be due to the formation of localized

TABLE 1: Effects of pretreatment with ultrasound bath with four different working modes and two frequencies on the secondary structure content (%) of zein.

Treatment	α -Helix	β -Sheet and β -Turn	Random coil
Control	79.6	7.8	7.3
33 kHz	83.7 (4.90%)	13.2 (40.90%)	11.1 (34.23%)
33 \pm 2 kHz	83.4 (4.55%)	13.2 (40.90%)	18.7 (60.96%)
68 kHz	80.6 (1.24%)	7.2 (-8.33%)	7.0 (-4.29%)
68 \pm 2 kHz	84.5 (5.79%)	12.4 (37.09%)	18.4 (60.32%)
33 & 68 kHz	84.5 (5.79%)	12.1 (35.53%)	18.7 (60.96%)
33 \pm 2 & 68 \pm 2 kHz	81.8 (2.69%)	9.7 (19.59%)	17.1 (57.30%)

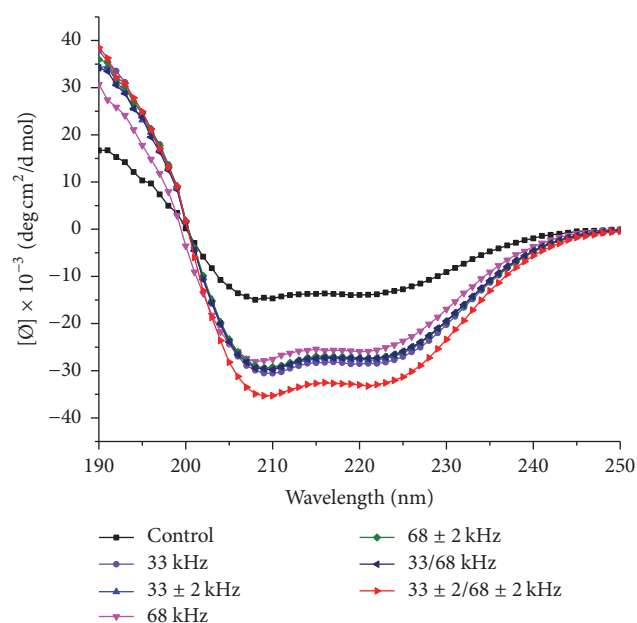


FIGURE 7: Effects of pretreatment with ultrasound bath with four different working modes and two frequencies on the circular dichroism spectra of zein.

hot spots upon the collapse of bubbles, shear forces created by shock, and microstreaming waves induced by ultrasound, which expose amino acid residues buried in the hydrophobic core of the zein protein and disrupt and rearrange interactions between protein molecule chains. However, with ultrasound treatment at a frequency of 68 kHz, the β -sheet, β -turn, and random coil contents decreased, which may suggest protein molecular aggregation. This indicates that a large number of chemical bonds may be buried inside the protein molecule and that the enzyme may have difficulty accessing the active site of zein after pretreatment with ultrasound at a frequency of 68 kHz. Notably, the corresponding release of peptides with higher ACE inhibitory activity was more limited with this treatment than with the other working modes and frequencies of ultrasound.

3.4. Conclusion. Ultrasound pretreatment at two frequencies using four working modes was found to significantly improve the angiotensin I-converting enzyme (ACE) inhibitory activity of zein hydrolysates. Zein hydrolysates pretreated with

dual sweeping frequency ultrasound at 33 \pm 2 and 68 \pm 2 kHz showed the highest ACE inhibitory activity. The ACE inhibitory activity resulting from single sweeping frequency ultrasound was higher than that resulting from single fixed frequency ultrasound. Dual fixed frequency ultrasound also resulted in a higher ACE inhibitory activity than single fixed frequency ultrasound. However, changes in the degree of hydrolysis (DH) of zein induced by ultrasound were not directly related to the ACE inhibitory activity of zein hydrolysates. Changes in the conformation of zein induced by ultrasound were confirmed by assessment of the solubility, hydrophobicity (H_0), ultraviolet-visible (UV-Vis) spectra, intrinsic fluorescence spectra, and circular dichroism (CD) spectra of zein pretreated by ultrasound, and these changes were consistent with the observed increases in ACE inhibitory activity. Ultrasound pretreatment can significantly improve the enzymolysis properties of proteins; our further studies should help to explain how the different working modes of ultrasound induce protein unfolding and aggregation, influence the enzymolysis properties of proteins, and thus affect the release of bioactive peptides.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

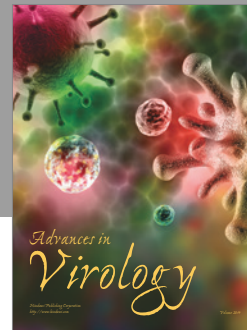
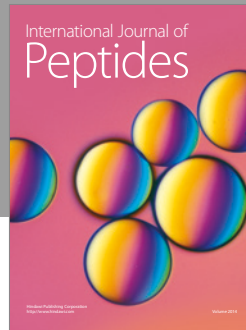
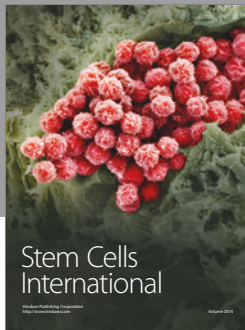
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