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Surface topography, nano-mechanics and secondary structure of wheat gluten pretreated by alternate dual-frequency ultrasound and the correlation to enzymolysis

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ABSTRACT

The effects of alternate dual-frequency ultrasound (ADFU) pretreatment on the degree of hydrolysis (DH) of wheat gluten (WG) and angiotensin I-converting enzyme (ACE) inhibitory activity were investigated in this research. The surface topography, nano-mechanics and secondary structure of WG were also determined using atomic force microscope (AFM) and circular dichroism (CD). The correlations of ACE inhibitory activity and DH with surface topography, nano-mechanics and secondary structure of WG were determined using Pearson's correlation analysis. The results showed that with an increase in either pretreatment duration or power, the ACE inhibitory activity of the hydrolysate also increases, reaching maximum at 10 min and 150 W/L, respectively, and then decreases thereafter. Similarly, AFM analysis showed that as the pretreatment duration or power increases, the surface roughness also increase and again a decrease occurs thereafter. As the pretreatment duration or power increased, the Young's modulus and adhesion of WG also increased and then declined. Young's modulus and adhesions average values were compared with ACE inhibitory activity reversely. The result of the CD spectra analysis exhibited losses in the relative percentage of α-helix of WG. Pearson's correlation analysis showed that the average values of Young's modulus and the relative percentage of α-helix correlated with ACE inhibitory activity of the hydrolysates linearly and significantly ($P < 0.05$); the relative percentage of β-sheet correlated linearly with DH of WG significantly ($P < 0.05$). In conclusion, ADFU pretreatment is an efficient method in proteolysis due to its physical and chemical effect on the Young's modulus, α-helix and β-sheet of WG.

1. Introduction

Wheat gluten (WG) is one of the main by-products of the starch industry with a high content of protein at about 76% (w/w). WG complex consists of gliadin and glutenins and it is insoluble in water due to large molecular size and extensive intermolecular interactions. Therefore, WG is used to produce bread floor, pet food and meat replacement as well [1]. During the past few years, numerous studies have reported that WG is a potential source of plant protein which could be used to produce antihypertensive peptides with strong angiotensin I-converting enzyme (ACE) inhibitory activity without side effects [2,3].

Power ultrasound (20–100 kHz) is widely used in improving functionality of enzymatic substrates by changing their physicochemical properties and structure conformations [4]. Cavitation effect during ultrasound pretreatment plays a main role in facilitating disintegration of enzymatic substrates while ultrasonic frequency influences liquid yields [5,6]. It has been reported that the yield of ultrasonic cavitation could be enhanced by dual-frequency sonication [7,8]. The dual-frequency sonication provides a wider range of frequencies than single frequency. The dual-frequency sonication can provide two operation modes, the alternate working mode and the simultaneous working mode. Jin et al. [9] reported that the alternate working mode have stronger effects on accelerate protein enzymolysis than simultaneous

Abbreviations: ADFU, alternate dual-frequency ultrasound; DH, degree of hydrolysis; WG, wheat gluten; ACE, angiotensin I-converting enzyme; AFM, atomic force microscope; CD, circular dichroism (CD).

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working mode of dual-frequency sonication, which enhances more mass transfer during protein pretreatment.

A number of studies have demonstrated that ultrasound pretreatment on plant protein before enzymatic hydrolysis could improve the degree of hydrolysis (DH) and ACE inhibitory activity of hydrolysates [10–12]. It has been reported that the improvement of DH and ACE inhibitory activities was caused by various changes in surface hydrophobicity, free sulfhydryl, disulfide bond, and secondary structures of protein after ultrasound pretreatment. Zhou et al. [7] reported that the main reasons for the improvement of ACE inhibitory activity of the hydrolysates after ultrasound pretreatment were changes in fluorescence intensity, surface hydrophobicity, free sulfhydryl (SHF) and disulphide bond (SS). Moreover, Ren et al. [4] reported that the sweeping frequency pretreatment could remarkably enhance the DH and ACE inhibitory activity of zein hydrolysis by altering the second structure and rupturing the smooth surface of zein. The changes in surface microstructure and chemical structure could be related to biomechanical change of protein. Very few data are available about the biomechanical properties of protein and specifically their correlation to ACE inhibitory activity during ultrasound pretreatment. Therefore, it is vital to study the micromorphology and biomechanical properties of proteins in attempts to explain physiological and chemical changes during ultrasound of protein.

Atomic force microscope (AFM) is a powerful tool to measure the surface morphology and nano-biomechanics of polymeric and biological samples [8–12]. The AFM provides information on both fundamental surface forces (via force curves) and particle geometry (via topographic images) [13]. In previous work, it is reported the dramatic change of surface roughness and biomechanical map of WG protein after ultrasound pretreatment. Hydrophobic regions of the protein were gathered and the protein stiffness reduced [14]. However, further study is needed in the dynamic monitoring of surface morphology and occurring of nano-mechanical properties in the process of ultrasonic pretreatment at different pretreatment durations and power levels.

The objectives for this research were to (1) investigate the effect of different ultrasound pretreatment conditions (treatment duration and power) on the DH of WG and ACE inhibitory activity of WG hydrolysate, (2) study the changes of surface morphology, nano-mechanical characteristics and secondary structural properties of WG after pretreatment with ultrasound and (3) explore relationships among surface roughness, nano-mechanical characteristics and secondary structural elements of WG and ACE inhibitory activity of the hydrolysates.

2. Materials and method

2.1. Materials

Wheat gluten powder (protein content, 76 g/100 g) was purchased from Xu Zhou OK wheat starch Co. (Jiangsu, China). Alcalase with an activity of 2.280×10^5 U/ml (by Folin Phenol method) [15], was purchased from Novozymes (China) Biotechnology Co., Ltd. (Tianjin, China). Angiotensin-I-converting enzyme (ACE) was extracted from pig lung according to the method described by Maruyama et al. [16]. Substrate hippuryl-His-Leu (HHL) was purchased from Sigma Chemicals Co. Ltd. (St. Louis, MO., USA). All other chemicals and solvents were of analytical grade.

2.2. Ultrasound pretreatment

Prior to enzymatic hydrolysis, the WG was pretreated by alternate dual-frequency ultrasound (ADFU). The ADFU equipment (Fig. 1a) was developed by our research team and manufactured by Meibo Biotechnology Co. (Zhenjiang, Jiangsu, China). The equipment consists of two ultrasonic probes, controllers, a reaction vessel, a liquid circulating system and a temperature controlling system. Each probe has a maximum output power of 300 W. The alternate operation of the equipment refers to operation with two kinds of ultrasound probe generating different frequencies. The frequencies were generated sequentially without an intermission between the treatments (Fig. 1b). Before ultrasonic treatment, an aliquot of 1 L WG suspension with substrate concentration of 30 g/L was stirred for 10 min and then put in the reaction vessel. The probes were submerged to a depth of 2.0 cm in the suspension. The initial temperature of the suspension was 30 ± 2 °C.

Ultrasound pretreatment was conducted at frequency of 20/35 kHz in an alternate working mode and the duration was 5 s for each treatment as optimized by our previous studies [17]. The first set of experiments was done under power density of 200 W/L for pretreatment durations of 5, 10, 15, 20 and 25 min. The second set of experiment were conducted at the power density of 100, 150, 200, 250 and 300 W/L for 10 min, respectively. For structural analysis, WG suspensions with and without ADFU pretreatment were centrifuged at 5000 rpm for 10 min. Then the supernatant was collected and freeze-dried (Freeze dryer ALPHA 1-2, Martin ChristGmbH, Osterode, Germany) for further structural analysis.

2.3. Enzymatic hydrolysis

The enzymatic hydrolysis was performed according to the methods by Dadzie et al. [1] with some modifications. A 1 L of ADFU pretreated suspension was adjusted to pH 9.0 using 1 M NaOH. A 0.6 ml of alcalase was added in the solution with enzyme-substrate ratio [E/S] of 4560 U/g. The enzymatic hydrolysis was performed at stable condition of pH 9.0 and temperature of 50 °C. After 30 min of reaction, the hydrolysate was boiled for 10 min to terminate the reaction. The hydrolysate was centrifuged at 5000 rpm for 10 min at 4 °C and the supernatant was stored at 4 °C for further analysis.

2.4. Determination of effect of enzymatic hydrolysis

2.4.1. Determination of degree of hydrolysis

Degree of hydrolysis (*DH*), which is defined as the percentage ratio of the number of peptide bonds broken (h) to the total number of peptide bonds in the substrate (h_{tot}), was calculated according to the pH-stat method of Adler-Nissen [18];

$$DH(\%) = \frac{h}{h_{tot}} \times 100\% = \frac{BN_b}{\alpha M_p h_{tot}} \times 100\% \quad (1)$$

where, B (mL) is the volume of NaOH consumed, N_b is the normality of the NaOH, α is the average degree of dissociation of the α-amino groups related with the pK of the amino groups at particular pH and temperature (α is 0.885 for alcalase), M_P (g) is the amount of the protein in the reaction mixture and h_{tot} (mmol/g) is the total number of peptide bonds in the protein substrate used in the experiment (h_{tot} is 8.33 for WG).

2.4.2. Determination of ACE inhibitory activity

ACE-inhibitory activity was measured according to Jia et al. [12]. A 25 μL of sample solution (sample in 0.1 M borate buffer containing 0.3 M NaCl, pH 8.3) was mixed with 25 μl of ACE (ACE in 0.1 M borate buffers containing the 0.3 M NaCl, pH 8.3) and pre-incubated for 10 min at 37 °C. The reaction was initiated by adding 45 μl of hippuryl-His-Leu (HHL) sodium borate buffer (6.5 mM HHL in 0.1 M borate buffer containing 0.3 M NaCl, pH 8.3) and was carried out at 37 °C for 30 min. The reaction was