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Mechanism study of dual-frequency ultrasound assisted enzymolysis on rapeseed protein by immobilized Alcalase

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ABSTRACT

The mechanism of ultrasound field promoting enzymolysis efficiency is difficult to study, because the reaction system mixes with enzymes, proteins and hydrolysates. Immobilized enzyme is a good option that can be used to investigate the mechanism by separating enzymes out from the system after enzymolysis. The objective of this study was by using immobilized Alcalase to investigate the effects and mechanisms of the promotion of dual-frequency ultrasound (DFU) assisted-enzymolysis on rapeseed protein. Based on single factor experiments, response surface methodology model with three factors – hydrolysis time, power density and solid–liquid ratio at three levels was utilized to optimize the degree of hydrolysis (DH). Circular dichroism (CD) was used to analyze the secondary structure change of the protein, scanning electron microscopy (SEM) was used to analyze the surface microstructure change of the enzyme. The results showed that with DFU assisted-enzymolysis, the DH increased by 74.38% at the optimal levels for power density 57 W/L, solid–liquid ratio 5.3 g/L and enzymolysis time 76 min. After DFU assisted-enzymolysis, the yield of soluble solids content, including protein, peptides and total sugar in hydrolysate increased by 64.61%, 40.88% and 23.60%, respectively. CD analysis showed that after DFU assisted-enzymolysis, the number of α-helix and random coil decreased by 10.7% and 4.5%, β-chain increased by 2.4%. SEM showed that the degree of surface roughness of immobilized Alcalase increased. The above results indicated that the improvement of hydrolysis by DFU assisted-enzymolysis was achieved by enhancing the solid solubility, changing the molecular structure of protein and increased the surface area of immobilized enzyme.

1. Introduction

Rapeseed protein meal is a byproduct of rapeseed oil with protein content as high as 35–40%. Rapeseed protein could be potentially used to prepare functional peptides because it has a high composition and well-balance of essential amino acid [1]. Therefore, rapeseed is a good and abundant resource for food industry.

In recent years, using ultrasound technology to improve enzymolysis efficiency of rapeseed protein to prepare peptides is a hot topic. Many studies have found that ultrasound has a significant effect on accelerating the rate of degree of hydrolysis (DH), however, the mechanism is not very clear. Some researchers reported that the enhancement was achieved by changing the spatial structure of substrate proteins into loose and stretch condition, which lead to the active sites of protein buried interior exposed [2,3]. The exposure increase the contact area between enzyme and substrates, and advantageous to improve the reaction efficiency [4,5]. On the other hand, some researchers reported that the enhancement was achieved by changing the structure of protease. It has been reported that short time exposure under ultrasound could increase enzyme activity [6,7], but too long time of exposure may inhibit the catalytic activity under high intensity of ultrasound [8]. Wang et al. [9] confirmed that a proper ultrasonic treatment could improve the activities of enzyme for both freely soluble enzyme and immobilized enzyme. Ma et al. [10] proved that appropriate ultrasonic treatment could increase the activity of Alcalse by changing the spatial structure of enzyme to accelerate the process of enzymatic reaction therefore improve the reaction rate.

Most of the studies used ultrasound to pretreat proteins or enzymes followed by enzymolysis and then investigated the mechanisms of ultrasound on the characteristics of proteins and enzymes. However, few studies investigated the characteristics of proteins and enzymes treated by simultaneous ultrasound and enzymolysis (called ultrasound assisted enzymolysis). The reason

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is that during enzymolysis, proteins and enzymes mixed, the changes of substrates, proteases and hydrates were hard to analysis separately. To solve this problem, immobilized enzyme was introduced in the study in order to separate the protease out from the enzymolysis reaction system. Under this condition, the information of the enzyme activities and protein structures can be analyzed individually.

The objectives of the study were to (1) determine the effect of parameters of dual-frequency ultrasound (DFU) assisted-enzymolysis on DH of protein and enzyme activity of immobilized enzyme, (2) optimize the enzymolysis condition and (3) investigate the mechanisms of DFU assisted-enzymolysis on enhancement of enzymolysis effect.

2. Materials and methods

2.1. Materials and reagents

Isolated rapeseed protein (particle size 0.42 mm, crude protein content 53 g/kg) was purchased from Weipu biotechnology Co. Ltd (Hubei, China). Alcalase 2.4 L FG with enzyme activity of 226,000 U/ml was purchased from Novozymes Biotechnology Co. Ltd (Tianjin, China). All reagents of analytical grade were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China).

2.2. Enzyme immobilization

The immobilized Alcalase was prepared according to description of Wang et al. [9]. Immobilized Alcalase suspension was prepared by mixing 30 ml, 3.1% of sodium alginate with 1.33 ml Alcalase. Then, the suspension was fully stirred at room temperature and flow through a peristaltic pump, dropped from 5 cm high into a 200 ml, 3.0% $CaCl_2$ solution to form gelatinous beads. The beads with uniform particle and regular shape were selected. After wash by deionized water until free Alcalase could not be detected, the beads were stored in refrigerator at 4 °C for the following experiments. The activity of the immobilized Alcalase was 5518 U/g. The activity yield of immobilized enzyme was 55.2%; the mass yield was 98.5%.

2.3. DFU assisted-enzymolysis

The DFU assisted-enzymolysis experiments were conducted in an ultrasonic bath reactor (vessel inner size: 362 mm × 294 mm × 502 mm; Shangjia Biotechnology Co., Wuxi, China) equipping with 5 different frequency free combined plates (Fig. 1A) and the maximum output acoustic power of each plate is 600 W. The equipment has the simultaneous dual frequency mode, which produces waves showing in Fig. 1B and the pulsed operation performs with on-time and off-time cycle.

Each mass of 0.25 to 2.50 g of rapeseed protein was dissolved in 100 ml of distilled water to obtain protein solutions with solid–liquid ratio of 2.5 to 25.0 g/L. Different amount of immobilized Alcalases were added into the protein solutions to prepared enzymolysis solutions with enzyme–substrate ratios of 1120 to 2450 U/g, respectively. Each sample solution was adjusted to pH 9.5, sealed in a high-pressure resistance plastic bag and placed in the ultrasound bath at 60 °C between the two plates. For DFU assisted-enzymolysis, different conditions including ultrasonic volumetric power densities from 20 to 70 W/L, ultrasonic frequencies with combinations from 22 ± 2 to 68 ± 2 kHz and treatment times from 30 to 150 min with ultrasonic pulsed on-time 10 s and off-time 3 s were performed. After the enzymolysis reactions, immobilized enzymes were filtered to separate from the enzymatic hydrolysates. DH of protein and activity of enzyme were measured.

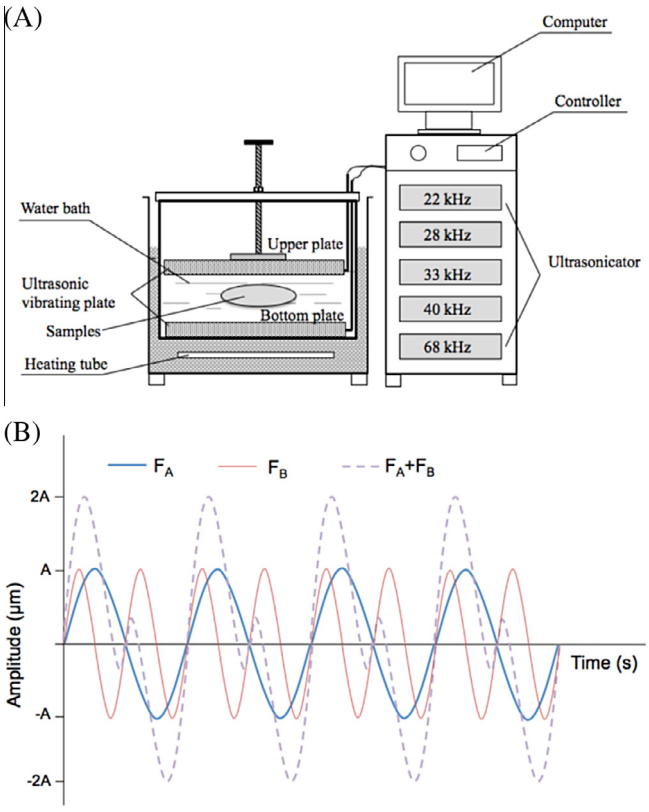

Fig. 1. Dual-frequency ultrasound equipment (A) and waveforms of simultaneous functional mode (B).

2.4. Determination of degree of hydrolysis

The degree of hydrolysis (DH), defined as the ratio of the number of peptide bonds cleaved by the total number of peptide bonds in a protein, was calculated from the consumption of base by the pH-stat method of Adler-Nissen [11]. The DH was calculated by Eq. (1):

$$\mathrm{DH} = \frac{B \times N}{\alpha \times mp \times h_{\mathrm{tot}}} \times 100\% \quad (1)$$

where, DH (%) is the degree of hydrolysis, B (ml) is the base consumption, N (mmol/ml) is the normality of the base, mp (g) is the mass of the protein ($N \times 0.625$), α is the average degree of dissociation of α-NH_2 groups in the rapeseed protein substrate, which is 0.885 for Alcalase and h_{tot} is the total number of peptide bonds per unit mass of protein (mmol/g), which is 7.8 for rapeseed protein.

2.5. Determination of enzyme activity

The activity of the enzyme was measured by using the Folin-phenol method described by Zhou [12]. The substrate for enzyme hydrolysis was casein. The absorbance was measured at A_{660} nm using an UV–visible spectrophotometer (Model Cary 100, Varian Inc., Palo Alto, USA) and the absorbance value was used to calculate the concentration of tyrosine released from casein. One unit of enzyme activity was defined as 1 μmol tyrosine released from the substrate per minute at pH 8.0 and 50 °C. The activities of enzyme were expressed as means ± SD of three determinations.

2.6. Experimental design and statistical analysis

The parameters for enzymolysis were optimized by response surface methodology (RSM) using Design-Expert Software (Version