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# Effects of ultrasound and ultrasound assisted alkaline pretreatments on the enzymolysis and structural characteristics of rice protein

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ABSTRACT

The objectives of this study were to investigate the effects of multi-frequency energy-gathered ultrasound (MFEGU) and MFEGU assisted alkaline pretreatments on the enzymolysis and the mechanism of two pretreatments accelerating the rice protein (RP) proteolysis process. The results showed that MFEGU and MFEGU assisted alkaline pretreatments improved significantly ($P < 0.05$) the degree of hydrolysis (DH) and the protein elution amount of RP. Furthermore under the same DH conditions, ultrasound and ultrasound assisted alkaline pretreatments were more save the enzymolysis time than the unpretreatment. The changes in UV–vis spectra, fluorescence emission spectra indicated unfolding and destruction of RP by MFEGU and MFEGU assisted alkaline pretreatments. The circular dichroism analysis showed that both pretreatments decreased α-helix but increased β-sheet and random coil of RP. Amino acid composition revealed that MFEGU and MFEGU assisted alkaline pretreatments could increase the protein elution amount and the ratio of hydrophobic amino acids. Atomic force microscopy (AFM) indicated that both pretreatments destroyed the microstructures and reduced the particle size of RP. Therefore, MFEGU and MFEGU assisted alkaline pretreatments are beneficial to improving the degree of hydrolysis due to its sonochemistry effect on the molecular conformation as well as on the microstructure of protein.



## 1. Introduction

Rice is cultivated primarily in Asia and is one of the most important staple food worldwide [1]. However, the protein component of rice (~8%) was usually discarded due to the starch component (~80%), which yielded greater commercial value including glucose and starch manufacturing [2,3]. Up until recently rice protein (RP) is considered valuable because it is rich in essential amino acids, highly nutritious, hypoallergenic, and particularly healthy protein source for human consumption [4]. Furthermore, some studies have shown that certain enzymatic hydrolysis products of rice protein exhibit hypocholesterolemic, antiatherosclerotic and inhibitory properties against particular cancer cells. Therefore they have potential as functional food [5–7]. Takahasi et al. [8] obtained a bioactive peptide with opioids antagonism effect from the tryptic digest of rice soluble protein. The structure of the peptide was Gly–Tyr–Pro–Met–Tyr–Pro–Leu–Pro–Arg, and it was named oryzatensin. Zhang et al. [9] and Adebiyi et al. [10] found antioxidants such as amino acids, peptides from rice endosperm. Recently our research team is focused on the study of antihypertensive peptides from rice protein, whose molecular distribution is about 200–1000 Da.

However, the poor solubility of rice protein and the special construction have led to the low degree of hydrolysis due to the difficulty of enzymes to attack the cleavage of the protein [11]. Thus, some efficient pretreated methods to overcome these shortcomings were designed, such as microwave radiation assisted technology [12], ultra-high pressure assisted technology and [13] ultrasound-assisted technology [14]. Therefore, our team adopts the ultrasound pretreatment technology, which is considered as a novel non-thermal physical processing technology, and its

*Abbreviations:* RP, rice protein; DH, degree of hydrolysis; PEA, protein elution amount; MFEGU, multi-frequency energy-gathered ultrasound; AFM, atomic force microscopy; CD, circular dichroism; HAA, hydrophobic amino acids; AA, amino acids; PBS, phosphate buffered solution.

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mechanism is attributed to the thermal, cavitation and mechanical efficacies. These roles can enhance mass transfer and increase the contact frequency between substrate and enzyme or change the substrate configuration [15,16]. So the ultrasound-assisted technology can improve the yield of proteolysis. Nonetheless the action of ultrasound-assisted pretreatment is limited to rice protein alone during previous experiments. With regards to the ultrasonic mechanism and the characteristic of RP, the pretreatment of ultrasound assisted alkali is proposed.

Ultrasound assisted alkali is widely used in food, chemical and biological processes [17–19]. Essentially, the ultrasound pretreatment process can be improved further by the application of alkali [20]. The principle is that ultrasound assisted alkaline pretreatment can decrease or disrupt the structure of protein more effectively. As a consequence, the accessibility of the attacking enzyme is substantially improved. However, this method was seldom tested for pretreatment of cereals and to the best of our knowledge, no research work has been reported on the enzymatic hydrolysis of ultrasound assisted alkaline pretreated rice protein. Moreover, little is known about the functional properties and biochemical characteristics of the rice protein processed by ultrasound assisted alkali before enzymatic hydrolysis.

Therefore, the objectives of this research were to (1) study the effects of the two pretreatments (ultrasound and ultrasound assisted alkaline) on the enzymatic hydrolysis, which can be reflected by the degree of hydrolysis (DH) and the protein elution amount of RP, (2) investigate the mechanism of ultrasound and ultrasound assisted alkaline pretreatments accelerating the RP proteolysis process in terms of changes in the molecular conformation as well as the microstructure of RP. It is also expected that the results of this research could provide the theoretical basis and technological support for further research in polypeptide production.

## 2. Materials and methods

### 2.1. Materials

Rice protein (particle size of 0.15 mm, crude protein content of 813 g/kg) was purchased from Zhengzhou tianshun food additives Co., Ltd. (Henan, China). Alcalase 2.4 L with an activity of 23,400 U/mL was purchased from Novozymes Co., Ltd. (Tianjin, China). All reagents used in the experiment were of analytical grade.

### 2.2. Methods

RP samples were pretreated by three kind modes, as shown in Fig. 1.

#### 2.2.1. Method 1(control)

Method 1 was the traditional enzymatic hydrolysis of RP. The enzymolysis apparatus consisted of a digital thermostat water bath (DK-S26, JingHong experimental apparatus Co., Shanghai, China), a pH meter (FE-20, Mettler Toledo Co., Shanghai, China) and an impeller-agitator (JJ-1, ZhongDa instrument Co., Jiangsu, China) at a speed of 100 rpm. The solution containing 32 g rice protein and 800 ml deionized water was stirred for 15 min at 50 °C. After pretreatment, one part (400 mL) solution was centrifuged at 5000 rpm to remove the supernatant, and the precipitate was vacuum freeze drying for 36 h to obtain the treated rice protein; the other part (400 mL) was adjusted to pH 8.5 by 1 M NaOH and Alcalase enzyme ($E/S$ = 1638 U/g) was added. The pH value was maintained constant by continuous addition of 0.5 M NaOH during the enzymolysis process. The enzymolysis time was 100 min.

#### 2.2.2. Method 2

Method 2 was the ultrasound-assisted enzymatic hydrolysis of RP. Prior to the enzymolysis reaction, the RP was pretreated by the multi-frequency energy-gathered ultrasound equipment (MFEGU). This apparatus was developed by our research team and manufactured by Meibo Biotechnology Co., Ltd. (Zhenjiang, Jiangsu, China). "Multi-frequency" means the apparatus has a pulsed probe equipped with five different frequency ultrasound generators (20 kHz, 28 kHz, 35 kHz, 40 kHz, 50 kHz). Based on our previous result, the frequency of ultrasound generator was set at 28 kHz. "Energy-gathered" is realized by designing the machine to low frequency (20–100 kHz) high power (10–1000 W/cm$^2$) and by avoiding the waste of energy, due to the direction of the ultrasound wave contrary to that of the solution [21].

The RP suspension with net protein content of 32 g soaked in 800 mL distilled water with a beaker, was placed in the hot water bath affiliated ultrasound device, which was set at 50 °C for keeping the temperature of RP suspension constant. The ultrasound facility had two peristaltic pumps, one was used to make the suspension flow into the reaction vessel, where probe had been submerged to a depth of 2.0 cm and the other was to transport the cooling water to keep the temperature of the reactants constant.

The conditions of the MFEGU processes were: ultrasonic power density 58 W/L and 28 kHz, ultrasound on-time 3 s, ultrasound off-time 2 s, total working time 15 min and the initial temperature 50 °C, and the flow rates of two peristaltic pumps were set at 200 mL/min.

After ultrasound, the solution was also divided into two parts averagely. Freeze drying and enzymatic hydrolysis were the same with the way of method 1.

#### 2.2.3. Method 3

Method 3 was the ultrasound assisted alkaline enzymatic hydrolysis of RP. It was almost the same as method 2 and the difference between them was that in the method 3 the alkali solution was used to adjust to pH 8.0 before pretreatment by the multi-frequency energy-gathered ultrasound equipment. The ultrasound parameters were the same with the method 2. After ultrasound, the solution was divided into two parts equally. Freeze drying and enzymatic hydrolysis were the same with the way of the method 1.

### 2.3. Determination of the degree of hydrolysis (DH)

The degree of hydrolysis (DH) was calculated according to the pH-stat method described by Adler-Nissen [22]:

$$\mathrm{DH}\ (\%) = \frac{h}{h_{tot}} = \frac{N_b \times V \times 100}{\alpha \times M_p \times h_{tot}} \tag{1}$$

$$\alpha = \frac{10^{pH-pK}}{1 + 10^{pH-pK}} \tag{2}$$

where, $N_b$ is the concentration of NaOH (mol/L); $V$ is the volume of NaOH consumed (mL); $M_p$ is the mass of protein to be hydrolyzed (g); $h_{tot}$ is the total millimoles of peptide bonds per gram of protein substrate, which is 7.72 mmol/g for rice protein; $\alpha$ is the average degree of dissociation of the $\alpha$-amino groups related with the $pK$ of the amino groups at particular $pH$ and temperature, which is 0.969 at pH 8.5 and 50 °C by Eq. (2).

### 2.4. Determination of the protein elution amount (PEA)

The protein elution amount of RP was determined by using the Folin-phenol method [23]. The protein absorbance in the reactor