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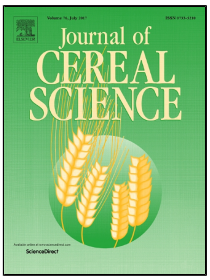

Influence of ultrasound-assisted alkali treatment on the structural properties and functionalities of rice protein

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Highlights

1. Ultrasound-assisted alkali (UAA) treatment could improve the solubility of rice protein.
2. UAA treatment seemed to unfold the protein internal structural conformation, which led to the exposure of buried functional groups, degradation of protein subunit and reduction of particle size.
3. Functionalities of rice protein, such as emulsifying and foaming properties, were improved by UAA treatment.

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Abstract

The poor solubility of rice protein (RP) limits its applications in food industry. In this study, the effects of ultrasound-assisted alkali (UAA) treatment on the solubility, structure and functional properties of RP were investigated. Using UAA treatment, the solubility of RP increased with increasing alkali concentration, reaching a maximum value of 19.79 mg/mL at an alkali concentration of 0.08 M. The solubility was improved by 230-fold compared to un-treated samples. In addition, a reduction in particle size and degradation of the protein subunit were observed. UAA seemed to unfold the protein internal structural conformation and expose buried functional groups, which are linked to good emulsifying properties and foaming properties. A decrease in zeta potential was also observed after UAA treatment, which could be the reason for the decreased stability of the emulsion. UAA treatment modified the protein structure and significantly improved solubility.

Keywords: rice protein; ultrasound-assisted alkali treatment; solubility; functional properties

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important crops in developing countries. It can provide 35%–59% of total caloric intake for more than 50% of the world's population (Juliano, 2016). In addition, rice is also an important raw material of the sugar industry because of the high starch content. After processing, about 50% of the raw rice becomes a byproduct called rice residue. In China, 10 million tons of rice residues were produced in the sugar industry every year. After the saccharification of starch, the protein content in the rice residue is increased to more than 50%. Because of the hypoallergenic properties and anti-cancer activity(Helm and Burks, 1996; Shoji et al., 2001), rice protein (RP) has gained increasing attention in the food industry. And the high content (66%–78%, w/w) insoluble glutenin resulted in the low solubility of RP (Wang et al., 2015a), which is a bottleneck for its extensive applications in food industry. Many treatments have been investigated to improve the solubility of RP. Various physical approaches, such as microfluidization (Xia et al., 2012a), hydrothermal cooking (Xia et al., 2012b) and high-pressure treatment (Kato et al., 2000) have been reported. Chemical methods, including enzymatic hydrolysis (Ahmadifard et al., 2016) and phosphorylation (Yi and Yao, 2005), have also been studied. Alkali solution is the most common means of extracting rice proteins, as the proteins show higher solubility in alkali media. However, the effects these treatments on the solubility of rice protein are still not satisfied.

Ultrasound has been used both to analyze food structure and composition at low ultrasonic intensities and high frequencies and to modify ingredients at high ultrasonic intensities and

low frequencies. In the food industry, power ultrasound has proved to be a highly effective food processing and preservation technology, and use of high-intensity ultrasound with or without heat may be used, for example, to homogenize or disperse two-phase systems such as emulsions or suspensions(Mason et al., 1996). Ultrasound has also been tested for improving solubility and modifying the functional properties of proteins, which is considered an environmentally friendly and innovative technology. Ultrasound treatment damages the protein quaternary structure through cavitations, producing a small molecular subunit and increasing solubility of RP (Gulseren et al., 2007). Jambrak et al. (2008) reported ultrasound frequency of 20 kHz, power of 600 W and 30 min duration can improve solubility of protein.

Ultrasound assisted alkali treatment has also been reported in the modification of protein. According to the reported studies, in the ultrasound assisted alkali treatment, the protein solution's pH shifting to 12 or higher, compact structure of protein is getting loose, and the simultaneous ultrasound treatment damaged protein's structure easier and increased protein's solubility (Jiang et al., 2017; Lee et al., 2016; Yildiz et al., 2017).

To date, improving the solubility of RP via ultrasound assisted alkali treatment has not yet been investigated. In this study, rice protein powder with 90 wt% protein content extracted from rice residue was used as a raw material, and the effects of UAA treatment on the solubility as well as structural and functionalities of RP were investigated. Through these experiments, we aimed to evaluate the potential of UAA treatment for improving the application of RP in food industry.

2. Materials and method

2.1 *Materials*

RP (90 ± 1.13 wt% protein content, wet basis) were purchased from Jinnong Biotechnology Co. (Wuxi, Jiangsu, China). SDS-PAGE kit was purchased from Solarbio Co. (Beijing, China) and used without further purification. All of the other chemicals were of analytical grade and purchased from Sigma-Aldrich Co. LLC (Beijing, China).

2.2 *Ultrasound assisted alkali treatment of rice protein*

RP was dispersed in NaOH solutions with the following concentrations: 0.02, 0.04, 0.06, 0.08, and 0.1 M. The final protein concentration was 50 g/L (w/v) in each solution. A 100 mL aliquot of RP solution was transferred to a jacket beaker(250 mL) and treated by ultrasound at 50°C for 60 min. The ultrasound experiments were carried out at 20 kHz using an ultrasound generator (Scientz Biotechnology Co., Ltd, Ningbo, China, Model: Scientz-950E) with a 12 mm vibrating titanium tip probe. The probe was immersed 2 cm into the liquid. The solution temperature was measured by the ultrasound generator built-in temperature sensors. The rating power of the ultrasound generator was 600 W and the ultrasound intensity was 19.3 W/cm^2 calculated as follows (Cárcel et al., 2007; Raso et al., 1999):

$$I_a = {}^{P_a}/_{S_A}, \text{ where } P = m \cdot c_p({}^{dT}/_{dt}) \quad (1)$$

where P_a (W) is the acoustic power, S_A is the surface area of the ultrasound emitting surface (1.13 cm^2), m is the mass of ultrasound treated solution (g), c_p is the specific heat of the

90 medium (4.18 kJ/gK) and dT/dt is the rate of temperature change with respect to time, starting
91 at t = 0 (°C/s).

92 After the UAA treatment, the samples were readjusted to pH 7.5, and centrifuged at 10,000 g
93 for 15 min. Supernatants were collected and freeze-dried for 12 h.

94 **2.3 *Solubility measurements***

95 Soluble protein in the supernatants was measured via the Bradford assay. Samples not
96 subjected to ultrasound treatment served as the control. The effectiveness of the treatment was
97 expressed as the accumulated concentration of the soluble protein content. Bovine serum
98 albumin (BSA) was used as a standard for the Bradford assay. Absorbance at 595 nm was
99 measured using a UV spectrophotometer (T6, Purkinje General Instrument Co. Ltd, Beijing,
100 China). Protein solubility was expressed as the concentration of water-soluble proteins.

101 **2.4 *Emulsifying activity and emulsion stability***

102 Emulsifying activity (EA) and emulsion stability (ES) were determined via turbidity
103 measurements. A 1% (w/v) aqueous protein suspension was adjusted to pH 7.5 using 1 M
104 hydrochloric acid. To 6 mL of the protein solution, 2 mL olive oil was added, and the mixture
105 was homogenized in a mechanical superfine homogenizer (FA25, Fluko Equipment Shanghai
106 Co., Ltd, China) at 10,000 r/min for 1 min to produce a full emulsion. After homogenizing, 50
107 μL aliquots of the emulsion were pipette at 0 min and 15 min and then mixed with 5 mL of
108 0.1% SDS solution respectively. The absorbance of the emulsion was measured at 500 nm
109 with a UV spectrophotometer. The absorbance that was measured at time 0 min (T_0) was

110 expressed as the EA of the proteins. The ES was determined as follows:

111 $$ES = T_0(\Delta t/\Delta T) \qquad (2)$$

112 where ΔT is the change in turbidity and Δt is the time interval (15 min). BSA was used as the
113 standard for emulsifying activity.

114 **2.5** ***Foam activity and foam stability***

115 The FA was expressed as the volume of foam immediately measured after foaming (10,000
116 r/min for 1 min) 40 mL of 1% protein solution containing 0.05 M phosphate buffer (pH 7.5)
117 in a glass tube. The foam stability (FS) was calculated as follows:

118 $$FS = V_0(\Delta t/\Delta V) \qquad (3)$$

119 where ΔV is the change in the volume of foam (V) occurring during the time interval Δt (30
120 min) and V_0 is the volume of foam at time 0 min. HEA was used as the standard for foaming
121 activity.

122 **2.6** ***SDS-PAGE***

123 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out
124 using the method described by Laemmli (1970). SDS-PAGE was carried out on a gel slab
125 comprised of 5% stacking gel and 15% separating gel in a SDS-Tris-glycine discontinuous
126 buffer system. Protein powder was prepared in RRP (control) and different UAA-RRP
127 (NaOH concentration 0.02, 0.04, 0.06, 0.08, and 0.1 M) conditions using a buffer solution
128 with 2-mercaptoethanol. Electrophoresis was performed at a constant potential of 200 V per

gel for approximately 2 h. The gels were stained with Coomassie Brilliant Blue R 250. The protein marker was purchased from Solarbio Co. (Beijing, China).

2.7 *Sulfhydryl and disulfide bond contents*

The free sulfhydryl group (SH_F), total sulfhydryl group (SH_T), and disulfide bond (S–S) content of the protein samples were determined according to the method described by Beveridge et al.(1974) with some modifications. RP (15 mg) was dissolved in 10 mL Tris–Gly buffer (pH 8.0) containing 0.086 M Tris, 0.09 M glycine, 0.004 M EDTA, and 8 M urea. The mixture was centrifuged at 10000 g for 10 min. For SH_F content determination, 50 μL of Ellman's reagent (DTNB in Tris–Gly buffer, 4 mg/mL) was added to 1 mL of protein supernatant, and the solution was mixed. After reacting for 5 min, the absorbance at 412 nm was measured. For SH_T content determination, 1 mL of the supernatant was treated with 4 mL of 15 g/L β-ME (β-ME in Tris–Gly buffer containing 8 M urea and 5 M GdnHCl) for 1 h, and then the protein was separated by precipitation with 12% TCA for 1 h. After centrifugation at 10,000 g for 10 min, the precipitate was collected and washed three times with 5 mL of 12% TCA, and dissolved in 10 mL of Tris–Gly buffer containing 8 M urea. To 4 mL of the protein solution, 40 μL of Ellman's reagent was added, and the absorbance was measured at 412 nm. The SH_F and SH_T content were determined by a standard curve, using cysteine hydrochloride monohydrate.

2.8 *FTIR*

Spectrum analyses were carried out using a Vertex 70 FTIR spectrometer (Bruker Co., Germany). Approximately1 mg of lyophilized protein powder was mixed with KBr, ground, and pressed into a pellet. The absorbance intensity was obtained at 2 cm^{-1} resolution in the wave number range of 4000–400 cm^{-1}. A total of 32 scans were measured and averaged. The KBr spectrum was taken as background. After background correction, all spectra were baseline corrected after analysis.

PeakFit 4.12 (SeaSolve Software Inc., USA) was used to deconvolve the amide I region (1700–1600 cm^{-1}) of the spectra. The deconvolved spectrum was iteratively fitted with Gaussian band shapes. The corresponding peaks of the fitted bands were assigned based on the results of a previous study (Byler and Susi, 1986). Peaks at 1640–1600 cm^{-1}, 1650–1640 cm^{-1}, 1658–1650 cm^{-1}, and 1700–1660 cm^{-1} were assigned to β-sheet, random coil, α-helix, and β-turn protein structures, respectively.

2.9 *Microstructure characterization*

The size and polydispersity index (PDI) of untreated and ultrasound treated rice proteins was measured by dynamic light scattering (DLS) with a Zetasizer Nano ZS90 (Malvern Instruments, UK). All measurements were carried out at 25 ± 1°C and at a scattering angle of 90°. The protein size values were reported as the average particle size and the standard deviation of three repeat measurements.

2.10 *Zeta potential*

Changes in surface potential of RP over time were measured by Doppler velocimeter and phase analysis of light scattering technology. Zeta potential was measured by ZetasizerNano ZS90. The refractive index and absorption parameters are 1.330 and 0.001 respectively. All the measurements were taken in triplicate.

2.11 Statistical analysis

Statistical analysis of data was performed using Statistical Analysis System IBM SPSS and Microsoft Excel. Student's t-test with a 95% confidence interval was used to assess the significance of the results obtained. Data with $P < 0.05$ were considered statistically significant.

3. Results and Discussion

3.1 *Solubility*

As shown in Fig. 1, the solubility of rice protein in the ultrasound treatment decreased after an initial increase, with a maximum solubility at 19.3 W/cm^2. Therefore, a follow-up experiment was conducted using the optimal solubility conditions. Alkali treatment considerably extends the protein tertiary structures (Jiang et al., 2009). After the alkali treatment, the solubility of RP increased gradually for concentrations of NaOH between 0.02 and 0.08 M. When NaOH concentrations were higher than 0.08 M, a slight decrease in protein solubility was observed. This was similar to the results reported by Wang et al. (2015a). Furthermore, RP's structure was more prone to getting loose and unfolding at high pH than at low pH, which explains the accelerated solubility after high pH treatment (Wang et al., 2015a). Ultrasound-assisted alkali

(UAA) treatment showed similar trends to the alkali treatment, but it improved the solubility of rice proteins more significantly. This might be attributed to that the unfolding or loose protein structure is easier to be damaged by the violent agitation and acoustic cavitations caused by ultrasound, which could cause chemical and physical changes in a viscous medium by cyclic generation and collapse of cavities, thereby the increased pressure in the vicinity of these cavities is the basis to cause physical damage contributing to increasing protein solubility and transforming the insoluble proteins into soluble proteins and enhance the impact of alkali treatment (Yildiz et al., 2017).

3.2 *Emulsion characteristics*

Emulsion characteristics are important functional properties of protein. Due to the amphipathic nature of protein, RP can spontaneously migrate to an oil-water interface, forming a high viscosity membrane that is conducive to the stability of an emulsion system. As shown in Table 1, after treatment, the emulsifying activity (EA) of rice proteins was markedly enhanced and significantly higher than the control. With increasing alkali concentration, EA of rice proteins increased greatly, peaking at 0.08 M. The same trend was observed for solubility. The peak value of EA (1363.81 m^2/g) was significantly higher than that of BSA (396.11 m^2/g). In addition, BSA has better emulsifying properties, and it is the most commonly used standard material for comparing protein emulsifying properties. Solubility of protein is an important factor that affects the EA. The results indicate that the hydrophilic and lipophilic abilities of modified rice proteins were improved with increasing solubility. However, each treatment showed declining emulsion stability. Improvement of

emulsifying properties may be due to the unfolding protein structure during alkali and heat treatment, exposing more hydrophobic regions and enhancing protein adsorption to the water-oil interface (Dickinson, 1997; Dickinson, 1999).

3.3 *Foaming characteristics*

Foaming ability (FA) and foaming stability (FS) of the protein are associated with the ability to reduce surface tension at the water-air interface and closely related to the protein structure. As shown in Table 1, the FA and FS values of treated samples were significantly increased by UAA. HEA is the most ubiquitous standard for comparing foaming properties of proteins (Symes, 1980). The FA and FS values of RP treated by ultrasound with a NaOH concentration of 0.08 M (56.33 mL and 67.1%, respectively) were significantly higher than that of HEA (28.5 mL and 22%, respectively). Wang et al. (2015a) observed an increase in FA and FS of protein after freeze-milling and alkali treatment. This result is similar to our study. The variation in foaming characteristics may be due to an increase in protein solubility and rapid unfolding into a cohesive layer around gas/air droplets (Tang et al., 2003). On the other hand, treated protein was easy to adsorb on the gas-water interface, forming three dimensional network structures. The treatment increased film thickness and mechanical strength, which improve the FA.

3.4 SDS-PAGE

According to the molecular weight of protein, the SDS-PAGE gel was divided into three regions (Fig. 2), mainly 43–97.4 KDa, 20.1–43 KDa and < 20.1 KDa, called band I, band II

and band III, respectively. The protein bands of the control sample were distributed in band II and band III. As shown in Fig. 2, three bands were observed, which were mainly distributed in the 35 KDa, 19 KDa and 13 KDa, respectively. In a study published by O'Sullivan et al. (2016), SDS-PAGE of rice protein also contains the same band distribution. Glutenin fractions mainly consist of acid subunits (Glutenin-AS 30-39 KDa) and basic subunits (Glutenin-BS 19-25 KDa) (Anne et al., 2006). The band at 13 KDa may be alcohol soluble glutenin. Compared with the control, the concentration of each band decreased gradually with an increase in alkali concentration. This indicates that UAA causes some insoluble protein aggregates to dissolve. UAA treatment generates some soluble protein aggregates and breaks down the protein subunits, in some cases. This may be the mechanism of improved solubility.

3.5 Sulfhydryl and disulfide bond contents

$SH_{F,}$ SH_T and S-S represent the free sulfhydryl group, the total sulfhydryl group and the disulfide bond, respectively. The total sulfhydryl contains sulfhydryl groups exposed to the molecular surface and entrapped inside the molecule. $SH_{F,}$ SH_T and S-S content of the control were 17.11 μmol/g, 1.09 μmol/g and 8.01 μmol/g, respectively. As listed in Table 2, SH_F, SH_T and S-S content of rice proteins by UAA were markedly higher than those of control. This result can be attributed to the exposure of buried groups during protein unfolding (Wang et al., 2015a). It could be inferred that UAA treatment exposed hydrophilic groups of the protein, which led to increased solubility.

3.6 ***FTIR***

Changes to the secondary structures of the denatured protein were more precisely observed with FTIR. Fig. 3 shows the FTIR spectra of RP after treatment with different NaOH concentrations and ultrasound treatment. As shown in Fig. 3, band width, intensity and the peak position change to some degree after treatment. We speculated the secondary structures of RP were changed by UAA treatment. A similar result was found in the study of Wang et al. (2015b), in which alkali and freeze-milling treatment led to a more polar surface of RP, resulting in improved solubility.

FTIR data were analyzed by PeakFit 4.12, and the content of the secondary structure was obtained. As shown in Table 3, the β-sheet and random coil of RP decreased after treatment. Both the α-sheet and the β-turns increased. This indicates that the ordered structure of the protein increased, and the disordered structure decreased. These structural changes are related to the degradation of the protein to small molecular peptides after the alkali treatment.

3.7 *Microstructure characterization*

After UAA treatment, RP particle size ranged from 219 to 249 nm (Table 4). According to Fig. 4, protein size distribution of the control was shifted to the left region, which indicates that the particle size of treated samples became smaller. As shown in Fig. 4, with increasing NaOH concentration, the degree of shift in peak II initially increased and then decreased. For peak I, the peak position was also shifted to a smaller size value. This indicates that the total number of soluble particles increased. Moreover, the changes in protein particles may also be the reason for increased protein solubility.

The PDI value of treated samples is smaller, which indicates that the particle size distribution range of protein dispersion system is smaller than that of the control and the particle dispersion is better (Gulseren et al., 2007). As listed in Table 4, UAA treatment led to PDI values of RP between 0.3 and 0.39, which are lower than the control (0.508). PDI of dispersion system initially decreased and then increased slightly with increasing NaOH concentration. This indicates that UAA treatment alters the dispersion range of particle size in the RP dispersion system. Overall, the range of particle size distribution was reduced, which enhanced protein particles disperse in water solution. When the NaOH concentration reached a certain value, the concentration of particles in the solution increased, and the dispersion of protein particles decreased slightly.

3.8 *Zeta potential*

For systems with higher zeta potential, the repulsive force between particles was larger, and aggregation did not readily occur. These systems were considered to be relatively stable. However, for systems with lower zeta potential, the repulsive force between particles was smaller, and the systems were unstable. The zeta potential of RP formed by UAA is listed in Table 5. RP has a negative charge at pH 7.5, and with increasing alkali concentration, the absolute zeta potential in RP decreased gradually. The absolute zeta potential in modified protein was lower compared to untreated protein (40.4 mV), which did not agree with the findings of previous studies by Shigeru et al. (1985). Hayakawa and Nakai (1985) observed that, with increasing deamidation, the surface charge of soy protein increased accordingly. Additionally, the result further proves the reasons for decreased ES in UAA treated RP. The

289 zeta potential of the protein emulsion after UAA treatment decreased, indicating lower
290 electrostatic repulsion between the oil droplets leads to lower stability of the emulsion.

291 **4. Conclusions**

292 UAA treatment can damage protein subunits and extend the tertiary structure, reducing the
293 compactness of protein. It can also cause degradation of the secondary structure, exposing
294 more polar groups and increasing the number of soluble protein particles significantly. As a
295 result, the interaction between water and protein as well as the solubility of RP was enhanced.
296 In addition, the emulsifying properties and foaming properties were remarkably improved and
297 were much higher than those of BSA and HEA, respectively. Therefore, UAA is a potential
298 treatment of insoluble protein with high quality and processing efficiency. This study also
299 provided valuable insights into the mechanism of accelerated solubility of insoluble protein
300 by UAA. Such information may benefit the processed food industry for developing an
301 economically feasible solubility system that results in high quality food products.

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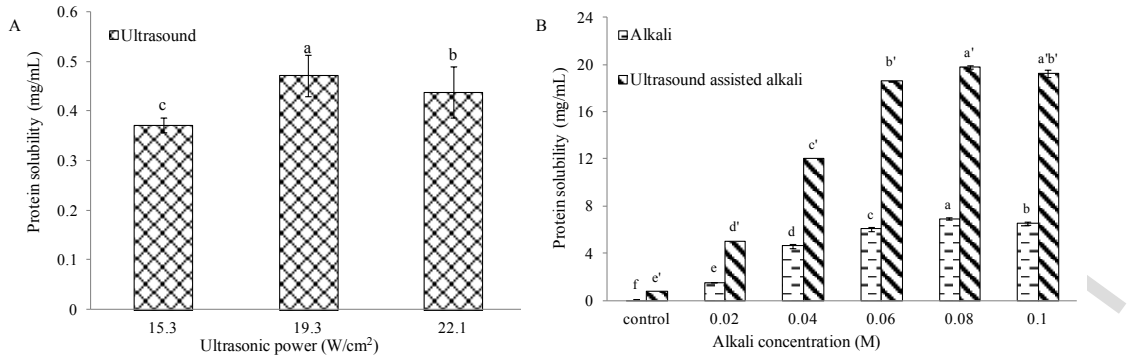

Fig. 1 Influence of different treatments on protein solubility. A: alkali and ultrasound assisted alkali treatment. B: ultrasound treatment. Different superscripts (a, b, c; a', b', c') in the same factor bar indicate significant difference ($P < 0.05$).

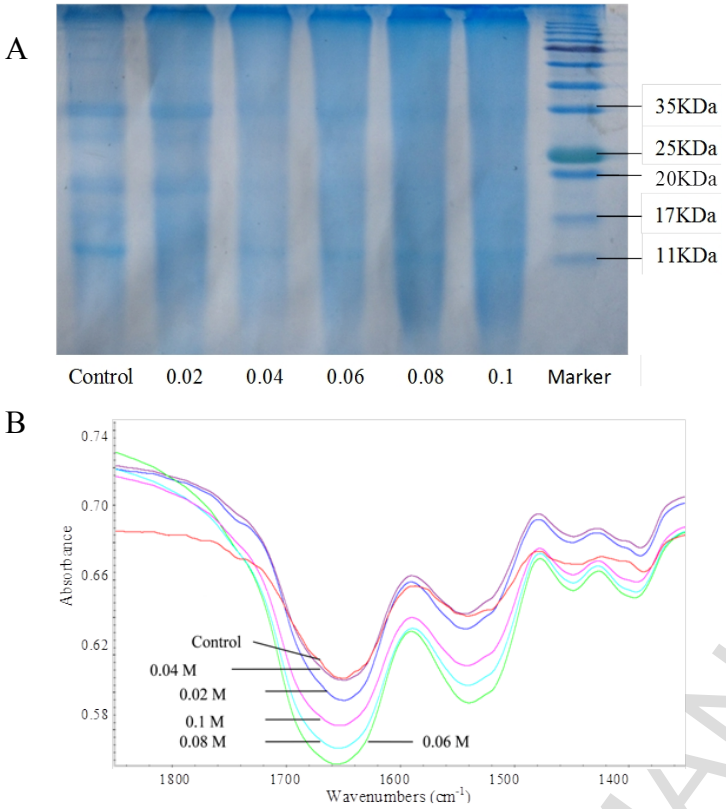

Fig. 2 SDS-PAGE profiles (A) and FTIR spectra (B) of ultrasound-assisted alkali-treated rice protein using different NaOH concentration

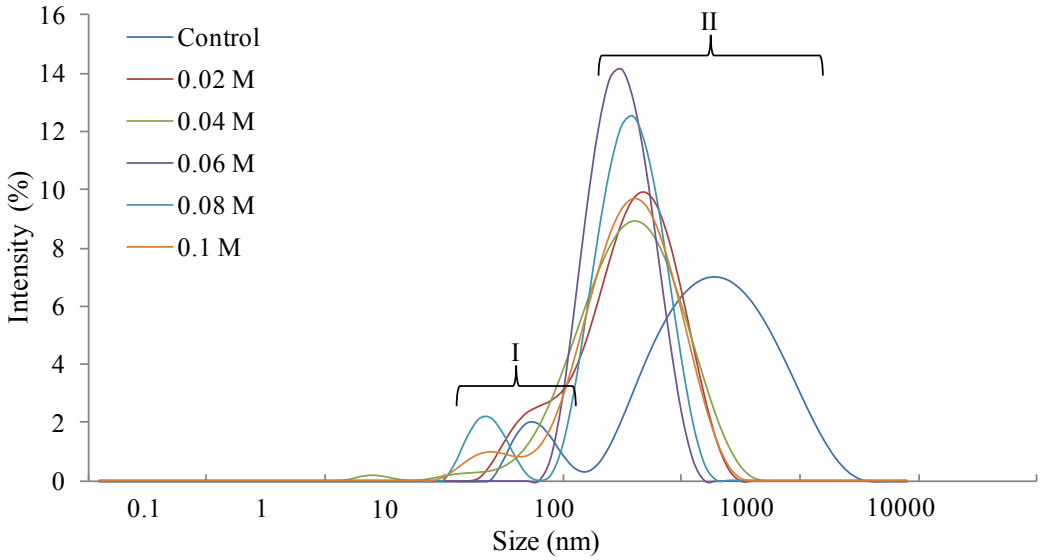

Fig. 3 Effects of NaOH concentration on particle size and intensity of RP dispersion system

Figure captions

Fig. 1 Influence of different treatments on protein solubility. A: alkali and ultrasound assisted alkali treatment. B: ultrasound treatment. Different superscripts (a, b, c; a', b', c') in the same factor bar indicate significant difference ($P < 0.05$).

Fig. 2 SDS-PAGE profiles (A) and FTIR spectra (B) of ultrasound-assisted alkali-treated rice protein using different NaOH concentration

Fig. 3 Effects of NaOH concentration on particle size and intensity of rice protein dispersion system

Table 3. Effect of ultrasound-assisted alkali treatment on the size, polydispersity index (PDI) and the zeta potential of soluble rice protein aggregates.

Treatment	Size (nm)	PDI	Zeta potential
control	486.4±7.1^{a}	0.508±0^{a}	−40.4±1.31^{a}
0.02	248.8±4.05^{b}	0.385±0.01^{b}	−28.7±0.67^{b}
0.04	231.1±14.2^{bc}	0.376±0.02^{bc}	−25.4±0.6^{c}
0.06	219.8±15.9^{c}	0.361±0.01^{c}	−15±0.21^{d}
0.08	223.3±2.85^{c}	0.309±0.01^{d}	−13.3±0.15^{e}
0.1	227.3±8.4^{bc}	0.313±0.01^{d}	−13.8±0.2^{e}

*Means ± standard deviation; values followed by a different superscript lower case letter on the same parameter in the same column are significantly different at $P < 0.05$.

Table 1. Effect of ultrasound-assisted alkali treatment on emulsifying properties and foaming properties of rice protein.

Methods	Emulsion capacity		Foaming capacity	
	Emulsion activity ($m^2 \cdot g^{-1}$)	Emulsion stability (min)	Foaming activity (mL)	Foaming stability (%)
control	225.16±16.38^{g}	92.38±27.07^{a}	43.33±6.11^{c}	32.99±0.96^{b}
0.02	590±43.38^{e}	47.28±14.56^{b}	41±4.58^{c}	64.67±5.03^{a}
0.04	720.3±8.53^{d}	45.62±10.43^{b}	49.33±2.08^{b}	56.39±3.76^{a}
0.06	1005.22±59.43^{b}	29.02±1.16^{b}	54.67±3.18^{a}	60.18±7.27^{a}
0.08	1363.81±59.28^{a}	34.06±1.84^{b}	56.33±1.53^{a}	67.1±4.26^{a}
0.1	799.17±16.56^{c}	31.29±7.41^{b}	53.33±2.37^{a}	64.7±18.41^{a}
BSA	396.11±17.16^{f}	29.10±0.44^{b}	—	—
HEA	—	—	28.50±0.96^{d}	22±2.9^{c}

*Means ± standard deviation; values followed by a different superscript lower case letter on the same parameter in the same column are significantly different at $P < 0.05$.

Table 2. Effect of ultrasound-assisted alkali treatment on the sulfhydryl (SH), disulfide bond (S-S) content and the secondary structure of rice protein.

Treatment	SH and S-S content (μmol/g protein)			Secondary structure (%)			
	SH_T	SH_F	S-S	α-sheet	β-sheet	β-turns	random coil
control	17.11±0.09^{e}	1.09±0.09^{d}	8.01±0.00^{d}	7.31±0.2^{c}	29.12±0.32^{a}	60.2±0.61^{b}	3.37±0.05^{a}
0.02	23.95±0.28^{d}	1.47±0.33^{d}	11.24±0.05^{bc}	15.68±0.09^{a}	18.1±0.11^{d}	66.21±0.15^{ab}	0^{c}
0.04	28.27±1.16^{c}	6.91±0.81^{c}	10.68±0.46^{c}	15.01±0.23^{a}	18.54±0.05^{d}	65.22±0.01^{ab}	1.24±0.02^{b}
0.06	31.05±0.40^{b}	8.09±0.58^{b}	11.48±0.46^{b}	14.02±0.08^{ab}	21.55±0.15^{c}	64.08±0.17^{ab}	0.35±0.01^{c}
0.08	33.99±0.67^{a}	9.42±0.67^{a}	12.28±0.46^{a}	12.17±0.12^{ab}	18.05±0.08^{d}	69.53±0.03^{a}	0.25^{c}
0.1	29.98±0.51^{b}	8.62±0.72^{ab}	10.68±0.46^{c}	10.51±0.15^{bc}	24.83±0.04^{b}	64.1±0.04^{ab}	0.57±0.01^{c}

*Mean ± standard deviation; values followed by a different superscript lower case letter on the same parameter in the same column are significantly different at $P < 0.05$.