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Effect of Different Stabilisation Treatments on Preparation and Functional Properties of Rice Bran Proteins

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Abstract

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The stabilisation treatments of rice bran were performed using microwave heating (100% power, 1–2 min) and dry heating (120°C, 10–20 min), respectively, and then protein was prepared by alkaline extraction (pH 9.5) and acid precipitation (pH 4.5). Stabilisation treatments resulted in a decrease in the protein yield, but an increase in the protein purity. Heat-stabilisation was effective in inhibiting the rancidity of rice bran, and microwave heating was more effective than dry heating. The functional properties of proteins such as the emulsifying properties and oil holding capacity were improved with the stabilised rice bran, while the foaming properties, water holding capacity and nitrogen solubility of protein were slightly impaired. By comparison, dry heating treatment at 120°C for 20 min was effective and suitable for the stabilisation of rice bran for long term storage, as well as improving some functional properties of rice bran proteins. These results could provide basic information for industrial preparation of rice bran protein and its application in various food formulas.

Keywords: dry heat stabilisation; functional properties; microwave stabilisation; rice bran proteins

Rice bran is a valuable by-product in the process of rice milling and constitutes approximately 10% of the weight of rough rice (Gul *et al*. 2015). It contains about 60% of the nutrients in raw rice such as proteins, carbohydrates, dietary fibre and phenolic substances, so rice bran is regarded as a natural nutrient resource (Zhou *et al*. 2004). However, rice bran is not consumed as food by humans due to possible hull contamination and its high fibre content. The most defatted rice bran is currently either used as fuel in boilers or as an ingredient in animal feed (Fabian & Ju 2011).

Rice bran contains 10–16% high-quality protein. In particular, rice bran proteins have unique hypoallergenic and nutritional properties (e.g., essential amino acids, protein efficiency ratio and digestibility) compared with other vegetable proteins from legumes and cereals (Juliano 1999; Rafe *et al*. 2014). Thus, it is suitable for use in infant formulas and nutraceutical food (Hamada 2000). Various methods have been used to extract rice bran protein including enzymatic, alkali and physical methods. Currently, the alkali method is the most common way to produce rice bran protein because NaOH can break hydrogen, amide, and disulfide bonds in proteins (Hamada 1997). For example, Zhang *et al* (2012) reported that alkaline extraction (pH 9.5) of defatted rice bran could result in a protein yield of 32.9% under conditions of continuous shaking at 300 *g* for 2.0 h at 50°C. However, the nutritional characteristics of protein would be changed at high pH conditions (Paraman *et al*. 2008), so a weak alkali

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concentration is required in order to obtain high-quality protein. In this work, based on our previous studies, a pH of 9.5 was used for the extraction of rice bran protein using the alkali method.

Although the nutritional value of rice bran proteins has been well confirmed, no large-scale commercial production is currently practiced (Gupta *et al*. 2008). The main limitation on the industrial preparation of protein is that rice bran becomes rancid soon after its detachment from the kernel (Garcia *et al*. 2012), which could destroy the nutritional value of rice bran protein. For this reason, stabilisation treatment is required to maintain high-quality rice bran. Various stabilisation methods such as dry heating, microwave heating, ohmic heating, extrusion and refrigeration have been reported (Kim *et al*. 2014). Dry heating treatment is simple and convenient, and it has potential for industrialisation (Sharma *et al*. 2004). Another method of heat treatment, the use of microwaves, has the virtue of high heat efficiency, short preparation time and no temperature grads (Ramezanzadeh *et al*. 1999). Therefore, these two stabilisation methods have been reported in previous studies. However, a major problem with the application of stabilisation treatments is that the functional properties of rice bran protein can be damaged (Dos Santos Conceicao Faria *et al*. 2012). Therefore, the purpose of this study was to explore the effect of the two stabilisation methods on the preparation and physicochemical characteristics of rice bran protein, and to provide basic information for application in food formulas.

MATERIAL AND METHODS

Preparation of rice bran protein. Fresh rice bran was obtained from Xing-Wang rice bran oil Ltd. (China). The rice bran was passed through a 60-mesh sieve to get rid of impurities, and then stored in a freezer at –20°C for future use. The protein content of rice bran was about 14.47 ± 0.80%, as determined using the Kjeldahl method (N × 5.95, wet basis). Low molecular weight protein marker and ethylenediaminetetraacetic acid (EDTA) were purchased from Sangon Biotech. Co., Ltd. (China). 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) was purchased from Sigma-Aldrich (USA). All other chemicals were of analytical grade.

Stabilisation treatment. Dry heating treatment with reference to the method of Sharma (2004) with some modifications. Firstly, the drying oven was preheated to 120°C to offer a constant initial temperature. Then, rice bran was placed in open shallow pans and uniformly spread (0.5 cm layer), and the pans were transferred to a drying oven maintained at 120°C and heated for 10 or 20 minutes. Microwave heating treatment was performed according to the method described by Ramezanzadeh *et al.* (1999) with some modifications. Firstly, the microwave oven (GZ-WXJ-III; China) was preheated at 800 W for 3 min; then, rice bran was spread uniformly (0.5 cm layer) in a microwave oven and heated at 100% power for 1 or 2 minutes. Stabilised rice bran and free rice bran were stored at room temperature in polyethylene bags, respectively. Rice bran was sampled every seven days and used for the determination of free fatty acids.

Defatting of rice bran. Stabilised rice bran was defatted by mixing bran with solvent (*n*-hexane) at a 1:10 ratio and stirring for 30 min in the beaker followed by centrifugation (H1850; Xiangyi Laboratory Instrument Development Co., Ltd., China) at 4000 *g* for 10 minutes. The stabilised, defatted rice bran samples were air-dried (25°C) for 8 hours.

Preparation of rice bran proteins. The stabilised defatted rice bran samples were dispersed in deionized water with a ratio of 1 : 15 (w/v). The pH of the slurries was adjusted and maintained at 9.5 using 0.1 M NaOH, and shaken at 200 *g* and 45°C in a thermostatic oscillation incubator for 120 minutes. Then, the slurries were centrifuged at 4000 *g* for 20 minutes. The supernatant was collected and the protein concentration was measured with the Bradford method using bovine serum albumin (BSA) as the standard. Rice bran protein was recovered by precipitation at pH 4.5. The protein concentration in the isolated material was determined using the Kjeldahl method, and the ratio of protein weight to isolate weight was termed protein purity. Protein yield was calculated as follows:

$$\text{Protein yield (\%)} = (\text{total protein content in supernatant} / \text{total protein content in stabilised defatted rice bran}) \times 100 \quad (1)$$

Free fatty acids. The free fatty acid (FFA) content was determined according to a titration method (Sharma *et al*. 2014). Exactly 5 g of rice bran samples and 50 ml benzene were added into a flask for extraction of free fatty acids. After stirring for 30 min, 25 ml extraction liquid was taken and mixed with a 25 ml 0.04% phenolphthalein ethanol solution in the flask. The mixture was titrated with 0.05 M

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KOH until a light pink colour appeared. FFA were calculated using the following equation:

$$\text{FFA (mg KOH/g)} = \frac{\Delta V \times 0.05 \times 56.1 \times (50/25) \times 100}{m \times (100 \times \text{M})} \quad (2)$$

where: ΔV – volume of titration (ml) with 0.05 M KOH; 56.1 – KOH milligrams equivalent; m – weight of rice bran sample (g); M (%) – percentage of rice bran sample moisture

Water holding capacity and oil holding capacity. Water holding capacity and oil holding capacity were determined according to Rao (2002). Protein samples were dispersed in distilled water or soybean oil and diluted to 10 mg/ml. The protein solution was centrifuged at 4500 *g* for 30 min, and then the supernatant was removed. The retention of water or oil in the protein sample was expressed as water absorption capacity or oil absorption capacity (g/g), respectively.

Foaming capacity and foaming stability. The foaming capacity of protein samples was determined by measuring the volume of foams immediately after homogenisation of 1% (w/v) protein solution. The ratio of the foam volume after 30 min to the initial foam volume was taken as foaming stability (Wang *et al*. 1999).

Emulsifying activity and emulsion stability. Emulsifying activity and emulsion stability were determined using the method of Yasumatsu *et al* (1972). Protein sample (2 g) was dispersed in a 100-ml mixture of distilled water and soybean oil (1 : 1). The mixture was emulsified with a homogeniser at 10 000 *g* for 2 minutes. The emulsion was centrifuged at 2000 *g* for 5 min in centrifuge tubes. Emulsifying activity (EA) was calculated using the following equation:

$$\text{EA (\%)} = (\text{height of the emulsified layer/height of the whole layer in the centrifuge tube}) \times 100 \quad (3)$$

The centrifuge tube was heated at 80°C for 30 min and then cooled to room temperature. The emulsion was centrifuged at 2000 *g* for 5 min in the centrifuge tube. The ratio of the height of the remaining emulsified layer to the initial emulsion layer was used to determine emulsion stability (ES).

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using a previously described method (Laemmli 1970). Concentrations of stacking gel and separating gel were 5% and 15%, respectively. The protein samples (2 mg) were dissolved in 500 µl sample buffer (0.125 M Tris-HCl buffer containing 20% (v/v) glycerol, 8 M urea, 1% SDS (w/v) and 2% 2-mercaptoethanol (2-ME, v/v), pH 6.8), and then were heated in boiling water for 5 minutes. After centrifugation at 10 000 *g* for 10 min, 10 µl of supernatants were loaded into gel pockets and run in an electrophoresis chamber. Gels were stained using Coomassie brilliant blue R-250 staining solution (0.25%, w/v) and destained in a methanol-water solution (acetic acid : methanol : water = 1 : 1 : 8, v/v/v).

Nitrogen solubility index. Each protein sample (1 g) was dispersed in 100 ml of distilled water, and the pH value of the solution was adjusted to 2–12.0 using 0.2 M HCl or 0.2 M NaOH. After stirring for 30 min using a magnetic stirrer (MS-H280-Pro; China), the suspension was centrifuged at 4500 *g* for 10 minutes. The protein concentration of the supernatant was determined using the Bradford method. The rate of total protein content in supernatant to total protein content in sample was used to determine the nitrogen solubility index (Wang *et al*. 2016).

Sulfhydryl and disulfide bond contents. The levels of free sulfhydryl groups (SH_F), total sulfhydryl groups (SH_T) and disulfide bonds (S-S) in protein samples were determined according to the method of Beveridge *et al.* (1974). Protein samples (30 mg) were suspended in 10 ml of Tris-Gly buffer (0.086 M Tris, 0.09 M glycine, 0.004 M EDTA, and 8 M urea, pH 8.0), followed by centrifugation at 8000 *g* for 10 minutes. For SH_F content determination, 2 ml of protein supernatant were taken and mixed with 80 µl of Ellman's reagent (DTNB in Tris-Gly buffer, 4 mg/ml), and then the absorbance was determined at 412 nm after binding for 5 min. For SH_T content determination, 2 ml of the supernatant were treated with 0.2% (w/v) 2-ME for 2 h, and then protein was separated by precipitation with 12% (w/v) TCA for 1 h and centrifugation at 8000 *g* for 10 minutes. The precipitate was collected and washed three times with 12% TCA, and then dissolved in 10 ml of Tris-Gly buffer. Aliquots (80 µl) of Ellman's reagent were added to 2 ml of the protein solution, and the absorbance was measured at 412 nm. The contents of SH_F and SH_T were calculated using the following equation:

$$\mu\text{mol SH/g} = 73.53 \times A_{412}/C \quad (4)$$

where: A_{412} – absorbance at 412 nm; C – protein concentration (mg/ml); 73.53 – derived from $10^6/(1.36 \times 10^4)$ (1.36 × 104 – Ellman's reagent molar absorptivity); S-S content was calculated as one half of the difference between SH_T and SH_F

Intrinsic protein fluorescence spectrum. The intrinsic fluorescence spectrum of proteins was deter-

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mined as previously described (Jiang & Zhao 2010). Each protein sample was dispersed in phosphate buffers (pH 7.0) to give a 0.15 mg/ml concentration. Internal fluorescent groups of protein molecules were used as a probe in the fluorescence spectrum analysis. In order to reduce the contribution of tyrosine, the excitation wavelength of the fluorescence spectrum was set at 290 nm. The scanning range of the divergence spectrum was 300–400 nm. The excitation slit and the emission slit width were set at 5 nm.

Statistical analysis. All of the tests were performed in triplicate and the results were expressed as means ± standard deviations. A *t*-test was used to evaluate significant differences ($P < 0.05$) between the means for each sample.

RESULTS AND DISCUSSION

Protein extraction. The protein extraction yield and the protein purity are shown in Figure 1. It can be seen that stabilisation treatment resulted in a significant reduction ($P < 0.05$) in protein extraction yield. The lowest extraction yield of protein was 41.89%, a reduction of 7.07%. This might be because stabilisation treatment led to protein denaturation and protein interaction with other components, making it more difficult to extract protein (Tang *et al*. 2003). Moreover, in the stabilised protein samples, dry heat rice bran protein had a better extraction yield as compared to that of microwave heat rice bran protein, which might be due to the high efficiency of microwave heating (Chen *et al*. 2016). Importantly, protein purity was improved with stabilisation treatment. The highest yield of protein purity was 73.34%, an increase of 11.08%.

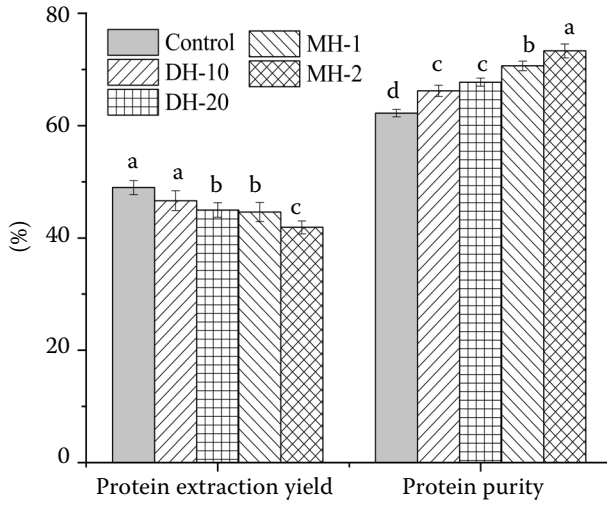

Figure 1. Protein extraction yield and protein purity

Control – unstabilised rice bran; DH-10 – dry heating treatment at 120°C for 10 min; DH-20 – dry heating treatment at 120°C for 20 min; MH-1 – microwave heating treatment at 800 W power for 1 min; MH-2 – microwave heating treatment at 100% power for 2 min

Free fatty acids. The change in FFA content could indirectly reflect the degree of rancidity in rice bran (Ramezanzadeh *et al*. 2000). As shown in Figure 2, the FFA content increased together with increasing storage time. Not surprisingly, the increase in FFA content for the unstabilised rice bran was much higher ($P < 0.05$) than for the treated rice bran. The FFA contents of the control, DH-10, DH-20, MH-1 and MH-2 were 39.51, 15.85, 14.47, 11.64 and 10.67 mg KOH/g after 35 days of storage, respectively. This indicates that the stabilisation treatment was effective in inhibiting enzymatic degradation. By comparison, microwave heating treatment was more effective in stabilisation of rice bran than dry heating treatment.

Water holding capacity and oil holding capacity. Water holding capacity, an important property of protein, was used to express the interaction between water and protein (Rao *et al*. 2002). As shown in Figure 3, the water holding capacity of rice bran protein was significantly decreased ($P < 0.05$) after stabilisation treatment. This was probably because heating treatment led to partial thermal denatura-

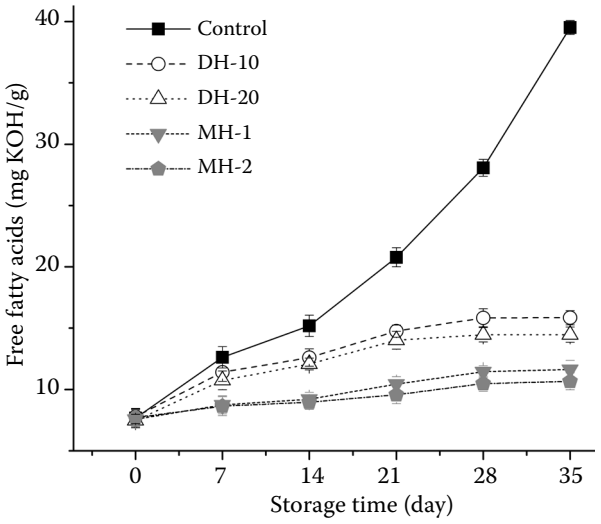

Figure 2. The contents of free fatty acids in rice bran (control, DH-10, DH-20, MH-1 and MH-2 samples were treated as described in Figure 1)

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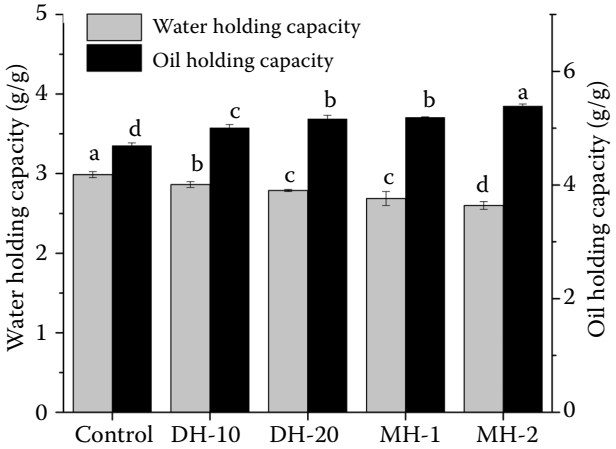

Figure 3. Water holding capacity and oil holding capacity of protein samples (control, DH-10, DH-20, MH-1 and MH-2 samples were treated as described in Figure 1; different letters on the bars indicate statistically significant differences between treatments – $P < 0.05$)

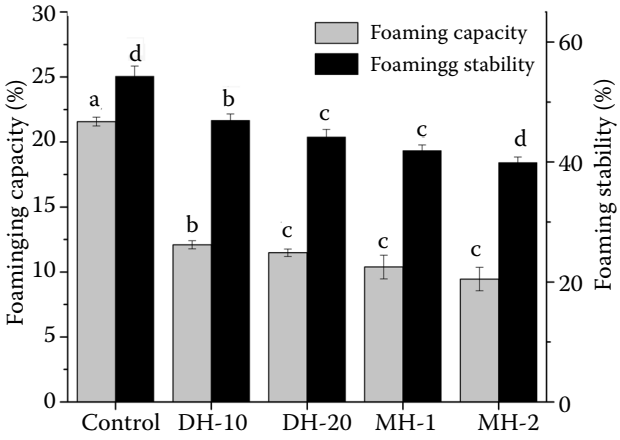

Figure 4. Foaming capacity and foaming stability of protein samples (control, DH-10, DH-20, MH-1 and MH-2 samples were treated as described in Figure 1; different letters on the bars indicate statistically significant differences between treatments – $P < 0.05$)

tion of proteins, as well as an opening of the spatial structure of the protein and a partial exposure of hydrophobic groups (Prakash & Ramanatham 1995). It could be seen that the destruction of water holding capacity by microwave heating treatment was more pronounced than in response to dry heating treatment, and heating times of longer duration also had an adverse effect on water holding capacity. However, previous research found that a water absorption capacity of protein in the range of 1.49–4.72 g/g should be considered ideal in viscous foods (Aletor *et al*. 2002), so these protein samples could still be regarded as possessing good water holding capacity for the application of various processed foods. High oil holding capacity is essential in food formulations such as cake batters or sausages. It was clear that the oil holding capacity of rice bran protein was significantly improved ($P < 0.05$) after stabilisation treatment, and the direction of the change in oil holding capacity was opposite to that of water holding capacity. This might be related to the increase in hydrophobic groups.

Foaming capacity and foaming stability. As an important functional property of protein, foaming properties reflect the ability of protein to reduce the surface tension of water and air (Bandyopadhyay *et al*. 2008). The foaming capacity and foaming stability of rice bran protein are given in Figure 4. Among all the protein samples, the unstabilised rice bran protein had better foaming capacity and foaming stability values, which reached 56.37 and 54.28%, respectively. Foaming capacity is dependent on the diffusion of protein at the air-water interface due to the unfolding of protein structure, while foaming stability is determined by the formation of a thick cohesive layer around the bubbles (Khan *et al*. 2011). A significant decrease ($P < 0.05$) in foaming properties was observed in the case of microwave or dry heating treatment, which might be because high temperature treatment caused partial aggregation of protein resulting in the formation of insoluble macromolecular aggregates and in thermal denaturation, making diffusion and adsorption of protein at the air-water interface more difficult (Hendrickx *et al*. 2006). From Figure 4, it can also be seen that an increase in heating time had adverse effects on the foaming properties of proteins.

Emulsifying activity index and emulsion stability. Good emulsifying properties are beneficial for proteins that are to be used as fat emulsifiers in food formulations. It has also been confirmed that the formation of emulsions is due to presence of hydrophilic and hydrophobic protein groups (Sogi & Chandi 2007). The emulsifying activity index and the emulsion stability of rice bran protein samples are presented in Figure 5. The emulsifying activity and emulsion stability of the unstabilised rice bran protein were 9.62 and 17.04%, respectively. It could be observed that the protein subjected to dry heat had better ($P < 0.05$) emulsifying activity and emulsion stability values than the control and microwave heating protein, and the highest value was obtained

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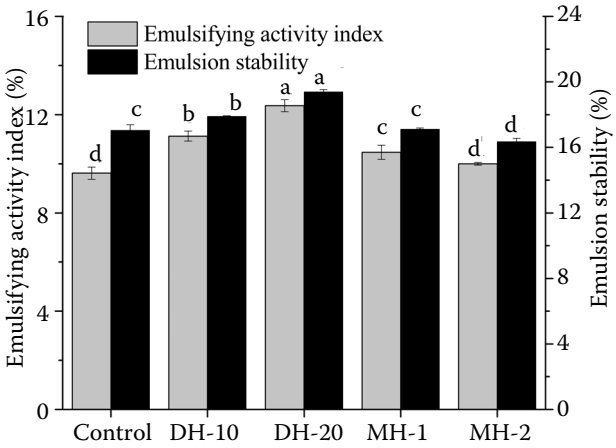

Figure 5. Emulsifying activity index and emulsion stability of protein samples (control, DH-10, DH-20, MH-1 and MH-2 samples were treated as described in Figure 1; different letters on the bars indicate statistically significant differences between treatments – $P < 0.05$)

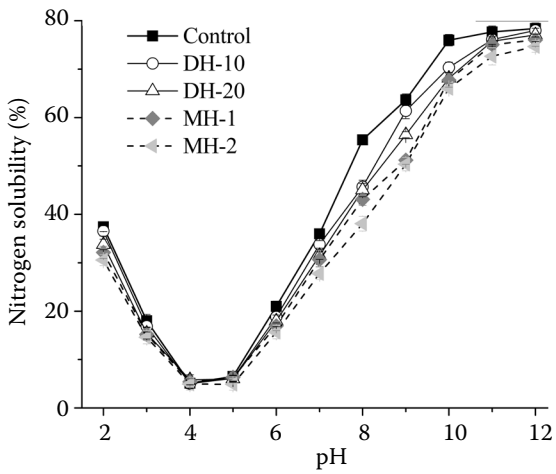

Figure 6. Nitrogen solubility of protein samples (control, DH-10, DH-20, MH-1, and MH-2 samples were treated as described in Figure 1; different letters on the bars indicate statistically significant differences between treatments – $P < 0.05$)

in the DH-20 protein sample. On the one hand, rice bran proteins can exhibit good emulsifying properties under alkaline conditions; on the other hand, heating treatment is conducive to the rapid expansion of protein molecules in the oil-water interface, thereby improving the emulsifying activity index and the emulsion stability of proteins (Sogi & Chandi 2007).

Nitrogen solubility index. Nitrogen solubility is an important parameter for the application of protein in the food industry because of its contribution to certain functional properties of the protein. The nitrogen solubility profiles of different rice bran samples in the pH range of 2–12 are shown in Figure 6. All protein samples showed similar solubility profiles. The lowest solubility of protein samples in water could be seen at pH 4–5, which might be due to the isoelectric region of proteins. The solubility increased gradually at pH values below 4 and above 6. Above pH 10, the solubility continued to increase but at a slower rate. This solubility pattern was consistent with previous reports by other researchers (Gnanasambandam & Hettiarachchy 1995). It was clearly seen that the nitrogen solubility of stabilised proteins showed a decrease to varying degrees, and nitrogen solubility was the lowest in the MH-2 sample. This might be because the heating treatment led to the exposure of some hydrophobic groups, resulting in a decrease in nitrogen solubility. It is interesting to note that the direction of change in nitrogen solubility was similar to that of foaming properties and water holding capacity, which indicated that foaming properties and water holding capacity might be related to nitrogen solubility (Ragab *et al*. 2004; Chandi & Sogi 2007).

Electrophoresis. SDS-PAGE is generally used to explore the subunit changes in proteins (Qiang *et al*. 2013). The electrophoresis patterns of rice bran protein samples are shown in Figure 7. However, in all five lanes, the molecular weight distributions of protein samples were almost identical, indicating that the microwave or dry heating treatment has no effect on the molecular weight distribution of protein samples. The molecular weight distribution of protein samples was mainly divided into three regions, i.e., 42.7–66.2, 31–42.7, and < 31.0 kDa.

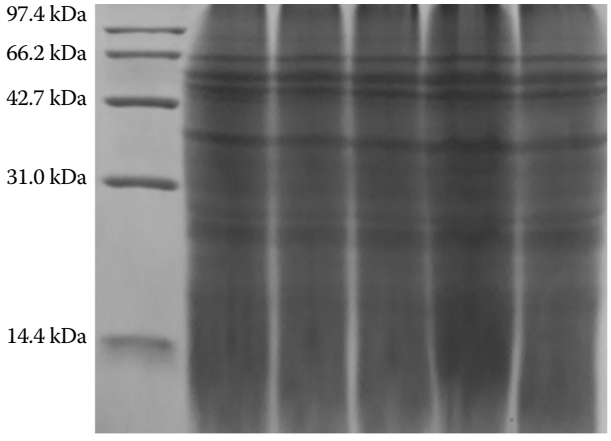

Figure 7. Electrophoretogram of rice bran protein (control, DH-10, DH-20, MH-1, and MH-2 samples were treated as described in Figure 1)

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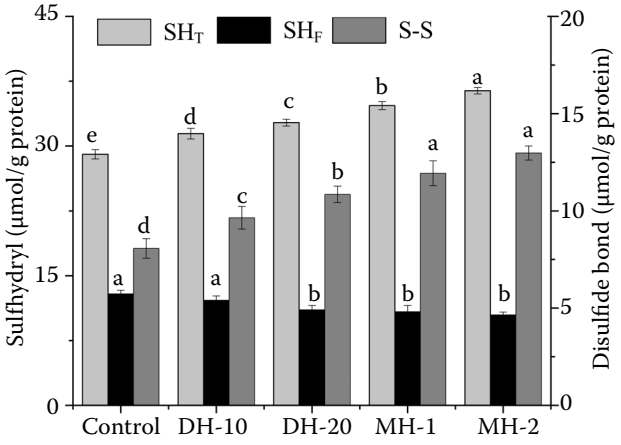

Figure 8. Sulfhydryl and disulfide bond contents of rice bran protein (control, DH-10, DH-20, MH-1, and MH-2 samples were treated as described in Figure 1; different letters on the bars indicate statistically significant differences between treatments – $P < 0.05$)

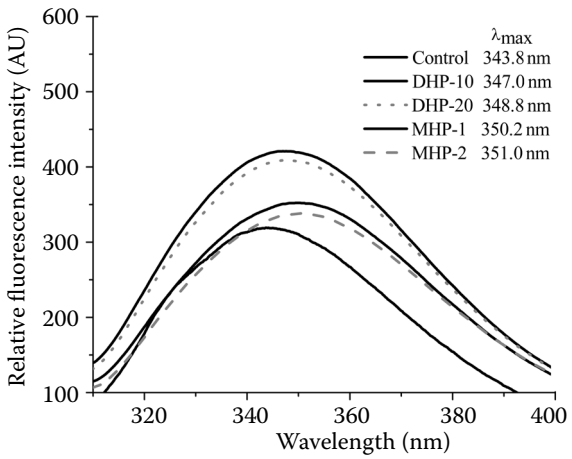

Figure 9. Intrinsic protein fluorescence spectrum (control, DH-10, DH-20, MH-1, and MH-2 samples were treated as described in Figure 1)

Additionally, the SDS–PAGE patterns of rice bran protein samples showed a slight difference to those from a previous report by Wang *et al* (2016), which might be attributed to the different protein samples used in that work.

Sulfhydryl and disulphide bond contents. Sulfhydryl and disulphide bond contents of rice bran protein samples are shown in Figure 8. The SH_T, SH_F and S-S contents of the control were 29.04, 12.87 and 8.07 μmol/g protein, respectively. By comparison, the SH_T contents of the DH-10, DH-20, MH-1 and MH-2 were markedly higher ($P < 0.05$) than that of the control, and the highest content of SH_T was observed in MH-2. This might be explained by heat treatment causing an unfolding of the protein leading to the exposure of buried groups (Wang *et al*. 2016). The oxidation of SH_F to S-S could result in an increase in S-S content and decrease in SH_F content (Tang & Ma 2009).

Intrinsic protein fluorescence spectrum. The intrinsic fluorescence spectrums of rice bran protein samples are shown in Figure 9. A red shift or blue shift in the spectrum can indicate a change in protein conformation. Red shifts were observed in stabilised rice bran protein, and the extent of the red shift reflected the degree of protein conformational change. Among all stabilised samples, the maximum value of λ_{max} (maximum fluorescence absorption wavelength) was in MH-2 and the minimum was in DH-10. The heating treatment resulted in exposure of the internal hydrophobic side chains of the rice bran protein to the polar environment of the molecular surface, followed by a subsequent change in the fluorescence spectrum of the tryptophan. Thus, the λ_{max} value of protein gradually increased with increasing heating time. The protein conformation in samples subjected to microwave heating exhibited more changes than in samples subjected to dry heating. This was mainly because of the high efficiency of microwave heating. In addition, the relative fluorescence intensity was mainly influenced by the concentration of the protein solution.

CONCLUSIONS

Microwave heating treatment was more effective in inhibiting the rancidity of rice bran than dry heating treatment, while the content of free fatty acids in unstabilised rice bran increased from 7.77 mg to 39.51 mg KOH/g after 35 days of storage. The emulsifying properties and oil holding capacity were found to be improved in protein from stabilised rice bran. Among all protein samples, DH-20 exhibited the best emulsifying properties, and MH-2 showed the best oil holding capacity. However, foaming properties, water holding capacity and nitrogen solubility of stabilised protein were destroyed by heating treatment, and the effects of microwave heating were more marked than those of dry heating. In addition, stabilisation treatments made it more difficult to extract protein from rice bran. By comparison, dry heating treatment at 120°C for 20 min was effective and suitable for the stabilisation of rice bran for long

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term storage and also improved some functional properties of rice bran proteins. The results of this study could provide basic information for industrial preparation of rice bran protein and its application in various food formulas.

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