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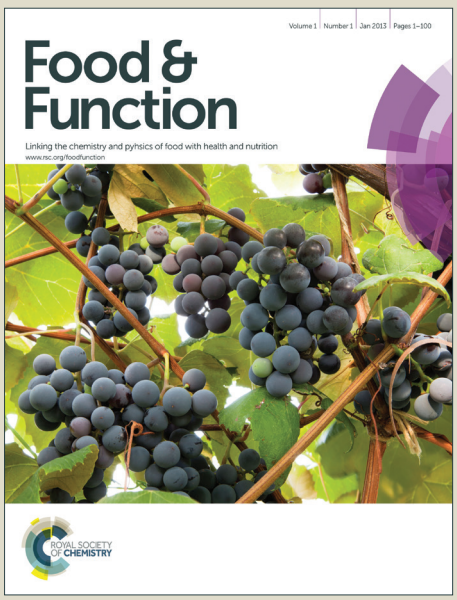

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Solubilization of Water-insoluble Subunits in Rice Proteins by Freeze-milling

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Abstract

This study investigated the effects of freeze-milling on the structural and functional properties of rice proteins (RPs). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed that freeze-milling slightly influenced the subunit bands of RPs. Secondary and tertiary structures were studied by analyses of Fourier transform infrared spectra, sulfhydryl and disulfide bond contents, and surface hydrophobicity. The freeze-milled RPs (FMRPs) may possess an unfolded conformation with exposed buried functional groups. In addition, the solubility of the FMRPs was higher than that of the control probably because of the exposure of water–protein interaction areas. Particularly, the solubility of the FMRPs treated at pH 12.5 was 42 times that of the control. Characterization of functionalities displayed that both emulsifying and foaming activities of FMRPs were improved by solubilization. However, functional stabilities were unaffected or deteriorated. Generally, the FMRPs showed better emulsifying activity and stability than bovine serum albumin while better foaming activity and stability than hen egg albumin. FMRPs may be of great interest to the food industry.

Introduction

Rice is a staple food in China, with a gross annual production of approximately 204 million tons (data collected in 2012 by the State Statistics Bureau of China). Despite their relatively low content (6%–15%) in milled rice/regular rice flour [1], rice proteins (RPs) cannot be neglected because of the large gross quantity of rice, particularly in the starch or glucose manufacturing industries. RPs are usually obtained through alkali extraction [2, 3] or enzyme-assisted preparation [4, 5]. Alkali extraction yields high protein recovery (>80%) and protein purity (>90%) by dissolving insoluble RPs in dilute alkaline solution and subsequently precipitating the proteins at their isoelectric point. The use of various carbohydrate-hydrolyzing enzymes also yields a high protein purity (>80%) by removing non-protein substances (mainly starch).

RPs are nutritional and healthy for human consumption. These proteins exhibit hypoallergenic [6], hypotensive [7], hypocholesterolemic [8], and anti-atherosclerotic [9] activities. Protein efficiency ratio (PER), an indicator of nutritional quality, ranges from 2.0 to 2.5 for RPs, compared to 2.5 for casein. RPs also have wide applications, including being suitable ingredients for infant food formulations, because of their >90% digestibility [10].

However, the commercial availability of RPs is limited by their poor solubility. This disadvantage can be principally attributed to gluten (*ca.* 80% of total proteins), the major storage protein of rice, which interacts with each other through excessive aggregation and/or extensive disulfide (S–S) bond cross-linking [11, 12]. Only 1%–5% of RPs (albumin) is water-soluble [13]. Solubility, a crucial physical property, substantially affects the functional properties (emulsification and foaming) of food proteins [14]. Treatments such as drying, extraction, and storage also influence protein functionalities. Zhao et al. [15] reported that spray-dried RPs exhibit better emulsifying and foaming activities than freeze-dried RPs. Other scholars observed the same trend [16, 17]. Paraman et al. [18] reported that alkali- or salt-extracted RPs (RP_A or PR_S) possess higher emulsifying and foaming properties than enzyme-extracted proteins (RP_E). This result may be attributed to the lower degree of thermal denaturation of RP_A or PR_S than RP_E. In addition, long-term storage may negatively influence the emulsification properties of proteins through denaturation [19].

Functional properties are highly dependent on solubility. Hence, physical treatments (*e.g.*, freeze–thaw, sonication, and ultra-high pressure) [20], enzymatic treatments [5, 21], and innovative methods (*e.g.*, hydrothermal cooking and microfluidization) [4, 22] have been utilized to enhance RP solubility. However, the effects of these methods on RP solubility are limited.

The increasing consumer demand for high-protein food products and the increasing cost pressure in the food industry require water-soluble RPs to be processed using an efficient and environmentally friendly approach. We have recently discovered

that combined freezing and milling can increase the solubility of RPs to such an extent that soluble RPs can be manufactured on an industrial scale [over 50% (w/w) of soluble RPs were obtained]. The simplicity and cost effectiveness of this combination treatment make it promising in the solubilization of other insoluble proteins aside from RPs. Therefore, this research aims to investigate the effects of freeze-milling on the structural and functional properties of RPs.

Materials and methods

Materials

RPs with a protein content of 90% ± 1.1% as measured by the Kjeldahl method were purchased from Jinnong Biotechnology Co. (Yichun, Jiangxi, China). Bradford kit, 5, 5′-dithio-bis-2-nitrobenzoic acid (DTNB), 1, 8-anilinonaphthalenesulfonate (ANS), bovine serum albumin (BSA), hen egg albumin (HEA), and low-molecular-weight protein markers were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). All other chemicals employed were of analytical grade.

Freeze-milling procedure

Fig. 1 shows the procedure for producing FMRPs using a flow diagram. Aqueous suspensions of RPs (1:20 w/v) were stirred and adjusted to different pH values by titration against a 1 M NaOH solution. Basic protein suspensions were frozen in a refrigerator (–20 ± 1 °C) for 24 h prior to immediate milling using a high-speed impact mill (XFB-500, Zhongzhou Co., China) at room temperature. Mechanical treatment was performed for 5 min. The treatment was paused after each minute at a time interval of 10 s. After freeze-milling, RP suspensions were readjusted to a neutral pH of 7.5 by titration against 1 M HCl. The water-soluble subunits of raw RPs were hardly detected at pH 7.0. Hence, pH 7.5 was used for solubility comparisons. The neutralized suspensions were centrifuged at 10,000 g for 10 min. Residues were washed and centrifuged twice. Supernatants were combined and collected before being lyophilized, and washed residues were resuspended and subjected to further freeze-milling. Freeze-milling was repeated twice, and each treatment was designated as one cycle.

Structural properties

Molecular weight (MW) characterization: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted following the method described by Laemmli [23]. Protein samples and standards were separately dissolved in suitable volumes of sample buffers [0.125 M Tris–HCl buffer, 1% SDS (w/v), 20% glycerol (v/v), with 2% 2-mercaptoethanol (2-ME, v/v), pH 6.8] and then heated at 95 °C for 5 min. Incubated samples were centrifuged at 10,000 g for 10 s, and aliquots (10 μL) of supernatants were dripped onto the narrow orifices of the electrophoresis chamber. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (0.05%) in methanol/acetic acid/water (25:10:65, v/v/v). Destaining was performed by soaking the gels for 24 h in acetic acid/ethanol/water (1:1:8, v/v/v).

Size-exclusion chromatography (SEC) was performed with a TOSOH TSK-G2000 SW_{XL} column (Yamaguchiken, Honshu, Japan) at 25 °C using an Agilent HPLC 1260 system (Agilent, USA). The mobile phase was 50 mM sodium phosphate (pH 7.5) containing 50 mM NaCl, and the flow rate was 0.5 mL/min. Protein samples were suspended in the mobile phase (0.5%, w/v), centrifuged at 10,000 g for 10 min, and then filtered through double 0.45 μm filters (Millipore, Fisher Scientific). Elution was monitored at a wavelength of 280 nm. The column was calibrated with ferritin (440 kDa), transferrin (77 kDa), apomyoglobin (16 kDa), and cytochrome c (12.4 kDa).

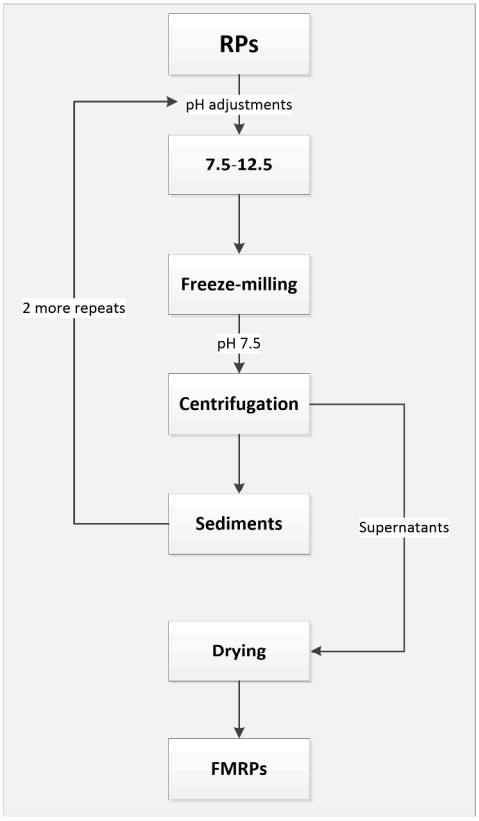

Fig. 1 Flow diagram of the procedure for producing FMRPs.

Secondary structural analysis: Protein conformation was analyzed via Fourier transformed infrared (FTIR) spectroscopy using a Nicolet iS10 FTIR spectrometer purchased from ThermoFisher Scientific (Marietta, OH, USA). Approximately 2 mg of protein powder was mixed with KBr, grounded, and then pressed into a pellet. Absorbance intensity was measured at 2 cm^{-1} resolution in the wavenumber range of 4000–400 cm^{-1}. Data processing was carried out according to the method described by Haque et al. [19]. Spectra of the amide I bands (1700–1600 cm^{-1}) were smoothed and baseline corrected using Omnic V8.1 (ThermoFisher Scientific, USA). PeakFit 4.12 (SeaSolve Software Inc., USA) was used to deconvolve the amide I region of the spectra. The deconvolved spectrum was iteratively curve-fitted with Gaussian band shapes. The resultant peaks were assigned to different secondary structures. Peak assignment of deconvolved amide I bands was conducted using the results of Prosa et al. [24] and Byler and Susi [25] as guides. Peaks at 1620 ± 20 cm^{-1}, 1645 ± 5 cm^{-1}, 1654 ± 4 cm^{-1}, and 1680 ± 20 cm^{-1} corresponded to β-sheet, random coil, α-helix, and β-turns in the fitting procedure, respectively.

Sulfhydryl and disulfide bond contents: The free sulfhydryl group (SH_F), total sulfhydryl group (SH_T), and disulfide bond (S–

S) contents of the protein samples were determined according to the method described by Beveridge et al. [26] with some modifications. Protein samples (15 mg) were suspended in 10 mL of Tris–Gly buffer (pH 8.0) containing 0.086 mol/L Tris, 0.09 mol/L glycine, 0.004 mol/L EDTA, and 8 mol/L urea, and then centrifuged at 10,000 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 binding for 5 min, the absorbance at 412 nm was monitored. For SH_T content determination, 1 mL of the supernatant was treated with 4 mL of 15 g/L 2-ME (2-ME in Tris–Gly buffer containing 8 M urea and 5 M Gdn HCl) for 1h, and then the protein was separated by precipitation with 12% TCA for 1 h. After subsequent centrifugation at 10,000 g for 10 min, the precipitate was collected and washed thrice with 5 mL of 12% TCA and then resolubilized in 10 mL of Tris–Gly buffer containing 8 M urea. Aliquots (40 μL) of Ellman's reagent were added to 4 mL of this protein solution, and the absorbance was measured at 412 nm. The contents of SH_F and SH_T were determined as follows:

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

where A_{412} is the absorbance at 412 nm, C is protein concentration (mg/mL), and 73.53 is derived from $10^6/(1.36 \times 10^4)$ (1.36×10^4 is Ellman's reagent molar absorptivity). S–S content was expressed as half of the difference between SH_T and SH_F.

Surface hydrophobicity (H_0): The H_0 of the protein samples was fluorometrically determined by ANS labeling according to the method described by Haskard and Li-Chan [27]. The samples were dispersed in 0.05 M sodium phosphate buffer (pH 7.5) and then centrifuged at 10,000 g for 10 min. Then, supernatant protein concentrations were adjusted to 0.0015%–0.015% (w/v). A 4 mL aliquot of the samples was titrated with a 10 μL aliquot of a 5.0 mM ANS solution. Fluorescence intensity was measured at excitation and emission wavelengths of 390 and 484 nm, respectively, using a Hitachi F-7000 fluorescence spectrofluorometer (Chiyoda-Ku, Tokyo, Japan). Under conditions with excess probe, the initial slope (S_0) of the fluorescence intensity versus protein concentration plot was obtained as the H_0.

Amino acid analysis: Amino acid analysis was conducted by measuring the absorption at 254 nm using an automatic amino acid analyzer (835-50, Hitachi, Japan) equipped with a PicoTag column. The samples were placed in sealed hydrolysis tubes and hydrolyzed with suitable volumes of 6 M HCl at 110 °C for 24 h. Amino acid content (expressed as g/100 g protein) was determined at a temperature of 38 °C and a flow rate of 1 mL/min. Tryptophan was not determined because it was degraded during hydrolysis.

Functional properties

Solubility characterization: Freeze-milling was performed at pH 7.5, 9.5, 11.5, and 12.5, respectively, and the process was repeated for two more cycles at each pH. After each treatment, the suspensions were readjusted to a neutral pH of 7.5 for soluble protein measurements. Samples of pH 7.5 suspensions not subjected to freeze-milling served as the control. The solubility of treatment cycles (n) was expressed as the *accumulated* percentage of soluble protein content of each cycle ($\leq n$). Unless otherwise noted, analysis was performed using lyophilized supernatants from the *first cycle* of freeze-milling. Content of water-soluble proteins was determined using Bradford assays, with BSA as the standard. Absorbance at 595 nm was measured using a Corona SH-1000 microplate reader (Hitachinaka-Shi, Ibaraki-Ken, Japan). Protein solubility was expressed as the percentage of water-soluble proteins/total proteins in raw RPs.

Emulsifying activity (EA) and emulsion stability (ES): EA and ES were turbidimetrically determined according to the method described by Pearce and Kinsella [28] with some modifications described below. One percent of aqueous protein suspension was adjusted to pH 7.5. Olive oil (2 mL) was added into the protein solution (6 mL) and homogenized in a mechanical homogenizer (T18BS25, IKA, Germany) at a setting gear of 5 for 1 min to produce full emulsion. After being homogenized, a 50 μL aliquot of the emulsion was pipetted at 0 and 15 min and then mixed with 5 mL of 0.1% SDS solution. The absorbance of the emulsion was measured at 500 nm with a spectrophotometer (UV-1800, Shimadzu, Japan). The absorbance that was measured immediately after emulsion formation (T_0) was expressed as the EA of proteins. ES was determined as follows:

$$\text{ES} = T_0\,(\Delta t/\Delta T)$$

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

Foam activity (FA) and foam stability (FS): The FA of the proteins was determined following the method described by Wang et al. [29] with some modifications. FA was expressed as the volume of foam immediately measured after the introduction of air (90 cm^3/min) for 15 s into 5 mL of 0.3% protein solution containing 0.05 M phosphate buffer (pH 7.5) within a glass tube (2.4 cm× 30 cm). FS was calculated as follows:

$$\text{FS} = V_0\,(\Delta t/\Delta V)$$

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

DSC: The thermal analysis of RPs was performed using a Q-2000 DSC thermal analyzer (TA Instruments, New Castle, DE, USA). 2 mg of protein powder was weighed into aluminum pans containing 10 μL of 50 mM phosphate buffer (pH 7.5). The pans containing protein suspensions were hermetically sealed and allowed to stand at room temperature for approximately 4 h to achieve complete hydration. The samples were heated in a calorimeter from 40 °C to 100 °C at a rate of 10 °C/min. A sealed empty pan was used as a reference. Denaturation temperature (T_d) and denaturation enthalpy (ΔH) were calculated by Universal Analysis Software, version 4.1D (TA Instruments-Waters LLC).

Statistical analysis

All experiments were performed in triplicate, and values are expressed as mean ± SD. Results were subjected to ANOVA, and differences between means were evaluated by Duncan's multiple range test.

Results and discussion

Structural properties

MW characterization: SDS–PAGE was performed to explore the effects of freeze-milling on RP subunits conditioned at various pH values (Fig. 2a). The RP fraction contributes to a wide variety

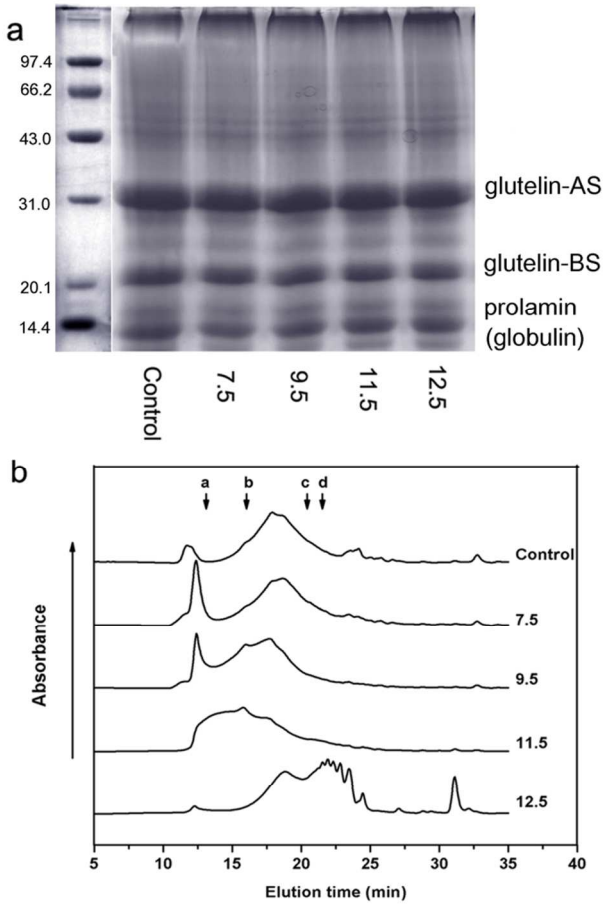

Fig. 2 MW studies of FMRPs. (a) SDS–PAGE profiles; (b) SEC profiles. Ferritin (440 kDa), transferrin (77 kDa), apomyoglobin (16 kDa), and cytochrome c (12.4 kDa) are designated by a, b, c, and d, respectively, with vertical arrows.

of subunits, several groups of which can be distinguished in the profile. Glutelin fractions are composed of acidic subunits (glutelin–AS, 30–39 kDa) and basic subunits (glutelin–BS, 19–25 kDa) [30], which constitute the most abundant components of RPs in this experiment. An intensive band and several slight bands close to 15 kDa were observed in each lane, which may be assigned to prolamin (13 and 16 kDa) [31] or globulin (15 kDa) [32].

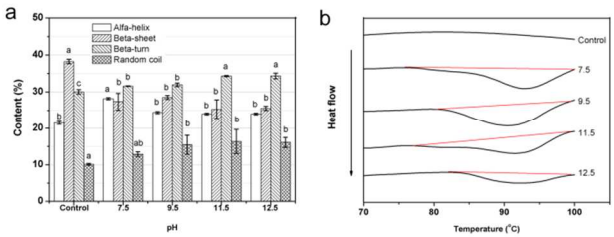

Fig. 3 Secondary structures and thermal analysis of FMRPs. (a) Secondary structure content. The same structure with different letters indicate significant differences; data are expressed as mean ± SD (n = 3). (b) DSC profiles.

Albumin fractions (18–20 kDa [33] and 40–55 kDa [34]) were not detected on SDS–PAGE graphs. This result may be attributed to fact that albumin was removed through washing during RP preparation.

Similar subunit bands were observed on SDS–PAGE where equal amounts of proteins were loaded. Thus, freeze-milling may slightly affect the major subunits of RPs.

Fig. 2b shows the SEC elution profiles of the protein samples at a wavelength of 280 nm. The loading solution contains low concentration of phosphate (50 mM) and NaCl (50 mM), and therefore the SEC profiles roughly reflect the water-soluble subunits in RPs. For the control, a major overlapped peak appeared at the elution time between 15 and 23 min. This result indicates that the MWs of the water-soluble subunits in the raw RPs exceeded 10.0 kDa. Compared with those of the control, the water-soluble subunits of the FMRPs treated at pH 7.5, 9.5, and 11.5 had higher MWs. Meanwhile, the number of low-MW subunits decreased as the elution located at 18–25 min declined. This result implies that the water-soluble parts of these samples aggregated.

SEC graphs show that the FMRPs treated at pH 12.5 possessed lower MWs than their counterparts. Notably, not all the subunits on SDS–PAGE were presented on SEC graphs because SEC graphs merely illustrated water-soluble parts of the proteins. Certain fractions appeared after over 23 min of the elution for the FMRPs treated at pH 12.5; these fractions corresponded to subunits with MWs lower than 10.0 kDa. Conversely, these fractions were not detected on the SDS–PAGE graphs. These data suggest that hydrolysis occurred after freeze-milling of the RPs treated at pH 12.5.

Table 1 SH and S–S contents and hydrophobicity (H_0)

Treatment	SH and S–S contents (μmol/g protein)			H_0 (× 10^6)
	SH_F	SH_T	S–S	
Control	25.68 ± 0.50a [a]	126.55 ± 0.76d	50.43 ± 0.52e	3.32 ± 0.025d
7.5	18.71 ± 0.38c	155.42 ± 0.76b	68.35 ± 0.33b	2.31 ± 0.026e
9.5	18.79 ± 0.29c	148.37 ± 1.04c	64.78 ± 0.38d	4.78 ± 0.055c
11.5	18.88 ± 0.25c	169.35 ± 0.76a	75.23 ± 0.36a	6.31 ± 0.019b
12.5	24.42 ± 0.50b	157.10 ± 0.87b	66.34 ± 0.50c	8.23 ± 0.052a

[a] Different letters in the column indicate significant ($P < 0.05$) differences among treatments. Values are expressed as mean ± SD (n = 3).

Secondary structures: Quantitative information concerning the secondary structures was derived from the FTIR spectra. The amide I region (1,700–1,600 cm^{-1}) mainly comprises the stretching vibration structure. α-Helix, β-sheet, β-turn, and random coil can be discerned through proper fitting of the amide I band [35]. In the present study, FTIR analysis of the amide I band

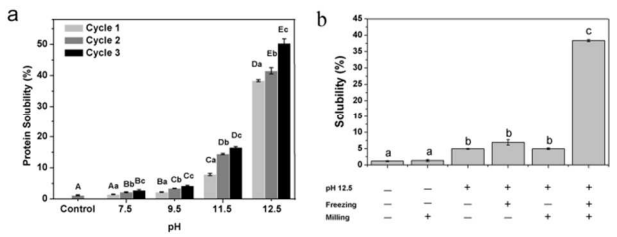

Fig. 4 Solubility characterization. (a) Protein solubility of FMRPs pre-treated at various pH values. Capital letters indicate significant differences ($P < 0.05$) among pH values within the same cycle; lower-case letters indicate significant differences ($P < 0.05$) among cycles at the same pH. Each value is expressed as mean ± SD (n = 3). (b) Solubility profiles of RPs in the presence (+) and absence (−) of pH, freezing, and milling.

exhibited that the secondary structural components, particularly β-sheet, considerably changed after freeze-milling (Fig. 3a). The content of β-sheet sharply decreased as a result of freeze-milling and pH conditioning. In contrast to β-sheet content, the relative fractions of α-helix, β-turn, and random coil were slightly increased. The turn structure is an unfolding product of any high-ordered structures, whereas anti-parallel β-sheet forms in folded protein molecules [36]. Zhao et al. [37] reported that, compared to spray-drying, freeze-drying significantly increases β-turns whereas reduces β-sheets and random coils of the proteins in rice dregs. They also correlated the sheet–turn transformation with partial unfolding. Based on these findings, we suggested that freeze-milling caused the unfolding of RPs.

Table 2 Amino acid analysis

Amino acid [a]	Control	7.5	9.5	11.5	12.5
Asp	7.6	6.8	6.9	8.0	7.7
Glu	16.7	10.8	11.5	13.9	15.1
Ser	4.1	3.1	3.4	3.8	3.9
His	2.1	1.4	1.4	1.8	2.1
Gly	4.0	4.1	4.7	5.3	4.6
Thr	3.2	2.3	2.4	2.8	2.8
Arg	7.5	5.4	5.8	6.9	8.1
Ala	5.1	4.2	4.4	4.9	4.8
Tyr	4.6	2.8	2.8	3.6	4.1
Cys-s	1.1	0.3	0.3	0.4	0.3
Val	6.0	4.3	4.4	5.3	5.7
Met	2.7	1.4	1.5	1.8	1.9
Phe	5.2	3.6	3.9	4.5	4.7
Ile	4.1	2.9	2.9	3.4	3.5
Leu	7.5	5.1	5.4	6.3	6.6
Lys	3.2	3.1	3.3	3.7	3.0
Pro	4.9	3.1	3.3	3.9	4.4
Trp	-[b]	-	-	-	-
Total	89.7	64.8	68.3	80.5	83.3

[a] Samples from the first cycle of freeze-milling were used.
[b] Not detected.

Sulfhydryl and disulfide bond contents: As listed in Table 1, the SH_T, SH_F, and S–S contents of the control were 126.55, 25.68, and 50.43 μmol/g proteins, respectively. The SH_F content of the FMRPs decreased. By contrast, the SH_T and S–S contents of the FMRPs were markedly higher than those of the control, which can be ascribed to the exposure of buried groups during protein unfolding. The oxidation of SH_F to S–S may also function in decreasing of SH_F and increasing of S–S bonds [38].

Surface hydrophobicity (H_0): High H_0 value indicates a high solubility and possible exposure of hydrophobic regions that are otherwise buried inside proteins [39]. Protein unfolding promotes the availability of hydrophobic zones that may be accessible to ANS fluorescence probe [40]. The H_0 (Table 1) of the FMRPs ($4.78–8.22 \times 10^6$ at pH 9.5–12.5) was significantly higher than that of the control (3.32×10^6). This result confirms that the proteins unfolded after freeze-milling. Furthermore, the extent of protein unfolding increased with rising pH, as indicated by an increased H_0.

Amino acid analysis: The amino acid content (g/100 g of protein) of the FMRPs is given in Table 2. The total amino acid content of the FMRPs increased with increasing pH. Approximately 10% of the raw RPs comprised non-protein constituents, and part of these components may be solubilized in the freeze-milled suspensions. The content of water-soluble proteins that were obtained during freeze-milling increased with increasing pH. This result may have contributed to the higher total amino acid content in the FMRPs treated at higher pH. The control and the FMRPs possess a high content of Glu, Asp, Arg, Leu, Val, and Ala. This result agrees with the findings of Xia et al. [23]. Arg content is an important factor that affects cholesterol metabolism and digestibility [41]. His, Thr, Val, Met, Ile, Leu, Trp, and Lys are essential amino acids required by FAO/WHO for weaned (2–5 years) children or adults [23]. Except for Met, these essential amino acids were retained by the FMRPs treated at pH 12.5 (Trp not measured). Cys-s content was reduced by freeze-milling. Other amino acids in the FMRPs treated at pH 12.5 were in the same range as those in the control.

Functional properties

Solubility characterization: As shown in Fig. 4a, the FMRPs had significantly higher solubility at all pH values, except at pH 7.5 (cycle 1), than the control. At each pH, freeze-milling significantly elevated the amounts of soluble RPs. In addition, increasing pH aggressively accelerated solubility. The solubility has rapidly and remarkably increased at pH 12.5. As a result, the RPs were 42 times more water-soluble after three cycles of freeze-milling at pH 12.5. In other words, 50.4% water-soluble RPs (vs. 1.2% water-soluble control) can be obtained using this protocol. To the best of our knowledge, this study is the first to improve RP solubility by tenfold. As shown in Fig. 4b, the solubilization of water-insoluble subunits in RPs was synergistically influenced by pH, freezing, and milling. The results suggest that alkali-assisted freeze-milling is a feasible technique for producing water-soluble RPs.

Proteins, which contain both polar and nonpolar amino acids, prefer to fold into structures with low free energy when water–polar group interactions are maximized and water–nonpolar group interactions are minimized [29]. Secondary structural analysis suggests that the FMRPs may present an extended structure, whereas the untreated proteins were folded and yielded compact bodies. In addition, the SH_T and H_0 values of the FMRPs were

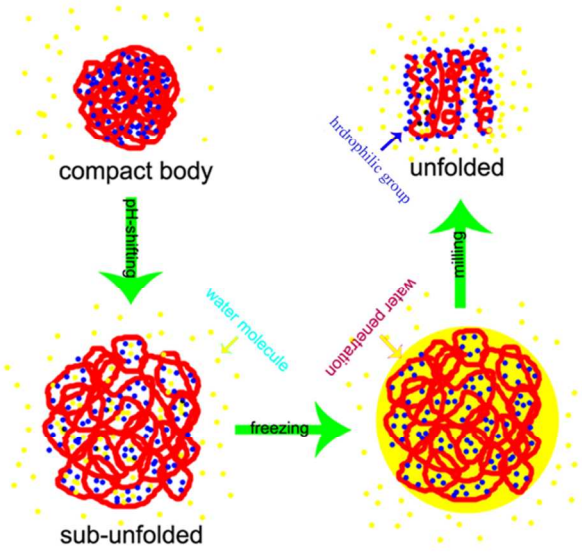

Fig. 5 Schematic diagram of protein unfolding by freeze-milling.

markedly higher than those of the control (Table 1). These findings suggest that the FMRPs were unfolded and that inner groups were exposed to an aqueous environment. Thus, we postulated that the FMRPs tended to expose their hydrophilic groups on the surface, which strengthened water–polar group interactions and possibly accounted for the solubilization. Moreover, SEC graphs may indicate that some subunits of pH 12.5 FMRPs were hydrolyzed into proteins with low MWs, which may have also partly contributed to solubilization.

The mechanisms underlying protein unfolding by freeze-milling remain vague. As shown in Fig. 4b, intense solubilization was achieved through milling and pH conditioning. However, the RPs were barely subjected to solubilization without freezing. Water molecules penetrate into the bulk water inaccessible area of macromolecules during freezing [42]. Alkali treatment considerably extends the protein tertiary structures [43, 44], thereby allowing water molecules to penetrate inward protein bodies because of decreased compactness. The penetrated water may accumulate in the inner part of protein bodies and form ice crystals. These ice crystals may transmit milling-induced mechanical energy and thus extend the structures of RPs. Furthermore, the RPs were more prone to unfolding at high pH than at low pH, explaining the accelerated solubility at high pH pre-treatment. Fig. 5 illustrates the schematic diagram of protein unfolding by freeze-milling.

However, the preferential solubilization of water-insoluble subunits in RPs is based on a high pH requirement. The alkali treatment of RPs may produce undesirable toxic substances, such as lysinoalanine [45]. Therefore, separation treatments such as dialysis are necessary to produce food-grade FMRPs.

Emulsifying activity (EA) and emulsifying stability (ES): Table 3 shows that EA was greatly enhanced by freeze-milling. The EA of the FMRPs was 0.42–0.58, which rapidly increased with increasing pH. Emulsifying properties not only depend on solubility but also on the hydrophilic–lipophilic balance (HLB) of a particular protein [46]. H_0 is an important factor that determines emulsifying properties [47]. Hydrophobic groups facilitate the interaction between proteins and oils, resulting in similar changes in emulsifying properties to those of H_0, as evidenced by the results in this study. The FMRPs presented significantly higher EA than BSA (0.36), which is a good emulsifier and the most frequently used standard for comparing emulsifying performance among proteins [29].

By contrast, all of the FMRPs, except for those treated at pH 12.5, presented lower ES than BSA. This result indicates that the emulsions formed by the FMRPs were vulnerable to environmental conditions. To stabilize the emulsion, proteins generate interfacial repulsion on the water–oil interface [48]. Water-insoluble subunits in PRs may be more efficient in reducing the interfacial tension and stabilising emulsions than the soluble subunits [49] in the FMRPs. Due to increased net charge, the FMRPs may develop frequent molecular rearrangement and result in flocculation of oil droplets.

Table 3 Results of emulsibility, foamability, and DSC

Treatments	Emulsibility		Foamability		DSC data	
	EA (abs.)	ES (min)	FA (mL)	FS (min)	T_P [b] (°C)	ΔH [c] (J/g)
Control	0.25 ± 0.015f[a]	44.69 ± 3.01b	10.6 ± 0.26d	0.81 ± 0.027a	-	-
7.5	0.42 ± 0.015d	23.23 ± 0.12d	9.93 ± 0.71d	0.11 ± 0.021d	93.98 ± 1.22a	2.242 ± 0.20a
9.5	0.48 ± 0.017c	19.56 ± 0.40d	14.57 ± 0.21c	0.21 ± 0.0092bc	91.26 ± 2.01a	1.589 ± 0.17b
11.5	0.53 ± 0.011b	19.79 ± 0.18d	22.47 ± 0.45a	0.19 ± 0.028c	92.87 ± 1.05a	0.561 ± 0.039c
12.5	0.58 ± 0.013a	55.76 ± 6.74a	22.07 ± 0.71a	0.25 ± 0.025b	92.41 ± 1.74a	0.633 ± 0.021c
BSA	0.36 ± 0.018e	29.10 ± 0.44c	-	-	-	-
HEA	-	-	18.50 ± 0.20b	0.22 ± 029bc	-	-

[a] Different letters in the column indicate significant ($P < 0.05$) differences among treatments. Values are expressed as mean ± SD (n = 3).

[b] T_P: Peak temperature.

[c] ΔH: Denaturation enthalpy.

Foaming activity (FA) and foam stability (FS): Protein-based foams are obtained based on the air–water interface-orienting diffusion, rapid conformational changes, and successive rearrangement at the interface. As a prerequisite for foam formation, proteins should solubilize in the aqueous phase and rapidly unfold into a cohesive layer of protein around gas/air

droplets [50]. The foaming results are presented in Table 3. The FA values of the control (10.6 mL) and the FMRPs treated at pH 11.5 and 12.5 (22.47 and 22.07 mL, respectively) were significantly lower or higher than that of HEA (18.5 mL), respectively. HEA is the most ubiquitously used standard for comparing the foaming properties among proteins [51]. The FMRPs had more flexible random-coiled structures, which may be attributed to the loss of complex secondary (β-sheet) structures. Therefore, the FMRPs may have undergone rapid conformational changes at the air–water interface with reduced surface tension, explaining their higher FA than the control.

The FS of the FMRPs was comparable or superior to that of HEA. The foaming results also suggest that the FMRPs can be used as a foaming agent in the food additive industry. However, the FMRPs presented interior FS to the control. FS requires the formation of a thick, cohesive, and viscoelastic film surrounding each gas bubble [29]. Protein-protein interactions of the FMRPs may be weakened due to reduced aggregation. Consequently, the air bubbles formed by the FMRPs were fragile and low FS was generated.

DSC: Fig. 3b shows the DSC thermograms of the FMRPs, and Table 3 shows the analytical data. The denaturation peak temperature (T_p) indicates the thermostability of proteins, and the enthalpy value (ΔH) correlates with the proportion of un-denatured protein or the content of ordered protein structure [52]. The FMRPs exhibited major endothermic peaks with T_p of 91.26–93.98 °C and ΔH of 0.633–2.242 J/g. The T_p values of the FMRPs were markedly higher than those of RPs prepared by enzyme-assisted microfluidization (66.46 °C) [22] and rice bran protein isolates prepared by enzyme extraction (83.4 °C) [29]. The high thermostability of the FMRPs may be attributed to their high content of disulfide bonds, which improve the thermostability of numerous proteins (Table 1) [53]. The control may have been completely denatured [54] during RP preparation, and the resultant protein molecules presented less ordered structures when hydrated. As a result, no endothermic peaks appeared. In this regard, solubility was facilitated because the FMRPs unfolded and became susceptible to forming high-ordered conformation in aqueous media, which promoted water–protein interactions.

Conclusions

We investigated the effects of freeze-milling on RPs. Freeze-milling may have exerted profound mechanical energy on RPs and facilitated the unfolding of these proteins. The unfolded conformation exposed buried functional groups to the solvent, which strengthened the water–protein interactions. Therefore, the solubility of the RPs was enhanced. Treatments involved in freeze-milling are commercially accessible, and therefore the protocol may be economically feasible for the preparation of water-soluble RPs. In addition, the emulsifying and foaming activities of the FMRPs were significantly higher than those of the control and were even superior to those of BSA and HEA, respectively. Unfolding-mediated improvements in HLB and conformational flexibility may have contributed to the advantageous emulsifying and foaming activities of the proteins, respectively. The data suggest that the FMRPs are potential functional agents.

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Notes and references

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