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The effect of rice bran stabilization on solubility and molecular weight distribution of protein fraction

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Abstract

This research was conducted through following stages stabilization of rice bran, defatting of rice bran, isolation of rice bran protein, protein fractionation, and molecular weight estimation using SDS PAGE. The percentage of soluble protein fraction of albumin, globulin, and glutelin (NaOH-soluble) of rice bran protein concentrate of stabilized rice bran (SRB) with no die double screw extruder higher than unstabilized rice bran (URB). Protein content of URB protein concentrates and SRB protein concentrates of Pandanwangi and Ciherang were 60.76%, 61.38%, 60.19%, and 60.23% respectively. The percentage of soluble protein of acid-soluble glutelin of Pandanwangi and Ciherang rice bran protein concentrate higher than that in albumin fraction. The percentage of soluble protein fraction of NaOH-insoluble protein of rice bran protein concentrate of SRB lower than URB. Protein fractionation resulted in glutelin acetic acid-soluble types α-glutelin (30-39 kDa) and β-glutelin (19-25 kDa). SDS PAGE of SRB protein concentrate fractions showed altered quantitatively and qualitatively due to SRB.

Introduction

Rice bran is an inexpensive and under-utilized by product of rice milling industries. Rice bran has a high nutritional value with 12-15% protein containing essential amino acids (Wang *et al*., 1999). Several studies have reported the potential of rice bran as a functional food (Kahlon *et al.*, 1992; Ryan, 2011) and protein sources (Wang *et al.*, 1999; Jiamyangyuen *et al*., 2005; Zhang *et al*., 2012).

The use of rice bran in food product is limited because the bran becomes easily rancid. Its instability caused by hydrolytic and oxidative rancidity. It is necessary to stabilize the bran in order to inactivate lipase and lipoxygenase. Lipase in the rice bran hydrolyzes triglycerols. Lipoxygenase specifically oxygenates polyunsaturated fatty acids and/or their esters and acylglycerols containing the cis, cis-1,4 pentadiene double bound system located between carbons 6-10 counting from the methyl terminus (Shastry and Rao, 1975). It causes off-flavor in food because of its reaction with unsaturated fatty acids.

Heat stabilization of rice bran has been reported by various methods, such as using parboiled (da Silva *et al.*, 2006), steam (Pourali *et al*., 2009), a single screw extruder (Malekian *et al.*, 2000; Kurniawati *et al*., 2014) and double screw extruder (Fuh and Chiang, 2001; Ubaidillah, 2010; Kusumawaty *et al*., 2013). Furthermore, Kusumawaty *et al*. (2013) obtained the optimum conditions for rice bran stabilization using no die double screw extruder that produced rice bran with higher oxidative stability. Protein concentrates from stabilized rice bran has the functional properties (emulsion and foam formation) similar to that of unstabilized one. However, some other properties such as amino acid composition, stability to heat, and the IR spectra were slightly different. Further study is needed to study the protein fractions of rice bran protein concentrates.

Research on rice bran extrusion and its protein fractions were limited and insufficient. Heating bean during the extrusion process are reported and may form and result in breaking of covalent bonds and non-covalent bonding (Shah, 2003) that will affect the protein solubility. Rice bran protein fractionation can be done by using a water to obtain fractions of albumin, 2% NaCl to obtain globulin fraction, 70% ethanol to obtain prolamin fraction, 0.1 M acetic acid and 0.1 M NaOH to obtain the glutelin fraction. Percentage of total protein (Kjeldahl) of albumin

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fraction of rice bran protein is the highest followed by globulin fraction, glutelin, and prolamin (Hamada, 1997). Different results were reported by Chanput *et al.* (2009) that the highest percentage of the total protein in the protein fractions of rice bran is the glutelin fraction, followed by the fraction of albumin, globulin, and prolamin. This difference may be due to differences in varieties of rice bran. According to Santos *et al.* (2013), differences in rice varieties affect albumin and glutelin fractions. This research aims to study the effect of rice bran stabilization on soluble and molecular weight distribution of protein fraction were fractionated from rice bran protein concentrates.

Materials and Methods

Materials

Paddy cultivars Ciherang and Pandanwangi were obtained from Paddy Milling Unit in Sumedang. Protein marker 7.5-230 kDa (Biorad) and other chemicals were of analytical grade.

Preparation of stabilized rice bran

Paddy was dehulled by a Yanmar Rice Huller (Model HW-60A, Yanmar Diesel Engine co. Ltd. Osaka, Japan) and debranned by a Satake Polisher for 60s to result in rice bran. Rice bran was stabilized with no die double screw extruder (Berto Industries) according to the method of Kusumawaty *et al.* (2013). This treated rice bran was called stabilized rice bran (SRB), while the control was called unstabilized rice bran (URB).

Preparation of rice bran protein concentrate (RBPC)

Rice bran was defatted twice using hexane (1:5 w/v) and the resulting defatted rice bran was suspended in distilled water (1:10 w/v). The pH of slurry was set at 8 using 5 N NaOH, stirred for 2 h at 50-55°C and centrifuged at 9.096 g (6000 rpm) for 15 min. The supernatant was adjusted to pH 4 using 5 N HCl, stirred for 30 min and allowed overnight for cold precipitation (4^{o}C). The supernatant was then decanted off and the precipitated was washed twice with 30% alcohol by centrifuging at 9.096 g for 15 min. The protein slurry was then resuspended in distilled water and netralized to pH 6.0. The slurry was kept at -20^{o}C before freeze drying (Zhang *et al*., 2012 with some modification). This product was called rice bran protein concentrate (RBPC). Protein contents were determined by the Kjeldahl method (conversion factor of 5.95) (AOAC, 2005).

Protein fractionation

Protein fractionation was performed according to the method of Hamada *et al*. (1997) with a slight modification. The method was based on the classical Osborne protein fractionation procedure. RBPC (10 mg) was extracted with 1 mL of deionized water by vortex for 5 min at room temperature followed by centrifugation at 20.021 g (11.000 rpm) at 4°C for 10 minutes.The residue from this step was extracted with 1 mL of 5% NaCl to obtain the globulin fraction, then the residue was further extracted with 70% ethanol to obtain the prolamin fraction. The residue after prolamin extraction was extracted with 0.2 M acetic acid to obtain glutelin fraction, then the residu was extracted with 0.1 N NaOH to result in the glutelin fraction. To improve the yield of the protein fractions, each extraction step was repeated twice and washing with deionized water to remove residual previous solvent. Protein fractions were stored at -20°C until analyzed. Soluble protein content in each fraction was measured using the Lowry method (1951). The percentage of soluble protein and insoluble protein residue calculated from the respective levels of the protein in the protein concentrate.

Analysis of molecular weight distribution

Analysis of molecular weight distribution of protein fraction was carried out by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS–PAGE was run using a 7.5-17.5% stacking gel and gradient separating gel. Twenty µL of protein fraction was mixed with bufer Laemlli (1:1 v/v), boiled 2 minutes then loaded into the well. Gels were run at 100 V for approximately 180 min and stained with 0.125% coomassie brilliant blue R-250 and destained with 25% ethanol and 10% acetic acid. The molecular weight of protein was estimated using protein marker ranging from 7.5 to 230 kDa (Board Range Catalog 161-0318 Biorad Prestained Molecular Standar). The protein bands of the destained gel were qualitated using ImageJ software 1.47. The molecular weight distribution of protein were calculated by plotting the log of the molecular weight of protein standards against the relative migration.

Statistical analysis

Test was performed 2 replications and values in average. Differences between treatments were analyzed by t-test on SPSS 16.0 software with a significance of $P < 0.05$ between the average value.

Results and Discussion

Protein content and yield of rice bran protein concentrate

The protein content and yield of RBPC from unstabilized rice bran (URB) and stabilized rice bran (SRB) Pandanwangi and Ciherang were presented in Table 1. The yield of protein concentrate from SRB was lower than that of from URB. This finding was in line with those reported by Gnanasambandam and Heitiarachchy (1995), that protein denaturation due to commercial heat stabilization caused the decreased in its solubility and thus impaired protein extractability.

Protein fractions in rice bran protein concentrate

Protein fractionation from RBPC consisted of 5 fractions, namely the water-soluble fraction of albumin, the NaCl-soluble fraction of globulin, the alcohol-soluble fraction of prolamin, the acetic acid-soluble fraction of glutelin, the NaOH-soluble fraction of glutelin, and the NaOH-insoluble residue. Soluble protein in the supernatant of each fraction was analyzed by the Lowry method. The percentage of soluble and insoluble protein residue was calculated from the respective levels of the protein in the protein concentrate (Table 2).

Tables 2 shows that the percentage of soluble protein fraction of albumin, globulin, and glutelin (alkali-soluble) of RBPC from SRB was higher than that from URB. Soluble protein fraction of NaOH-insoluble protein of RBPC from SRB was lower than that from URB. According to Shah (2003), extrusion may result in breaking and re-binding of covalent and non-covalent bonds, and the formation of protein aggregates owning negative or positive charge. This results show the importance of optimization of temperature and screw speed in rice bran stabilization.

High levels of soluble protein in the glutelin fraction maybe caused by the use of solvent 0.2 M acetic acid (pH 3) and 0.1 M NaOH (pH 12.5) in the fractionation process. According to Wang et al. (1999), there was increased protein solubility in strong acidic and alkaline condition.The percentage of soluble proteins in the glutelin fraction of Pandanwangi RBPC was higher significantly than that from Ciherang. The percentage of soluble protein in the albumin and glutelin fractions showed significant differences between the varieties.

Chanput *et al.* (2009) reported that the levels of total protein in the rice bran proteins from highest was glutelin fraction (22.7%), albumin (21.6%), globulin (17.4), and prolamin (8.1%). Different results were reported by Hamada (1997) that the levels of total protein (Kjeldahl) in the rice bran proteins from highest was albumin fraction (32-39.5%), globulin (12.8-17.45), acetic acid soluble glutelin (6-14.7%) ,and prolamin (5.3-7.8%). Approximately 24.2-42.2% residual protein soluble in NaOH.

Table 1. Protein content and yield of RBPC

Rice Bran Protein Concentrate	Protein (%)	Yield (%)
URB Pandanwangi	60.76	23.45
SRB Pandanwangi	60.19	16.80
URB Ciherang	61.38	21.63
SRB Ciherang	60.23	16.61

URB, unstabilized rice bran; SRB, stabilized rice bran

Tables 2 shows that the percentage of soluble protein fractions albumin, globulin, and glutelin protein concentrate of SRB higher than URB. According to Shah (2003), extrusion may result in breaking and re-binding of covalent and non-covalent bonds, and the formation of protein aggregates that negative or positive charge. This shows the importance of be optimized of the temperature and screw speed in the process of stabilization of rice bran

High levels of soluble protein in the glutelin fraction maybe caused by the use of solvent 0.2 M acetic acid (pH 3) and 0.1 M NaOH (pH 12.5) in the fractionation process. According to Wang et al. (1999), increased protein solubility in acidic and alkaline extremes. The percentage of soluble proteins in the glutelin acid-soluble fraction of Pandanwangi protein concentrate is higher than Ciherang and showed a significant difference. The percentage of soluble protein in the albumin and glutelin showed significant differences between the varieties.

Chanput *et al*. (2009) reported that the levels of total protein (Kjeldahl) in the bran proteins from highest to lowest is glutelin fraction (22.7%), albumin (21.6%), globulin (17.4), and prolamin (8.1%). Different results were reported by Hamada (1997) that the levels of total protein (Kjeldahl) in the bran proteins from highest to lowest albumin fraction(32-39.5%), globulin (12.8-17.45), acetic acid soluble glutelin (6-14.7%), and prolamin (5.3-7.8%). Approximately 24.2-42.2% residual protein soluble in NaOH.

Cao *et al.* (2009) reported that the levels of total protein in the albumin fraction (42.71% +2.47) and glutelin fraction (soluble in NaOH) (40.25% +2.55) from different rice bran protein were not significant. It showed that the percentage of the total protein content of the albumin and glutelin fraction of rice bran protein concentrate varied. This result was supported by Santos *et al.* (2013) that rice varieties affected the protein levels of albumin and glutelin fractions.

For qualitative profile of protein fractions from each RBPC can be seen through molecular weight by

Table 2. The percentage of protein in rice bran protein fractions

Rice Bran Protein Concentrate	Albumin (%)	Globulin (%)	Prolamin (%)	Glutelin-acid soluble (%)	Glutelin – alkali soluble (%)	NaOH-insoluble protein (%)
URB Pandanwangi	14.3±0.01[a]	4.7±0.01[a]	2.5±0.0002[a]	51.6±0.01[a]	7.1±0.005[a]	19.8±0.02[a]
SRB Pandanwangi	15.6±0.001[a]	6.6±0.01[b]	2.3±0.002[a]	53.1±0.032[a]	8.8±0.01[a]	13.7±0.01[a]
URB Ciherang	12.4±0.08[b]	5.6±0.09[a]	2.8±0.08[a]	40.0±0.05[b]	3.4±0.02[b]	35.7±0.02[b]
SRB Ciherang	14.8±0.03[b]	9.80±0.08[d]	2.7±0.02[a]	40.0±0.01[b]	8.6±0.02[c]	24.1±0.01[c]

[a,b,c,d] *Figures in the same column followed by different letters indicate significant differences (p<0.05)*

SDS PAGE. In this study, rice bran protein isolation used water as a solvent, therefore the supernatant fraction of alcohol-soluble (prolamin) was not further analyzed.

Molecular weight distribution of protein fraction

The results of SDS PAGE analysis showed the effect of stabilization on the molecular weight distribution of the rice bran protein fraction of albumin (Figure 1), globulin (Figure 2), and glutelin (soluble in acetic acid) (Figure 3) of RBPC. Santos *et al.* (2013) reported that rice protein containing albumin with a molecular weight in the range of 15-96 kDa to 18-20 kDa as major polypeptide (Cao *et al.*, 2009) and globulin with a molecular weight in the range of 23-118 kDa. Glutelin differentiated into α-glutelin (30-39 kDa) and β-glutelin (19-25 kDa).

According to Santos *et al.* (2013), β-glutelin has a bad resolution in SDS PAGE, so the protein bands can not be separated. In this study, analysis of the molecular weight of each fraction was performed using gradient gel with 7.5-12.5% in the stacking gel and the separating gel. This method is used to obtain a clear resolution of glutelin separation and prevents the migration of protein in a homogeneous gel. Analysis SDS PAGE showed that the protein fractions of RBPC of Pandanwangi SRB indicated that the albumin fraction contained protein with a molecular weight of 17 kDa and 30.90 kDa. Globulin fraction showed no band. Glutelin fraction (acid soluble) showed protein bands with a molecular weight of 11.44 kDa, 13.92 kDa, 15.86 kDa, 21.99 kDa, 30.48 kDa, 37.08 kDa, 48.14 kDa and 66.74 kDa (band 8).

Analysis of the protein fractions of RBPC of Pandanwangi SRB showed that albumin fraction contained proteins with a molecular weight of 17 kDa, 30.90 kDa and 32.96 kDa. Globulin fraction contained proteins with a molecular weight of 48.53 kDa and 76.21 kDa.The results of SDS PAGE analysis showed differences in the band pattern of each protein fraction. Effect of stabilization seen in the albumin and globulin fractions because there was

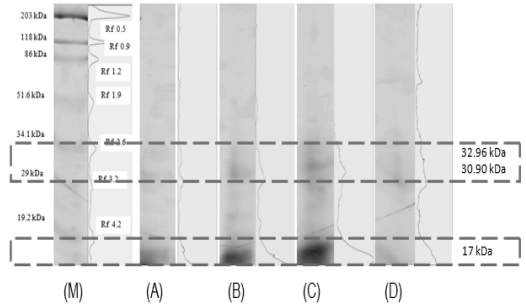

Figure 1. Electrophoretogram (A) URB Pandanwangi albumin fraction, (B) URB Ciherang albumin fraction, (C) SRB Pandanwangi albumin fraction, (D) SRB Ciherang albumin fraction, and (M) molecular weight of protein standards

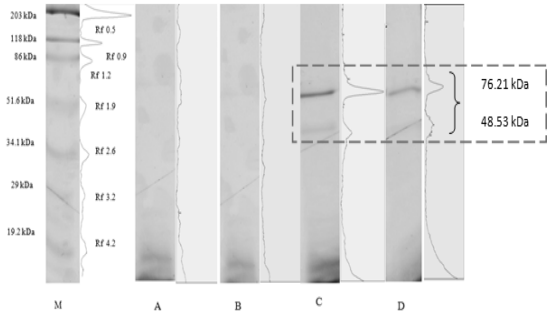

Figure 2. Electrophoretogram (A) URB Pandanwangi globulin fraction, (B) URB Ciherang globulin fraction, (C) SRB Pandanwangi globulin fraction, (D) SRB Ciherang globulin fraction, and (M) molecular weight of protein standards

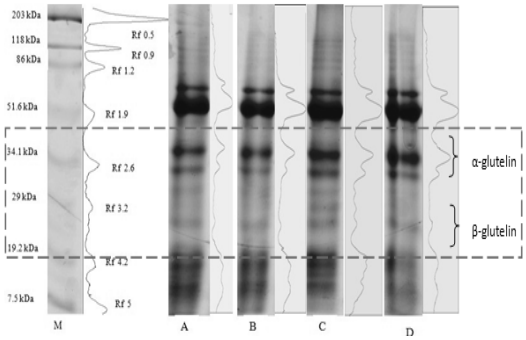

Figure 3. Electrophoretogram (A) URB Pandanwangi prolamin fraction, (B) URB Ciherang prolamin fraction, (C) SRB Pandanwangi prolamin fraction, (D) SRB Ciherang prolamin fraction, and (M) molecular weight of protein standards

a presence of the protein band which had a greater molecular weight.The presence of the protein band also showed in glutelin fractions of Pandanwangi SRB in the range of 21-30 kDa. According to Ummadi *et al.* (1997) and Shah (2003), extrusion may cause breaking disulfide bonds leading to depolymerization which results in lower molecular weight protein.

The presence of the protein bands in the glutelin fraction showed the formation of low molecular weight proteins (<65 kDa) that might be caused by depolymerization. This suggests that the extrusion temperature settings and contact time on the given material affect the formation and breaking disulfide bonds. The use of high temperatures can increase the formation of disulfide bonds resulting in lower protein solubility and produce more insoluble protein (Ummadi *et al.*, 1997). However, it still need further study related to the possibility of polymerization and depolymerization protein.

Protein fractionation resulted in glutelin acetic acid-soluble types α and β. Protein fractionation in a NaOH solvent did not produce bands due to the low levels of soluble protein (results not shown). This may be due to many soluble proteins in acetic acid solvent. Research in the α-and β-glutelin protein in rice bran is very limited compared to the rice protein. Differences in the levels of soluble protein and protein molecular weight distribution in the glutelin fraction between varieties in line with those reported by Khan *et al.* (2010) that the rice bran varieties affect the intensity and distribution of molecular weight glutelin.

Conclusions

Fractionation of proteins from RBPC from SRB yielded five protein fractions, namely albumin, globulin, prolamin, glutelin acid-soluble, and glutelin alkali-soluble. Analysis of soluble protein content and molecular weight profile of each protein fraction showed that stabilization of rice bran effect on albumin, globulin, and glutelin in rice bran protein concentrate is indicated by an increase in soluble protein in each fraction and more band in SDS PAGE.

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