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Separation and molecular mass distribution of rice proteins by size-exclusion high-performance liquid chromatography in a dissociating buffer

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Abstract

Rice bran proteins were isolated using a strongly dissociating buffer containing 0.1 *M* acetic acid, 3 *M* urea, and 0.01 *M* cetyltrimethylammonium bromide (AUC). Proteins were eluted by AUC from a diol-coated silica gel (30 cm×20 mm I.D.) with exclusion limit of $1.5 \cdot 10^5$. The size-exclusion high-performance liquid chromatography procedure was optimized for the preparative separation and quantitative determination of the relative proportions of the major size classes of the polypeptides of rice proteins from several varieties. Resolved peaks contained 8, 17, 11, 13, 14, 7, 13, 8, 5 and 6% of the injected protein and had relative molecular masses of 1.05, 0.84, 0.54, 0.39, 0.29, 0.23, 0.14, 0.07 and $<0.07 \cdot 10^5$, respectively. This reproducible separation procedure, based on size exclusion in the presence of a detergent and a chaotropic agent, allows the determination of the molecular mass distribution of rice proteins and further fractionation and characterization of the well-defined, individual polypeptides.

Keywords: Proteins; Molecular mass determination

1. Introduction

Poor solubility of rice proteins is due to the presence of a substantial amount of insoluble glutelin polypeptides. However, the exact reasons for the insolubility of rice glutelin are unknown. Many investigators believe that extensive aggregation [1], disulfide bonding and glycosylation [2] are among the forces responsible for the insolubility of the rice glutelin fraction. To allow the study of structure of rice proteins and understand the reason for their insolubility, a thorough characterization of these proteins is needed. This requires the availability of rice proteins in a pure, unreduced form. Effective, efficient methods have not been developed for isolating and solubilizing rice proteins, nor have the proteins, particularly the glutelin fraction, been well-characterized.

Size-exclusion chromatography separates molecules on the basis of their molecular size. Iwasaki et al. [3] characterized the two soluble rice proteins, albumin and globulin, by gel-permeation on Sephadex G-100 [4]. Relative molecular masses (M_r) of albumin ranged from $1.0 \cdot 10^4$ to $2 \cdot 10^5$ and three out of four globulins ranged from $1.6 \cdot 10^4$ to $1.3 \cdot 10^5$. Snow and Brooks [5] used Sepharose CL-6B [6] to fractionate the polypeptides of rice glutelin in 8 *M* urea or 0.5% SDS in Tris buffer (pH 9.0) into four broad unresolved fractions that eluted over a very wide range of M_r. The majority of the glutelin polypeptides was still aggregated with relative molecular mass up to $4 \cdot 10^6$, based on the fractionation range of the gel column (M_r range $1 \cdot 10^4$–$4 \cdot 10^6$). This was a major obstacle for the separation of

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polypeptides of rice glutelin into well-resolved size classes. Therefore, there is a need to use a strongly dissociating solvent containing both a detergent and a chaotropic agent to isolate and fractionate rice proteins. This may provide well-defined protein fractions with their established molecular size identities. Meredith and Wren [7] used the dissociating solvent AUC (acetic acid, 0.1 *M*; urea, 3 *M*; cetyltrimethylammonium bromide, 0.01 *M*) to fractionate wheat proteins on Sephadex G-200 gel [4] into glutenin, gliadin, albumin, and nonprotein nitrogenous material. Hamada et al. [8] used AUC solvent and Sephadex G-150 [4] to fractionate the proteins of several varieties from the hard red spring (HRS) class of wheat. Separated wheat protein fractions were as follows: glutenin (first peak), $M_r > 1 \cdot 10^5$; gliadin (second peak), M_r $1 \cdot 10^5 - 2.5 \cdot 10^4$; albumins (third peak), M_r $2.5 \cdot 10^4 - 1 \cdot 10^4$; and nonprotein nitrogenous material, to $< 1 \cdot 10^4$. Ma [9] characterized alkali-extracted protein concentrates from two oat cultivars by size-exclusion chromatography on Sephacryl S-200 [4], using AUC as eluting buffer. Chromatograms showed that five peaks were resolved with apparent relative molecular masses ranging from $9.5 \cdot 10^4$ to less than $1.0 \cdot 10^4$. The chromatograms of oat protein concentrates from the two cultivars were not markedly different.

In this paper, we used HPLC to develop a new chromatographic procedure for the separation of rice proteins, based on size exclusion in the presence of a detergent and chaotropic agent. The objectives of this research were: (1) to optimize the isolation of the major polypeptides in rice bran proteins by large-scale preparative purification techniques using HPLC; and (2) to determine and compare the molecular mass distribution of proteins in six different varieties.

2. Experimental[1]

2.1. Materials

Six bran samples from six different varieties were used in this study. Rice varieties were Bengal, Cypress, Della, Mars, Maybelle and Toro-2. Unless noted otherwise, laboratory-grade chemicals and reagents as well as protein markers were purchased from Sigma (St. Louis, MO, USA). Aldolase was purchased from Pharmacia LKB Biotechnology (Piscataway, NJ, USA).

2.2. Preparation of defatted rice brans

To 10.0 g of bran sample, 50 ml ethyl ether was added in a 60-ml Sorval Omni Mixer Cup and the suspension was homogenized using a 10-mm sawtooth blade assembly at 4000 rpm and 20°C for 1 h. Soluble fat was removed by centrifugation at 5000 *g* at 20°C for 20 min. Bran was extracted again with 30 ml ethyl ether using the same method. Defatted bran was left overnight in the hood to remove the residual solvent.

2.3. Protein extraction

A strongly dissociating solvent composed of 0.1 *M* acetic acid, 3 *M* urea, and 0.01 *M* cetyltrimethylammonium bromide (AUC) was used to extract the proteins in a rice bran sample. Ten milliliters of AUC were added to 1.0 g rice bran in a 60-ml Sorval Omni Mixer Cup and the suspension was homogenized using a 10-mm sawtooth blade assembly (to avoid foaming) at 12 000 rpm and 20°C for 3 min. The solubilized protein was recovered after centrifugation at 30 000 *g* at 20°C for 30 min. Bran was washed four times with deionized water using the same method of homogenization and centrifugation described above. The bran precipitate was left overnight in the hood to dry for later protein analysis. Aliquots of AUC-extracted proteins were filtered through a 0.45-μm Millex-HV Filter (Millipore, Bedford, MA, USA) before their injection.

2.4. HPLC analysis

The preparative chromatography system 'Delta Prep 3000' from Waters (Div. of Millipore, Milford, MA, USA) was used for this investigation. Delta Prep 3000 consisted of a solvent delivery system operated by a system controller and sample injection by a Rheodyne 7012 injector with a 5-ml loop. Elution with AUC buffer was monitored at 280 nm

[1] Commercial firms are mentioned in this publication solely to provide specific information. Mention of a company does not constitute a guarantee or warranty of its products by the U.S. Department of Agriculture nor an endorsement by the Department over products of other companies not mentioned.

by a Model 481 Lambda-Max spectrophotometer detector connected to a Baseline 810 chromatography workstation (Waters) for the identification and the integration of the proportions of various eluted peaks. Fractions were collected every three minutes using a Foxy fractionator (ISCO, Lincoln, NE, USA).

2.5. Size-exclusion chromatography

AUC-extracted proteins from six different varieties were separated by GP-HPLC on a 30 cm×20 mm I.D. Shodex 'Protein WS-2003', steel column packed with a bonded diol-coated silica gel (Waters), using AUC as eluent. This prepacked column has an effective fractionation range of 1000 to $1.5 \cdot 10^5$. Two levels of injections (0.3 and 0.6 ml) and flow-rates (0.5 and 1.0 ml/min) were used to study the effect of sample load and flow-rate on elution volume, protein content, and % peak area. Aliquots of the six injected samples (600 μl in AUC buffer) were eluted with AUC buffer at a flow-rate of 0.5 ml/min. Fractions were pooled based on the integrated peaks of the chromatogram containing more than 5% of the total protein. Pooled fractions were kept at −20°C until analyzed.

2.6. Calibration of size-exclusion column

Blue Dextran (M_r 2 000 000) was injected at 2.0 and 4.0 mg levels to determine the void volume of the column. Two levels of protein markers mixture (5 and 10 mg in 0.6 ml AUC solvent) were used to calibrate the column. The apparent relative molecular masses of these proteins as listed by the manufacturer, Sigma (St. Louis, MO, USA) are indicated in parentheses after each protein. In Fig. 4, standard proteins included aldolase (158 000), alcohol dehydrogenase (141 000) [10], bovine serum albumin (BSA) (15% dimer of 133 000 and 85% 66 400 monomer) [11], ovalbumin (45 000), glyceraldehyde-3-phosphate dehydrogenase monomer (36 000), carbonic anhydrase (29 000), trypsinogen (24 000), soybean trypsin inhibitor (20 100), bovine milk lactalbumin (14 200), cytochrome C (12 400), and aprotinin (6500).

2.7. Protein analysis

The protein contents of brans, AUC extracts, and pooled peaks were measured by the macro or micro method of Lowry et al. [12] using 'DC protein assay reagent' from Bio-Rad Laboratories (Hercules, CA, USA). Protein content of brans was determined also by a standard Kjeldahl method using 5.95 as a nitrogen conversion factor.

2.8. Statistical analysis of variance

Multi-factor analysis of variance (ANOVA) of the variables, determined in duplicates, was performed using the software package of Statgraphics (Rockville, MD, USA), driven from the statistical analysis system described by Barr and Goodnight [13]. Tukey's multi-range test ($P=0.05$) was used to compare the means of the different levels of each factor and to group the levels of a factor together if the different levels were not significantly different, i.e. homogeneous.

3. Results and discussion

3.1. Protein extraction

Ethyl ether was used to extract the fat from brans prior to protein extraction. The amount of fat that was removed from the brans of Bengal, Cypress, Della, Mars, Maybelle and Toro-2 were 19, 16, 19, 23, 21 and 22%, respectively. Rice proteins were prepared by extraction of defatted rice bran by AUC buffer. An average of 80% and a range of 67–94% of the proteins of defatted brans from six different varieties were extracted (Table 1). This solvent extracted most proteins of Cypress but was unable to extract 16–33% of proteins of the other varieties. This could be due to differences in the protein conformation of the varieties. AUC is a strongly dissociating solvent but it could not overcome the forces responsible for the insolubility of the high molecular mass protein fractions in some varieties, especially Toro-2, in which up to one third of the proteins were insoluble. Evidently, the AUC-in-

Table 1
Extraction of rice bran proteins from several varieties using the strongly dissociating buffer AUC[a]

Variety	% Total protein	Extracted protein (mg/100 mg)	% Extraction
Bengal	16.4	12.9	79
Cypress	16.3	15.3	94
Della	15.7	13.1	84
Mars	16.7	13.4	80
Maybelle	16.7	12.7	76
Toro-2	15.2	10.1	67
Average	16.1	13.0	80

[a] Buffer contained 0.1 *M* acetic acid, 3 *M* urea, and 0.01 *M* cetyltrimethylammonium bromide.

solubility of the high molecular mass protein fractions in these varieties is due to forces other than aggregation.

Extensive cross-linking through disulfide bonds is the likely force responsible for protein insolubility since AUC used by Meredith and Wren [7] was very effective in solubilizing up to 98% of wheat proteins. Protein insolubility was also obvious in some wheat classes such as Hard Red Spring wheats. In certain types of wheat, AUC solubilize a smaller percentage of the protein, e.g., 77–91% [14]. Further, it is possible that some of the unextracted protein may be in the form of lipid–protein complex as some lipids, phospholipids, are not ether-soluble. Although the impact of residual lipids may be negligible, a polar solvent such as *n*-butanol should be tried to compare the fat extraction using both solvents.

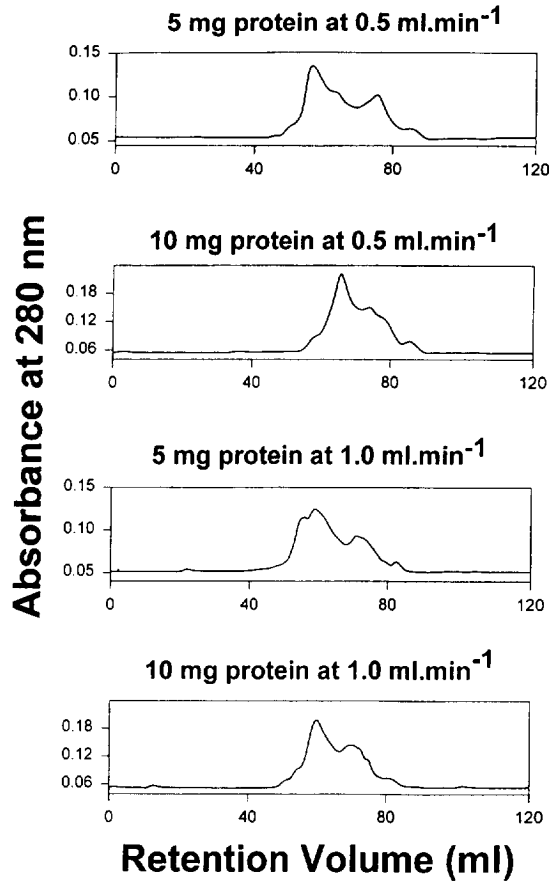

Fig. 1. Size-exclusion (GP) HPLC separation of Cypress bran proteins in the highly dissociation buffer AUC with two levels of protein loads (5 and 10 mg protein) and flow-rates (0.5 and 1.0 ml/min).

3.2. Effect of protein load and flow-rate on size-exclusion HPLC separation of a bran sample

Since the extraction of Cypress bran was the highest (94%), it was used for the optimization of a size-exclusion (GP) HPLC procedure, for the preparative separation and quantitative determination of the proportions of the major size classes of the polypeptides of rice protein. The separation of the AUC-extracted bran proteins from Cypress on the gel column in AUC buffer at 0.5 ml/min is presented in Fig. 1. Using this GP column under the separation conditions (the osmotic pressure caused by AUC), it was determined that 0.6 ml was the maximum amount of sample load that could be used without damaging the gel. This 0.6-ml injection contained 10 mg protein. GP-HPLC separation of this sample gave nine peaks that contained 10, 14, 9, 17, 15, 18, 7, 6 and 5% of the injected proteins. The % area for these peaks were 4, 5, 11, 24, 37, 16, 4, 0.3, and 0.2%, respectively (Table 2).

The effects of sample size (5 and 10 mg protein loads) and flow-rate (0.5 and 1.0 ml/min) on recovery of each fraction, expressed as % protein and % peak area, and on retention volume (V_e) were investigated. Runs of 5-mg loads at 0.5 ml/min

flow-rate and runs of 5- or 10-mg loads at 1.0 ml/min flow-rate (Fig. 1) gave also the same number of peaks described above for the 10-mg protein load and 0.5 ml/min flow-rate. Table 2 presents the elution volumes and the proportions of the peaks eluted from the gel column by AUC at the two levels of flow-rates and protein loads. Statistical analysis of variance revealed that % area and % protein for the peaks of each sample were independent of flow-rate and protein load. However, protein load and flow-rate significantly affected V_e. Increasing flow-rate increased the retention of the peaks by 9%. Protein injection load increased the V_e values of the peaks by 6% (Table 2). Vlaanderen et al. [15] investigated the effect of protein load and flow-rate on the HPLC fractionation of crystallins on Superose-6 (fractionation range $1.0 \cdot 10^4$ to $4 \cdot 10^6$ according to the manufacturer: Pharmacia LKB Biotechnology). They did not observe any effects of flow-rates ranging from 0.15 to 0.5 ml/min at a protein load of 4.4 mg. However, they found that lowering the protein load of injected sample decreased the V_e value and subsequently the determination of M_r. Decreasing protein injection from 175 to 1.75 mg/ml shifted the M_r of one of the peaks from 220 000 to 130 000.

3.3. Separation of proteins of rice brans from several varieties

The procedure of using the dissociating solvent AUC for the extraction and GP-HPLC fractionation of Cypress bran proteins was applied to the other five rice bran samples, which came from the five different varieties listed in Table 1. The GP separation of these samples as well as the Cypress sample at 0.5 ml/min flow-rate and 0.6 ml injection of the extract is presented in Fig. 2. This 0.6-ml aliquot contained 9 mg protein for Cypress, 8 mg protein for Bengal, Della, Mars and Maybelle varieties and 6 mg protein for Toro-2. Runs of the other five varieties showed ten peaks, of which nine peaks had the same elution characteristic as those obtained for Cypress. The proteins of these varieties had one peak that was not observed for the Cypress sample. Table 3 gives the elution volumes of the peaks separated from the bran proteins of these five varieties as well as Cypress. The peak that was missing from Cypress fractions (V_e=69) is numbered as 6 in Table 3. Statistical analysis of variance of rice protein fractions revealed that except for Toro-2, there was no significant difference among varieties in elution profiles includ-

Table 2
Elution volumes and proportions of the peaks of Cypress bran proteins eluted from the gel column using the strongly dissociating buffer AUC at two levels of flow-rate and protein injection

Peak No.[a]	Elution volumes (ml)[b]				Average[c]	
	0.5 ml/min		1.0 ml/min		% Area	% Protein
	5 mg protein	10 mg protein	5 mg protein	10 mg protein		
1	47	50	51	58	4[e]	10[c]
2	50	53	54	61	5[e]	14[b]
3	53	57	57	63	11[d,e]	9[c]
4	57	60	61	68	24[b]	17[a]
5	60	64	66	72	37[a]	15[b]
6	68	70	74	78	16[c]	18[a]
7	78	80	84	86	4[e]	7[d]
8	86	88	93	96	0.3[f]	6[d]
9	94	98	100	104	0.2[f]	5[d]

[a] Nine homogeneous groups for the nine peaks.
[b] Two statistical homogeneous groups for the effect of flow-rate and two homogeneous groups for the effect of load. Statistical homogeneous groups are based on multiple-range analysis.
[c] Statistical homogeneous groups are based on multiple-range analysis.

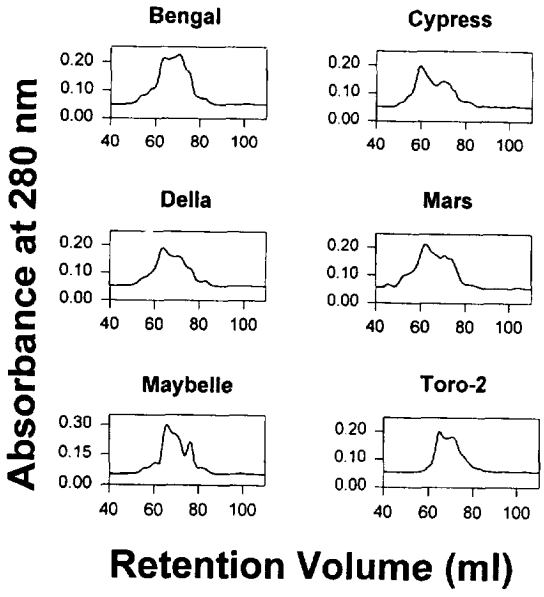

Fig. 2. Polypeptide peaks of bran proteins from several varieties separated by GP-HPLC in the highly dissociation buffer AUC.

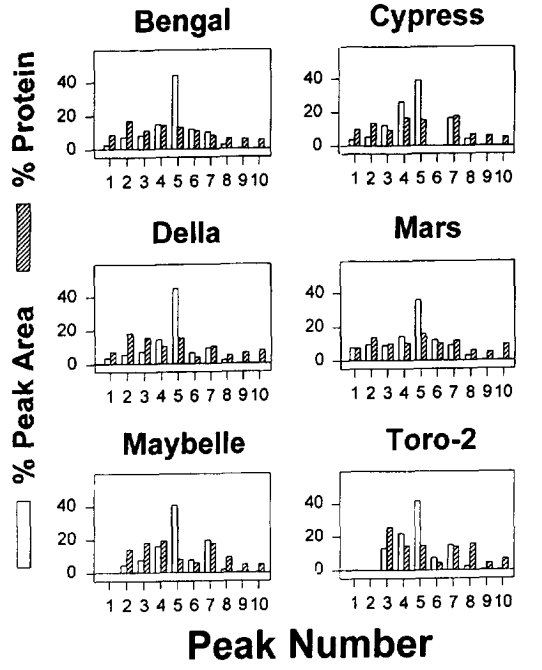

Fig. 3. Percent of peak area and protein of GP-HPLC polypeptide peaks of bran proteins from several varieties.

ing the start, end, retention and pooled volumes of the ten peaks. Elution volumes for Toro-2 peaks were smaller than other peaks. This could be due to the effect of protein load on retention volume since protein injection here was lower than all other samples.

As with Cypress, fractions were also expressed as percent of peak area and protein (Fig. 3). The % area and % protein were highly correlated and were not significantly different from each other if compared together. However, values of area and protein of each peak were compared separately in the following statistical analyses. Except for peaks 1, 8, 9 and 10, resolved peaks varied significantly in their peak areas

Table 3
Elution volumes of the peaks of bran proteins from several varieties as eluted from the gel column using the strongly dissociating buffer AUC

Peak No.	Elution volumes (ml)						
	Bengal	Cypress	Della	Mars	Maybelle	Toro-2	Average[a]
1	50	50	51	50	–	–	50
2	55	54	55	55	53	–	54
3	58	57	57	58	58	56	57
4	60	60	60	60	61	59	60
5	66	64	66	64	65	62	64
6	68	–	69	69	69	66	69
7	71	71	70	72	72	70	71
8	78	79	79	80	79	74	79
9	90	89	91	92	91	88	90
10	98	98	99	100	99	97	98

[a] Multiple-range analysis for elution volume by peak placed peaks into ten homogeneous groups.

and protein contents. When all peaks were combined into a single statistical analysis, no significant differences among varieties in % area or % protein were found. However, when a separate analysis of variance was conducted for each of the ten peaks, varietal difference were identified for peaks 1–8 for both % area (Table 4) and protein content (Table 5). Percent area for the six samples averaged 5, 7, 9, 18, 39, 9, 10, 2, 0.3 and 0.2%, respectively, for the ten peaks. Protein averaged 8, 17, 11, 13, 14, 7, 13, 8, 5 and 6%, respectively, for the ten peaks.

3.4. Calibration of the column

Average void volume of the column was 45.0 ml using 4 mg of blue dextran in AUC buffer for runs of 0.5 ml/min. The column was calibrated with standard proteins of known relative molecular mass solubilized in AUC. The calibration curve (Fig. 4) was constructed by plotting the $(V_e-V_o)/V_o$ of protein markers against log M_r. The relative molecular mass of the yeast alcohol dehydrogenase was considered as 141 000 (comprised of four subunits of 35 000) [10]. HPLC of BSA solubilized in AUC showed the presence of two major polypeptide peaks containing 15 and 85% of the injected proteins. It

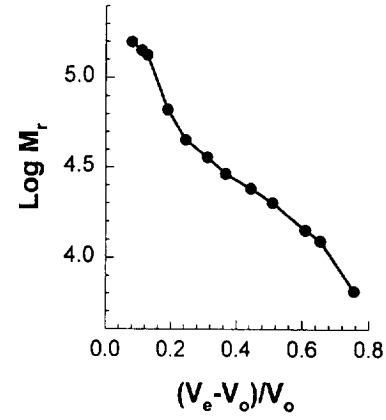

Fig. 4. Calibration of the gel column using AUC as eluent. Standard proteins included aldolase (158 000), alcohol dehydrogenase (141 000), bovine serum albumin (BSA) (133 000 and 66 400), ovalbumin (45 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), carbonic anhydrase (29 000), trypsinogen (24 000), soybean trypsin inhibitor (20 100), bovine milk lactalbumin (14 200), cytochrome C (12 400) and aprotinin (6500).

Table 4
Analysis of variance for varietal difference in percent area for individual peaks[a]

Peak	1	2	3	4	5	6	7	8	9	10
Bengal	2^{b}	$7^{b,c}$	8^{b}	15^{c}	44^{a}	12^{a}	10^{c}	$2^{c,b}$	0.3^{a}	0.2^{a}
Cypress	4^{b}	$5^{b,c}$	12^{a}	26^{a}	$39^{b,c}$	0^{c}	16^{b}	4^{a}	0.3^{a}	0.2^{a}
Della	3^{b}	6^{a}	7^{b}	15^{c}	45^{a}	7^{b}	9^{c}	2^{c}	0.3^{a}	0.2^{a}
Mars	8^{a}	10^{a}	9^{b}	15^{c}	36^{c}	12^{a}	9^{c}	3^{b}	0.3^{a}	0.2^{a}
Maybelle	0^{c}	5^{c}	7^{b}	16^{c}	41^{b}	8^{b}	19^{a}	2^{c}	0.3^{a}	0.2^{a}
Toro-2	0^{c}	0^{d}	13^{a}	22^{b}	42^{a}	7^{b}	15^{b}	2^{c}	0.3^{a}	0.2^{a}

[a] Mean values associated with different letters for a given peak are significantly different according to the least significant difference (LSD) test at a 95% level of significance.

Table 5
Analysis of variance for varietal difference in percent protein for individual peaks[a]

Peak	1	2	3	4	5	6	7	8	9	10
Bengal	$9^{a,b}$	17^{a}	11^{c}	14^{b}	13^{a}	11^{b}	8^{c}	$6^{c,d}$	$6^{a,b}$	5^{b}
Cypress	10^{a}	14^{b}	9^{c}	$17^{a,b}$	15^{a}	0^{d}	18^{a}	7^{c}	$6^{a,b}$	5^{b}
Della	7^{c}	18^{a}	16^{b}	10^{c}	15^{a}	4^{c}	10^{c}	5^{d}	7^{a}	$8^{a,b}$
Mars	7^{c}	14^{b}	10^{c}	10^{c}	16^{a}	10^{a}	12^{c}	$6^{c,d}$	$5^{b,c}$	10^{a}
Maybelle	0^{d}	14^{b}	18^{b}	19^{a}	8^{b}	6^{c}	17^{a}	9^{b}	$5^{b,c}$	5^{b}
Toro-2	0^{d}	0^{c}	26^{a}	14^{b}	14^{a}	4^{c}	14^{b}	16^{a}	4^{c}	$7^{a,b}$

[a] Mean values associated with different letters for a given peak are significantly different according to the least significant difference (LSD) test at a 95% level of significance.

appears that these two peaks are the dimer and the monomer of BSA as part of BSA (15% here) survived in the AUC mobile phase as a dimer. Their relative molecular masses in nondissociation buffer were reportedly 133 000 and 66 4000, respectively [11]. Other markers were aldolase, alcohol dehydrogenase, BSA dimmer, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase monomer, carbonic anhydrase, trypsinogen, soybean trypsin inhibitor, bovine milk lactalbumin, cytochrome C and aprotinin of relative molecular masses of 158, 141, 133, 66.4, 45, 36, 29, 24, 20.1, 14.2, 12.4 and $6.5 \cdot 10^3$, respectively. The effects of flow-rate and protein load on V_e were taken into consideration in the calibration of the column. The V_e values used to construct Fig. 4 for these markers were the averages for runs of a 10-mg total protein load and a 0.5 ml/min flow-rate.

Table 6
Molecular mass distribution of size exclusion peaks of rice bran proteins separated in a strongly dissociation buffer[a]

Peak No.	Relative molecular mass$\times 10^3$			
	Start	End	Retention	Homogeneous groups[b]
1	150.0	111.3	104.5	a
2	107.5	62.3	84.4	b
3	61.5	45.0	54.0	c
4	45.0	32.8	38.5	d
5	31.9	25.0	29.0	e
6	25.0	16.7	22.5	e,f
7	16.2	11.8	14.0	f,g
8	11.6	5.7	7.3	g
9	5.3	ND[c]	ND[c]	–
10	ND[c]	ND[c]	ND[c]	–

[a] Calculated using the calibration curve of Fig. 4.
[b] Based on multiple-range analysis.
[c] Not determined because values were out of fractionation range that can be calculated from calibration curve.

3.5. Protein size classes and molecular mass distribution of the bran proteins from several rice varieties

The relative molecular masses of the peaks of GP separation of bran samples were estimated using the calibration curve of Fig. 4. The relative molecular mass of each peak at its start, end and maximum retention was calculated from elution volumes and is presented in Table 6. Resolved peaks had apparent relative molecular masses of 1.05, 0.84, 0.54, 0.39, 0.29, 0.23, 0.14 and $0.07 \cdot 10^5$, respectively, for the first eight peaks. The M_r values for the last two peaks were not calculated because they were out of the calibration range of the standards. However, this range was listed as within the fractionation range for this column by the column supplier. The prepacked column has been marketed by Waters as having an effective fractionation range from 1000 to $1.5 \cdot 10^5$. Accordingly, the last three contained small peptides and nonprotein nitrogen of M_r of less than 10 000. This optimized separation procedure allows the accurate determination of rice polypeptides as separated into several peaks based on their molecular size and shape. Further studies of the isolated rice polypeptides will provide information pertaining to their structures and will enable the detection of similarities in sequential peaks so similar peaks, if any, may be combined.

Subunits of rice proteins are similar to those of soy and oat proteins as determined by SDS-PAGE [2]. Ma [9] used conventional size-exclusion column with exclusion limit of 200 000 in AUC buffer to fractionate alkali-extracted oat protein concentrates into five peaks with apparent relative molecular masses of 95 000, 38 000, 23 000, 14 000 and a nonprotein nitrogen peak of less than 10 000. Ma [9] did not observe any substantial difference in the chromatograms of oat protein concentrates from the two cultivars used in the study. In this study, we also found no significant varietal differences among the peaks of the rice bran proteins when all peaks were combined into a single statistical analysis. However, when a separate analysis of variance was conducted for each of the ten peaks, varietal difference in % area and % protein were identified for peaks 1 through 8. Although our M_r values are identical to those for oat proteins, there is one exception, that is, Ma [9] reported that peak-1 had M_r of 95 000 but we resolved this peak into two peaks (peaks 1 and 2) with M_r values of 105 000 and 84 000 thus averaging 95 000 for both. Snow and Brooks [5] separated the polypeptides of rice glutelin by Sepharose CL-6B [6] in 8 *M* urea or 0.5% SDS in Tris buffer (pH 9.0) into four broad unresolved fractions that eluted over a very wide range of M_r covering the whole V_t of the column. Their chromatography of rice glutelin poly-

peptides was unable to separate the polypeptides into resolved peaks with well-defined molecular sizes. This confirms that the use of a combination of a detergent and chaotropic agent in one buffer (AUC) was necessary to solubilize and fractionate rice polypeptides into sharp peaks.

This HPLC procedure, which utilizes AUC, can be a powerful separation technique despite the fact that the fractions are not homogeneous nor have a clean separation of electrophoretic components or Osborn solubility classes. This procedure provides a meaningful classification of rice proteins that other methods do not. This new chromatographic procedure, based on size exclusion in the presence of a detergent and a chaotropic agent can be used for the preparative separation of the rice protein either from bran or other parts of the kernel. This separation procedure should contribute useful information concerning the precise nature of the links involved in making up the rice polypeptides after they are fractionated into several peaks based on their molecular size and shape. Also, the isolated peaks could be further investigated by SDS-PAGE and circular dichroism to gain information about their differences in secondary structures and the subunit composition of the polymers of each peak.

4. Conclusions

Rice proteins are difficult to purify and study because of the insolubility of their major components in the buffers normally used for protein isolation. Although extensive aggregation through hydrogen bonds and hydrophobic interactions play the major role in insolubility, extensive disulfide bonding can be a major element contributing to insolubility of the proteins of some rice varieties. Research was undertaken to isolate rice bran proteins using a strongly dissociating buffer and to study their molecular mass distribution by size exclusion using high-performance liquid chromatography in this dissociation solvent. This procedure was optimized and used for the preparative separation and quantitative determination of the relative proportions of the major size classes of the polypeptides of rice proteins for several varieties. The separation is reproducible and thus allows the determination of the molecular mass distribution of rice proteins and further characterization and fractionation of the individual polypeptides.

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