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EXTRACTION AND COMPOSITION OF RICE ENDOSPERM GLUTELIN*

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Key Word Index—*Oryza sativa*; Gramineae; rice extraction and composition; endosperm glutelin; protein subunits.

Abstract—"Crude" glutelin was prepared from milled rice (*Oryza sativa*) flour by sequential extraction of the albumin-globulin fraction with 0.5 M NaCl and prolamin with 70% ethanol–0.6% β-mercaptoethanol. The solvent, 0.5% sodium dodecyl sulphate (SDS)–0.6% β-mercaptoethanol, extracted 91% of the endosperm glutelin without gelatinizing starch granules, whereas chaotropic solvents such as urea and guanidine caused extensive gelatinization. The *S*-cyanoethyl glutelin (Ce-glutelin) prepared by SDS extraction of the "crude" glutelin (9.5% protein) of IR480-5-9 rice gave three major subunits with MW 38000, 25000 and 16000 in the ratio 2:1:1 as determined by SDS polyacrylamide gel electrophoresis. A similar preparation from "crude" glutelin of a lower protein containing rice had the corresponding subunits in the ratio of 16:3:1. The MW 38000 subunit was unique to glutelin and was not present in C3-albumin-globulin or prolamin; the subunits were only partially purified by SDS Sephadex G-150 gel-filtration. The C3-glutelin was also prepared from a crude glutelin–prolamin preparation from IR480-5-9 by NaOH extractions followed by precipitation at pH 10 and ethanol extraction of the precipitate (C3-glutelin). This preparation had the same three major subunits and in the same ratio as C3-glutelin prepared by the SDS method. The subunits of the former preparation were separated by carboxymethyl Sephadex C-50 chromatography; the MW 38000 subunit eluted between pH 6.2–8.5, the MW 25000 in an impure state at pH values above 9, and the MW 16000 subunit was eluted at pH 8.6–9.2. Amino acid composition of the Ce-glutelin preparations were similar to each other. The MW 38000 and 16000 subunits had lower lysine contents than whole C3-glutelin, whereas the MW 25000 subunit had a higher lysine content.

INTRODUCTION

The major storage proteins of most cereal grains are glutelin and prolamin. In rice (*Oryza sativa* L.) unlike most other cereals, however, glutelin is the only major protein of the endosperm, comprising at least 80% of the total protein and prolamin accounts for less than 5% [1]. Because of its insolubility except in dilute alkali, its high MW and its heterogeneity [2–4], very little work has been done to characterize rice glutelin. Rice endosperm protein is present mainly in the form of protein bodies [5,6], which have the same glutelin content and aminogram as total endosperm protein [5].

Using a purified glutelin preparation, Sawai and Morita [7] demonstrated that the polypeptide subunits of rice glutelin are bound together by disulphide bonds. Under reducing conditions they found one major subunit with MW of *ca* 20000 and two minor subunits with MW of *ca* 35000 and 43000 [8] in the ratio of 8:1:1 [7]. In contrast to these findings, reduced glutelins from corn [9], oat [10], wheat [10–12] and sorghum [13], have two or more major polypeptide subunits as established by SDS polyacrylamide gel electrophoresis. Similarly, Sephadex gel-filtration in the presence of guanidine or urea of reduced and alkylated glutelins from different cereals separated several major components which were not completely resolved—six with corn [9], three with wheat [12], and five with sorghum [13].

Because of the importance of milled rice as the major source of protein in the diets of tropical Asia [1], breeding programmes are in operation to improve the protein content of rice. High protein rices from this programme, such as IR480-5-9 [14] are ideal materials for studying rice glutelin because of their higher glutelin content [15]. This paper compares methods used to isolate rice glutelin and describes the characteristics of the principal subunits of reduced and Ce-rice glutelin from IR480-5-9 rice. Alkylation was undertaken to prevent disulphide interchange occurring after isolation of glutelin.

RESULTS AND DISCUSSION

Extraction of glutelin

Extraction of commercial rice flour containing 6.7% protein, first with 0.5 M NaCl and then by 70% EtOH–0.6% β-mercaptoethanol resulted in the extracted flour (crude glutelin preparation) containing 4.8% protein. Since crude glutelin-prolamin preparations contained 5.0% protein, the protein of the original rice flour containing 6.7% protein consisted of 25% albumin–globulin, 3% prolamin, and 72% glutelin. Extraction of IR480-5-9 milled rice flour containing 10.6% protein with

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Table 1. Efficiency of extraction of protein from milled-rice powder and crude glutelin preparations by various solvents*

Sample	Solvent (with 0.6% β ME)	Extraction efficiency (%)
Milled rice†	0.1 N NaOH	98
Milled rice†	0.5% SDS	94
Crude glutelin–prolamin‡	0.5% SDS	94
Crude glutelin§ (70% EtOH denatured)	0.1 N NaOH	98
	0.5% SDS	81
	0.5% SDS, 0.5 M NaCl, 0.1 M borate, pH 10	75
	0.5% SDS, 0.5 M NaCl, 0.1 M borate, pH 10, 0.5% $NaBH_4$	78
	H_2O	11
	0.5 M NaCl, 0.1 M borate, pH 10¶	6
Crude glutelin§ (2 hr exposure to 70% EtOH)	0.5% SDS	91
	0.5% SDS, 0.1 M NaPi, pH 7.2	91

* 150 mg in 2 ml solvent for 1–2 hr at room temp. † 6.6% protein (N × 5.95). ‡ 4.98% protein (N × 5.95). § 4.82% protein (N × 5.95). ¶ No β-mercaptoethanol added.

0.5 M NaCl left a residue (crude glutelin–prolamin preparation) with 9.8% protein. Short duration extraction of this with 70% EtOH made 0.6% with β-mercaptoethanol gave a crude glutelin preparation with 9.5% protein. Thus, glutelin constituted 89% of the protein of IR480-5-9. These results are consistent with previous observations that differences in the level of rice endosperm protein were mainly due to differences in glutelin content [15].

A survey of protein extractants, including chaotropic solvents [16] indicated that only 0.5% SDS–0.6% β-mercaptoethanol approached 0.1 N NaOH in efficiency of protein extraction from rice (Table 1). Chaotropic agents such as 6–8 M guanidine, 8 M urea, 4 M NaSCN and 4 M $NaClO_4$ caused immediate gelatinization of starch giving viscous gels. When lower concentrations of these solvents were used which did not extensively gelatinize the starch granules, i.e. 1.5 M $NaClO_4$, 1 M NaSCN, 3 M urea or 3 M guanidine, only 40–60% of the glutelin present was extracted.

The solvent 0.5% SDS–0.6% β-mercaptoethanol extracted 81% of the protein from a crude glutelin preparation from which prolamin had been removed previously by prolonged exposure to 70% EtOH. The same solvent extracted 91% of the protein from a similar preparation of crude glutelin, which had been extracted with 70% EtOH for only 2 hr (Table 1). Since the SDS-β-mercaptoethanol solvent extracted 94% of the protein of milled-rice it appears that prolonged exposure of glutelin to EtOH results in its partial denaturation. Landry *et al.* [17] also found that SDS-β-mercaptoethanol extracted 94% of the protein of barley with a similar reduction in the efficiency of extraction by SDS-β-mercaptoethanol after prolonged exposure of the barley flour to EtOH.

Ce-glutelin prepared by sodium dodecyl sulphate extraction

Ce-glutelin prepared from IR480-5-9 rice by the SDS-β-mercaptoethanol method was insoluble in water and in most solvents except SDS-β-mercaptoethanol and chaotropic solvents. It was sparingly soluble in non-ionic detergents, e.g. 1% aqueous solutions of Brij 35, Tween 80 and Triton X-100, an observation in agreement with that of Makino *et al.* [18] that Triton X-100 was not as good a solvent as SDS for denaturing proteins.

SDS-polyacrylamide gel electrophoresis showed that this preparation contained three major subunits with MW 38000, 25000 and 16000 in the ratio of *ca* 2:1:1 (Table 2). A similar preparation containing 4.8% protein from a commercial lower protein rice had the corresponding subunits in the ratio 16:3:1. Sawai and Morita [7] reported a ratio of 8:1:1 for the subunits of their rice Ce-glutelin.

Better resolution was obtained with 12% gels than with 10% gels and the MW 16000 subunit was readily detected in the former but was not fully resolved from the bromophenol blue front in the latter. In addition, a white precipitate was apparent in the MW 16000 band even before staining and this may lead to an over-estimate of the concentration of this subunit based on absorbance of the Naphthalene Black 12B stain. This MW subunit was not observed when reduced alkylated glutelin was dissociated with some SDS preparations.

Only the MW 38000 band was observed when the MW 38000 subunit was extracted from three gels, the extracts combined, protein precipitated with 3 vol. of acetone and after solubilization, re-run on SDS-polyacrylamide gel electrophoresis. Hence, the MW 25000 and 16000 subunits were not derived from the MW 38000 subunit by reduction with β-mercaptoethanol.

Addition of 8 M urea, 6 M guanidine, or 4 M NaSCN to the glutelin 1 hr before alkylation did not change the electrophoretogram pattern of Ce-glutelin obtained subsequently and alkylation was, therefore, carried out in the absence of these chaotropic solvents.

Comparison of the SDS-polyacrylamide gel electrophoresis patterns of reduced and alkylated rice protein fractions showed that the major subunits of the "albumin" or water-soluble fraction of a salt extract, i.e. Ce-albumin-globulin had MW's 25000, 19000 and 16000, whereas its "globulin" or water-insoluble fraction had major subunits with MW's 35000, 25000 and 14000 (Table 2). The Ce-prolamin had a single major subunit with MW 23000. Thus, the major subunit of Ce-glutelin MW 38000, is not common to the other protein fractions. A major globulin protein of rice endosperm has been reported with a MW of 25000 [19].

The SDS-β-mercaptoethanol extract of "crude" glutelin–prolamin had the same electrophoretogram as that of Ce-glutelin. The removal of the 6% protein from the

Table 2. MW of subunits of Ce-glutelins compared with those of Ce-albumin-globulin and prolamin*

Ce-protein	MW of subunits × 10^{-3}	
	Major	Minor
Glutelins		
SDS-β-mercaptoethanol method	38 25 16	42 35 27 19 14
NaOH–NaCl method	38 25 16	42 35 19 14
0.14 M NaCl, pH 7–10 precipitate	35 27 25	38 32 22 18
Albumin–globulin		
"Albumin"	25 19 16	42 35
"Globulin"	35 25 14	16
Prolamin	23	

* By SDS-polyacrylamide gel electrophoresis of derivatives of protein fractions from IR480-5-9 rice.

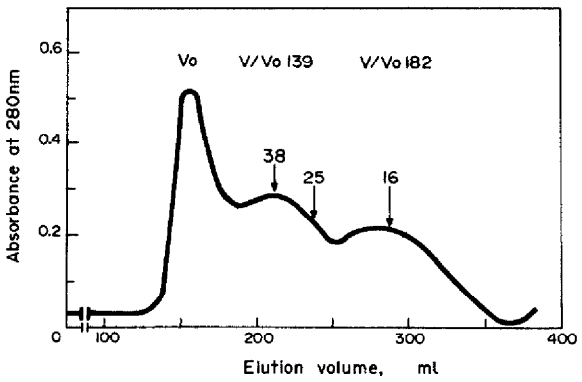

Fig. 1. MW fractionation of Ce-rice glutelin prepared by the SDS-β-mercaptoethanol method on a Sephadex G-150 column (3.0 × 60 cm) with 0.05 M Tris–HCl, pH 8.6 with 0.5% SDS as eluting solvent.

Ce-glutelin which was soluble in 0.5 M NaCl–0.1 M borate–NaOH, pH 10 buffer [4] also did not affect the electrophoretogram pattern of the residual glutelin.

Fractionation of Ce-glutelin prepared by the SDS-β-mercaptoethanol method using SDS Sephadex G-100 chromatography gave incomplete separation of the MW 38000 subunit from material eluting with the void volume.

Ce-glutelin by the NaOH–NaCl method

Because of the difficulty of isolating pure subunits from Ce-glutelin prepared by the SDS-β-mercaptoethanol method, Ce-glutelin was also prepared from "crude" glutelin–prolamin of IR480-5-9 by using a modification of the method of Sawai and Morita [2,7]. Protein precipitates obtained by further acidification of the 0.14 and 0.5 M NaCl solutions from pH 10 to 7 were also collected; 7 times more glutelin was obtained by precipitation between pH 10 and 7 than at pH 10 as in the Sawai and Morita method. The Ce-derivatives of these glutelin preparations gave almost identical electrophoretograms, except for the slightly lower content of the MW 16000 subunit in the pH 10 glutelin. Sawai and Morita [2] considered a similar preparation to be pure glutelin. Denatured protein in the surface layer obtained during the centrifugation of these glutelin preparations also had a similar electorphoretogram.

The electrophoretogram showed the three subunits with MW 38000, 25000 and 16000 in the ratio of *ca* 2:1:1; the same ratio as obtained with Ce-glutelin prepared by the SDS method. However, glutelins when prepared by the SDS-β-mercaptoethanol method had a minor subunit MW 27000 which was not seen in Ce-glutelin prepared by the NaOH–NaCl method. The protein which remained in solution in 0.14 M NaCl at pH 10 but which was precipitated at pH 7 in the NaOH–NaCl method had this MW 27000 band as a major subunit, in addition to subunits with MW 25000 and 35000 (Table 2). This step removed contaminating protein which may have been deposited in the protein bodies during grain development and which was not accessible to 0.5 M NaCl during extraction of the meal with this solvent, since glutelin constitutes at least 80% of the rice endosperm protein [1]; it was only dissolved when the glutelin was dispersed. Probably most of the minor subunits found in the glutelin preparation are from contaminating proteins since they correspond in MW to subunits found in the albumin–globulin fraction (Table 2). The status of the MW 25000 and 16000 subunits may be different from the other minor subunits since they tended to be present at higher levels relative to the MW 38000 subunit in glutelin from high protein rice IR480-5-9 as compared to similar glutelin preparations from low protein rice; the albumin and globulin fractions do not increase proportionately to the glutelin with an increase in the protein content of rice [15,20]; varietal differences in the ratio of glutelin subunits have also been reported for wheat [21].

All three subunits showed no reaction with periodic acid–Schiff's reagent after separation on gels by SDS-polyacrylamide gel electrophoresis, indicating that rice glutelin has little or no carbohydrate content. Sawai and Morita [2] reported only 0.32% carbohydrate in glutelin prepared by the NaOH–NaCl method, although Tecson *et al.* [4] have reported a total carbohydrate content of 2.8% in a similar preparation.

CM-Sephadex C-50 chromatography of Ce-glutelin (NaOH-NaCl method) by means of a pH gradient in 6 M urea [7] resolved the major subunits (Fig. 2). Protein unadsorbed at pH 5.2 consisted mainly of the MW 14000 subunit. The first major protein peak which eluted as a clear solution between pH 6.2 and 8.5 was the MW 38000 subunit. The opalescence of Ce-glutelin solution which was adsorbed by the ion-exchanger was eluted out by the NaOH eluant at pH 8.6–9.2 in the second protein peak. SDS-polyacrylamide gel electrophoresis showed this peak to be mainly the MW 16000 subunit.

The MW 25000 subunit, which eluted only above pH 9, was contaminated by other subunits. Protein still adsorbed on CM-Sephadex C-50 at pH 9.3 and extracted by 0.5 M Na_2CO_3–6 M urea (pH 11.4) was also principally the MW 25000 subunit. A purer preparation of this subunit was obtained by SDS-Sephadex G-150 chromatography of Ce-glutelin prepared by the SDS-β-mercaptoethanol method, but it had only 1.1% N.

Rechromatography of the two major peaks from CM-Sephadex C-50 chromatography on the same ion-exchanger led to further purification. The MW 38000 subunit eluted as a peak between pH 6.4 and 7.0 and contaminating subunits were mainly those with MW 42000, 35000 and higher; sub-fractions that eluted above pH 7 were contaminated with lower MW subunits.

The MW 16000 subunit eluted on rechromatography at pH 8.7. It still showed traces of the two major subunits

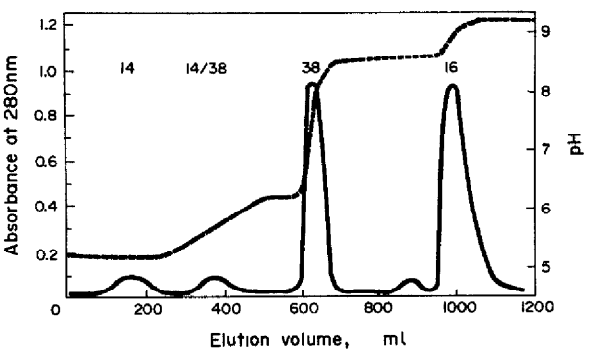

Fig. 2. Chromatography of Ce-rice glutelin prepared by the NaOH–NaCl method on a CM-Sephadex C-50 column (2.5 × 40 cm) using a non-linear pH gradient.

Table 3. Amino acid analysis in g per 16 g N (dry wt basis) Ce-rice glutelin and its major subunits as compared to the data of Sawai *et al.* [8]

Amino acid residues	Ce-glutelin (SDS-ME)‡	Ce-glutelin (NaOH–NaCl)‡	Subunits 38000	Subunits 16000	Subunits 25000	Ce-glutelin	Sawai *et al.* [8] Major subunit MW 20000	Sawai *et al.* [8] Minor subunit MW 43000
Asp	10.08	10.28	8.85	10.10	13 95	9.28	7.91	11.74
Thr	3.31	2.91	2 82	2.99	2 46	3.03	3.10	3.40
Ser	4.49	4.43	4.55	4 30	5 33	4.80	5 33	4 58
Glu	16.10	18.15	18.15	16 00	16 55	17.00	21.55	10 90
Pro	4.00	3 63	3.46	3.62	3 42	3 74	3.73	3.43
Gly	3 40	3.15	3.52	2.97	4.51	3.37	3.89	2 65
Ala	4.76	4.38	3.24	4.56	5.61	4.01	3.40	4 81
Val	6.04	5.69	5.10	5.45	6.98	5 89	5.20	6 60
Met	2.12	1.25	0.68	1.49	1.50	1.08	0.58	1.30
Ile	4.04	4.18	3.17	4.85	4.65	4 34	3 60	5 37
Leu	7.92	7.70	6.41	7.69	8 48	6 79	6 72	6 91
Tyr	4.94	5.35	4.09	5 39	5.20	4 90	4 64	5.62
Phe	5.34	5.59	5.96	5.21	5.61	5.40	6.00	5.08
His	2 16	2.14	2.16	2.09	2.60	2.42	2 26	2.67
Lys	3 19	2.66	2 20	2.17	4.10	3.21	2 30	4 50
Arg	8 02	8 53	9 26	7.63	8.62	9.08	8 93	9.34
$\frac{1}{2}$-Cys	1.69	n.d.*	1.20	0.84	n.d *	1.31	1 95	0.83
Try	0.67	1 79	n.d.	n d.	n.d	1.15	1 30	0.69
Total	92.27	91 81	84 82	87 35	99.57	90.80	92.39	90.42
Total N (g/100 g dry protein)	14 3	17 8	18 2	16 9	1.17†	17.9	17 5	17 3

n.d. = not determined. * Poor separation of S-carboxyethyl cysteine from glutamic acid. † Amino acid nitrogen.
‡ Method of preparation see text for details.

on SDS-polyacrylamide gel electrophoresis and still gave an opalescent solution. Protein that eluted up to pH 9.2 was principally the MW 16000 subunit as shown by SDS-polyacrylamide gel electrophoresis.

Sawai and Morita [7] also separated the three protein subunits of Ce-glutelin by CM-Sephadex C-50 chromatography, but with MW 20000, 35 000 and 43000 as calculated from amino acid analyses; they were in the ratio of *ca* 8:1:1. The difference in the ratio of the two sets of results may be varietal, since glutelin from lower protein rice had a ratio of 16:3:1 which is similar to Morita's ratio of 8:1:1. Sawai *et al.* [8] verified that the MW of the major subunit was 20000 by sedimentation analysis in 8 M urea. However, when the MW 38000 subunit of this present investigation was electrophoresed on the SDS-polyacrylamide gel system in the presence of 8 M urea, it did not dissociate.

Amino acid composition of Ce-glutelins

The amino acid composition of Ce-glutelins from IR480-5-9 rice and their three major subunits are given in Table 3, together with the results obtained for similar preparations by Sawai *et al.* [8]. The amino acid composition of glutelin prepared by us by the alkaline extraction method is very similar to that obtained by Morita and by Tecson *et al.* [4,8] especially since some contaminating protein was also removed in our preparation. Thus, both preparations showed high levels of aspartic and glutamic acid and low levels of sulpho-amino acids. Glutelin prepared by the SDS-β-mercaptoethanol method is also similar to that prepared by alkaline extraction, although the SDS-β-mercaptoethanol protein contained more non-protein material than did the alkaline preparation. However, the SDS-β-mercaptoethanol preparation had higher levels of lysine, threonine and lysine and lower levels of arginine, glutamic acid, tryptophan, tyrosine and phenylalanine. The amino acid composition of the major component subunit, component 1, of Morita's data [8], is higher in glutamic acid relative to its parent glutelin and contains twice as much methionine. Our major subunit was only slightly higher than glutelin in its content of glutamic acid but as with the Morita data it contained one-half as much methionine as did the parent glutelin.

The 16000 subunit is relatively lower in glutamic acid and arginine and higher in methionine, possibly explaining the different content of these residues in glutelin and its major subunit. The MW 38000 and 16000 subunits had lower contents of lysine than the NaOH–NaCl Ce-glutelin they were derived from.

The MW 25000 subunit was the only one isolated with low purity. It was the only one derived from SDS-Sephadex G-150 chromatography of Ce-glutelin. It had higher contents of lysine, aspartic acid and glycine and lower contents of threonine, proline and methionine than whole glutelin.

Our general conclusion is that our preparations are similar to those of Morita but that both may still contain small amounts of contaminating protein.

EXPERIMENTAL

Materials. Rice from the 1974 dry season harvest of IR480-5-9, a high-protein rice [14], was obtained from the International Rice Research Institute's farm. It was dehulled and milled in Satake machines and ground to a 60-mesh flour in a UDY cyclone mill. Preliminary experiments used a Crestona ground rice of Italian origin (Crestona Ltd., Worksop, England) which contained 6.7% protein.

Preparation of crude glutelin–prolamin and crude glutelin. Ground rice, 50 g in 100 ml 0.5 M NaCl, was blended at maximum speed in an Oster blender for 2 min and transferred to a centrifuge bottle with 50 ml more 0.5 M NaCl. After shaking for 1.5 hr the suspension was centrifuged at 1000 *g* for 10 min and the supernatant discarded. The rice was again extracted 2 × with 150 ml portions of 0.5 M NaCl for 1 hr after which the residue was washed 3 × with H_2O and was termed "crude" glutelin–prolamin. "Crude glutelin" was prepared from "crude" glutelin–prolamin by extraction 2 × for 0.5 hr with 70% EtOH containing 0.6% β-mercaptoethanol. The residue

was then washed 2 × with H_2O. Resulting crude glutelin was air-dried at room temp. and ground to a fine powder in a mortar and pestle.

Efficiency of various protein extractants. Duplicate samples of crude glutelin or rice powder (150 mg) were extracted at room temp. with 2 ml extractant containing 0.6% β-mercaptoethanol for 2 hr and then centrifuged at 1000 *g* for 10 min. The residue was washed once with H_2O and extracted for 30 min with 2 ml 0.1 N NaOH which was used to remove any protein left unextracted by the method. Protein content of extracts was estimated by the method of Ref. [22] using BSA as a standard. Protein content of the starting samples was determined on a fr. wt basis by using the micro-Kjeldahl method and a conversion factor of 5.95 [20]. Some extracts were alkylated with 24 μl acrylonitrile for 45 min [23], acidified with HOAc, and dialysed overnight against H_2O. The precipitated protein was subjected to SDS-polyacrylamide gel electrophoresis.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The discontinuous system of Ref. [24] was used except that the small pore gel had 12% acrylamide. Protein samples were dissolved by boiling for 3 min in 4% SDS in 0.067 M Tris–HCl buffer, pH 6.8, in the presence of β-mercaptoethanol. Electrophoresis was carried out with a current of 4 mA/tube for 2.5–3 hr. Gels were stained with 0.1% Naphthalene Black 12B in 7% HOAc. Reference proteins were: myoglobin, egg albumin, BSA fraction V and trypsin. Some gels were washed in 10% TCA–10% sulphosalicyclic acid for 1.25 hr, washed 7 × with 7% HOAc and stained for carbohydrate with periodic acid–Schiff's reagent [25]. Stained gels were scanned at 580 nm and the area corresponding to protein bands weighed in order to determine the amount of protein on the gels.

Preparation of Ce-glutelin from crude glutelin. Crude glutelin (50 g) was extracted with 360 ml 0.5% SDS 0.1 M Pi buffer, pH 7–0.6% β-mercaptoethanol at room temp. for 1.5 hr and centrifuged at 17500 *g* for 10 min. Residue was re-extracted with 140 ml solvent for 1 hr and the combined extracts were kept at room temp. for at least 8 hr, and then alkylated with 1.2% acrylonitrile with occasional shaking for 45 min. Ce-glutelin was precipitated by the addition of $(NH_4)_2SO_4$ and stored as a wet ppt. at −20°. Addition of 8 M urea or 6 M guanidine during reduction and alkylation did not affect the results obtained subsequently.

Preparation of Ce-albumin-globulin and prolamin. The albumin–globulin fraction obtained by 0.5 M NaCl extraction of rice flour and the prolamin obtained by extraction of rice flour with 70% EtOH–0.6% β-mercaptoethanol were also converted to Ce-derivatives. The opalescent 0.5 M NaCl extract was made 0.5% with SDS and 0.6% with β-mercaptoethanol, shaken for 1.5 hr at room temp. and alkylated with 1.2% acrylonitrile for 45 min [23]; it was then dialyzed against H_2O. The "globulin" or H_2O-insoluble fraction was collected by centrifugation and "albumin" protein collected from the supernatant by precipitation with $(NH_4)_2SO_4$. The prolamin extract was kept at room temp. for another hr and directly alkylated with 1.2% acrylonitrile, acidified with HOAc and then dialyzed against H_2O. The protein ppt. was collected by centrifugation.

Sephadex G-150 gel chromatography in sodium dodecyl sulphate. Ce-glutelin prepared by the SDS β-mercaptoethanol method was fractionated on a 3 × 60 cm column of Sephadex G-150 (40–120 μm) using 0.05 M Tris–HCl buffer, pH 8.6, with 0.5% (w/v or 0.0174 M) SDS sulphate as eluant [26]. The sample to be applied to the column was dispersed in 0.05 M Tris–HCl buffer, pH 8.6, made 2.5% with SDS and 0.6% with β-mercaptoethanol by heating to 100°. Fractions of 3–5 ml were collected with a flow rate of 20 ml/hr and protein monitored at 280 nm. Identification of the protein fractions was by SDS-polyacrylamide gel electrophoresis.

Preparation of Ce-glutelin from "crude" glutelin-prolamin. The procedure was a modification of the method of Refs. [2] and [7]. Crude glutelin–prolamin (100 g) was extracted with 720 ml 0.05 N NaOH for 1 hr at 0–4°, centrifuged at 17500 *g* for 10 min and re-extracted with 400 ml 0.05 N NaOH. Combined extracts were made 0.14 M with NaCl and the pH adjusted to 10 with N HCl. A fluffy ppt. and surface scum formed on standing 18 hr at 0° After centrifuging at 1000 *g* for 10 min, surface scum and ppt. were collected. The slightly turbid supernatant liquid was adjusted to pH 7, kept at 4° for 18 hr, and the ppt. collected by centrifugation at 17000 *g* for 10 min. The glutelin ppt, was washed once with 0.14 M NaCl and redispersed in 1 litre 0.05 N NaOH at 0–4°. This suspension was made 0.5 M with respect to NaCl and the pH adjusted to 10. On standing for 18 hr at 0–4°, a glutelin ppt., a scum and a very turbid supernatant liquid were obtained by centrifugation at 1000 *g*. The supernatant liquid was acidified to pH 7, and after 1 hr at 0–4°, a voluminous ppt., together with the scum, were collected by centrifugation at 1100 *g*. The pH 10 and pH 7–10 glutelin preparations were washed 2 × with 200 ml portions of 0.5 M NaCl, then 5 × with H_2O, once with 66% EtOH and then 2 × with H_2O. Purified glutelins were dispersed in 6 M urea, 0.1 M Na Pi buffer, pH 7, with 0.6% β-mercaptoethanol and alkylated by adding 1.2% acrylonitrile and shaking for 45 min. The solns were diluted with 5 vol. of H_2O and $(NH_4)_2SO_4$ added to ppt. the protein. The precipitations were collected by centrifugation at 17500 *g* for 10–15 min and kept at −20° until used.

Carboxymethyl Sephadex C-50 chromatography of Ce-glutelin. The procedure of Ref. [7] was employed using pH 7–10, *S*-cyanoethyl glutelin. A 2.5 × 40 cm column of CM-Sephadex C-50 was prepared with 6 M urea–0.05 M Tris maleate, pH 5 buffer and 4 g of wet, pH 7–10, Ce-glutelin dispersed in 120 ml of solvents. The column was then eluted at room temp. stepwise with 0.05 M Tris–maleate, pH 5.2 buffer, 0.05 M Tris–maleate buffer, pH 8.6, 0.05 M Tris–maleate, pH 9.5, 0.05 M Na_2CO_3, and 0.05 M NaOH each containing 6 M urea. Pooled fractions were dialysed against H_2O and subjected to SDS-polyacrylamide gel electrophoresis. The two major protein peaks were purified by rechromatography on 2.5 × 30 cm columns of CM-Sephadex C-50. The protein which eluted at pH 6.2–8.5 from the first column was re-chromatographed on a column equilibrated in 6 M urea 0.05 M Tris–maleate buffer, pH 5.8 and eluted with 6 M urea 0.05 M Tris–maleate buffer, pH 9.5. The second protein which eluted at pH 8.5–9 from the first column was rechromatographed on a column equilibrated with 6 M urea–0.05 M Tris–maleate buffer, pH 8.2 and eluted with 6 M urea–0.05 M Na_2CO_3, pH 11.4. Only the major protein peak from these columns was characterized.

Amino acid analysis. Protein samples for amino acid analysis were exhaustively dialysed against H_2O and freeze-dried. The method of hydrolysis and the amino acid analyses were carried out as described previously [27]. Cysteine was eluted as *S*-carboxyethyl cysteine which was sometimes not completely resolved from glutamic acid [8]. Tryptophan was determined by $Ba(OH)_2$ hydrolysis and short column chromatography [28].

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