Efficient Extraction and Some Properties of Storage Proteins (Prolamin and Glutelin) in Ancient Rice Cultivars

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Summary Two typical storage proteins (prolamin and glutelin) in a total of 14 samples of ancient rice cultivars were examined for their accumulation in the endosperms during days after flowering (DAF), extraction efficiency with various solvents, and variations in polypeptide components or amino acid compositions. There was little difference in accumulation profiles between this and previous observations, in which glutelin appeared on 5–7 DAF and prolamin on 7–9 DAF. As for the extraction of prolamin and glutelin, it was most effectively attained by sequential usage of 55% propanol and 2% SDS. As a result of SDS-PAGE, prolamin and glutelin proved to be composed mainly of a single polypeptide with a molecular weight (MW) of 15 kDa and two subunits with MWs of 21 kDa (α) and 32 kDa (β), respectively. A comparison between the ancient and modern rice cultivars revealed the same physicochemical properties in either case of prolamin or glutelin. Additionally, a good relationship was observed on the total protein and glutelin (but not prolamin) contents in the ancient rice cultivar.

Key Words ancient rice cultivar, storage protein, prolamin, glutelin, efficiency of protein extraction

Ancient rices, wild form *Oryza sativa* L., indigenous to various districts of Japan are now potential sources requisite for genetic improvement in nutritional quality (1) and allergenicity (2). They have received only limited attention for their basic physicochemical characteristics (3, 4). Storage proteins in most cereals are usually composed of prolamin and glutelin (5). As for rice grains, glutelin predominant in storage proteins comprises more than 80% of the total proteins, of which several percent are exchanged for prolamin (6). However, these contents are significantly variable according to rice cultivars (7, 8) or extraction procedures (9, 10). The extraction conditions affect not only the recovery of total proteins, but also their polypeptide components (11).

Although we have referred to ancient rice proteins in a preceding report (12), their extraction efficiencies and characteristics have not been investigated so much. To understand the biochemical properties of ancient rice proteins further, we aimed at finding the most effective and reproducible extraction procedure, and thereby elucidated some features of storage proteins in ancient rice grains.

In this paper, we describe the extraction efficiency of storage proteins with solvents, the protein accumulation in seed after flowering, and the multiplicity of polypeptide components of storage proteins on the basis

of comprising a total of 14 samples of ancient rice grain with modern rice cultivars.

MATERIALS AND METHODS

Rice seeds (*Oryza sativa* L.). Fourteen kinds of ancient rice cultivars including Midorimai, Benimiyako, Takaramitsu, Kaori-eigou, Kaori-mochi, Benihikari, Akita-kuro, Kunitsukasa-chukann, Mangetsu-mochi, Kuro-mochi, Kuro-60, Tsushimazairai, Murasaki-kuro, Kuro-73 and nine kinds of modern rice cultivars including Koshihikari, Akitakomachi, Kinuhikari, Hiyoku-mochi, Takasago-mochi, Aizen-mochi, Nobentadiablanco, Mitsuyou-23 and Bluebonnet were supplied by Uwa Rice Museum (Uwa-cho, Ehime 797–0015, Japan) as described previously (12). The rice seeds were dehulled and milled by a motor machine, ground into a 60-mesh flour, stirred twice with acetone for 5 h at room temperature, and air-dried.

Assay for seed protein accumulation. Japonica Kuro-mochi and Tsushimazairai (ancient rice cv.) were pot-cultured between June and late October in 1995 at the Laboratory of Food Science in the Faculty of Education at Ehime University (Matsuyama, Ehime 790–8577) as reported previously (12). The individual panicles were tagged at mid-panicle when anthesis occurred. After flowering, the developing panicles were harvested at in-

Table 1. Efficiency^a of prolamin extraction from ancient and modern rice cultivars with 4 aqueous-alcohols.^b

Rice cultivars	70% E-OH	55% P-OH	60% iP-OH	55% tB-OH	Total protein (%) ^c
<i>Kaori-mochi</i>	1.86	4.18	2.79	4.07	8.45
<i>Kunitsukasa-chukann</i>	3.14	7.40	4.37	4.93	8.87
<i>Kuro-mochi</i>	2.39	7.80	4.89	3.64	9.58
<i>Kuro-60</i>	3.92	5.16	4.33	4.54	9.64
<i>Tsushimazairai</i>	2.54	4.73	3.33	3.06	11.36
<i>Hiyoku-mochi</i> ^d	1.44	3.27	1.57	2.75	7.56
<i>Nobentadiablanco</i> ^d	1.62	4.37	2.49	3.24	7.91
<i>Koshihikari</i> ^d	1.71	5.49	1.83	2.56	8.15
<i>Akitakomachi</i> ^d	1.18	3.01	1.51	2.80	9.22
<i>Mitsuyou-23</i> ^d	1.65	3.94	2.84	3.30	10.71

^a Data are the means of triplicate determinations.

^b 70% ethanol (E-OH), 55% propanol (P-OH), 60% isopropanol (iP-OH), 55% tert-butanol (tB-OH).

^c Percent on a dry weight basis.

^d Modern rice cultivars.

tervals of 3 or 4 d during a period of 40 d after flowering (DAF). The harvested panicles were immediately frozen at -20°C until analysis by SDS-PAGE.

Extraction of prolamin and glutelin. The respective defatted rice flours from 10 cultivars (Table 1) were suspended with 10 volumes (v/w) of 20 mM phosphate buffer (pH 7.3) containing 0.5 M NaCl and stirred for 2 h at room temperature. After centrifugation at $21,000\times g$ for 20 min, the pellets were washed twice with water and freeze-dried. Then, prolamin was extracted as follows: the salt-insoluble pellets were separately stirred with 10 volumes (v/w) of aqueous alcohols such as 70% ethanol, 55% propanol, 60% isopropanol, and 55% tert-butanol for 1 h at room temperature, and centrifuged at $21,000\times g$ for 20 min at 4°C . The supernatants were dialyzed against tap water for 48 h and against distilled water overnight. Prolamin fractions were obtained as pellets by centrifugation. These prolamins were washed twice with water, freeze-dried and kept at -20°C .

The aqueous alcohol-insoluble pellet was washed three times with acetone and air-dried. The pellet thus obtained was looked upon as crude glutelin. The precipitation of glutelin was done as follows: the crude glutelin preparations from *Koshihikari*, *Tsushimazairai* and *Hiyoku-mochi* were stirred with 10 volumes (v/w) of 2% SDS and/or 5 M urea with or without 2-mercaptoethanol (2-ME) or 10 volumes (v/w) of 0.1% NaOH or 0.05% acetic acid for 1 h (but 30 min in the case of urea) at room temperature. The extracts were centrifuged at $21,000\times g$ for 20 min at 4°C (0.05% CH_3COOH or 0.1% NaOH) or 15°C (2% SDS and/or 5 M urea). The supernatants were neutralized and dialyzed against distilled water overnight and the resulting glutelin precipitates were freeze-dried and kept at -20°C . Supernatants of the SDS and urea extracts were

mixed with two volumes of acetone and allowed to stand for several hours at room temperature. The resulting glutelin sediments were washed three times with acetone, air-dried and stored at -20°C .

SDS-PAGE and two-dimensional gel electrophoresis (2-DE). SDS-PAGE was done with 15 or 25% polyacrylamide gels prepared essentially according to the method of Laemmli (13). The gels were stained with Coomassie blue R250 and destained with 10% acetic acid-20% isopropanol. 2-DE was done according to the method of O'Farrel et al. (14), in which DE was first conducted by electrofocusing with ampholine (Pharmacia) at a pH of 3.5–10 in a vertical column (90×2 mm) and secondly by SDS-PAGE with 15% polyacrylamide gel.

Chemical analysis. Protein was determined by the micro-Kjeldahl method with the conversion factor of 5.95 (15), or by Lowry's method (16) using bovine serum albumin as the standard. Amino acid composition was determined with a Hitachi 835 amino acid analyzer after acid hydrolysis in the usual way.

RESULTS AND DISCUSSION

Accumulation profiles of storage proteins in ancient rice cultivars

We first tried to study the maturing process of rice seed proteins in the endosperm. For this reason, the profiles of storage protein accumulation during the grain-filling period of *Kuro-mochi* and *Tsushimazairai* were analyzed by SDS-PAGE (Fig. 1A and B). On about 5 DAF, several distinct polypeptides with MWs of 52, 32, 21, and 15 kDa appeared. It has been reported by Kim et al. (17) that rice protein accumulation occurs between 3 and 8 DAF, accompanied with a dramatic increase in 32, 21 and 15 kDa. These accumulation profiles were also identical to the findings of Yamagata et al. (18), Luthe (19) and Zhao et al. (20), indicating that both 32 and 21 kDa subunits corresponded to rice glutelin α and β , respectively. The 15 kDa corresponded to rice prolamin, which was apparently one of the major polypeptides in *Kuro-mochi* (Fig. 1A), while *Tsushimazairai* showed two prolamin species with MWs of 15 and 13 kDa (Fig. 1B). A similar variation was also reported by Ogawa et al. (9), who observed that prolamin was composed of three subunits with MWs of 10, 13, and 16 kDa in *Nihonsakari*. It remains to be further investigated whether or not prolamin is inherently monomeric or polymeric.

Extraction efficiency of prolamin and glutelin from various rice seeds

The efficiency of extracting prolamin preparations from 5 ancient rice cultivars and 5 modern ones with 4 kinds of aqueous alcohols are compared in Table 1. Although there was a wide range of total protein contents (7.6–11.4%) among the cultivars, the most efficient was 55% propanol, by which 3.3–7.8% of the total protein was extractable. With 55% tert-butanol, 2.6–4.9% of the total protein was extracted. Neither 70% ethanol nor 60% isopropanol was very efficient in the extraction of rice prolamin. Shewry and Mifflin (5)

have referred to 50% propanol>55% isopropanol>60–70% ethanol in order of extraction efficiency. Sugimoto et al. (10) demonstrated that 55% propanol was the most effective in the extraction of rice prolamin among 5 kinds of aqueous alcohols. SDS-PAGE of prolamin preparations from *Kuro-mochi* by the 4 kinds of aqueous alcohols revealed only one polypeptide component with a MW of 15 kDa (Fig. 2), suggesting that the 15 kDa

subunit was in common with the aqueous alcohol-soluble “prolamin” component.

Crude glutelin preparations were obtained from 3 cultivars different in protein content (in %): *Tsushimazairai* (11.4), *Koshihikari* (8.2) and *Hiyoku-mochi* (7.6%). Glutelin was extracted with the various solvents shown in Table 2. More than 87% of the total protein could be extracted with NaOH, SDS, SDS+2-ME or SDS+urea+2-ME; above all, a combination of 2% SDS+5 M urea+0.5% 2-ME was the most effective in glutelin extraction from 3 rice cultivars, which was actually improved slightly by the addition of 2-ME. Cereal glutelin fractions usually contain starch, which gelatinizes the cereal sample in the presence of urea and thereby makes the glutelin unextractable (21, 22).

SDS-PAGE patterns about glutelin from these ancient rices are compared in Fig. 3A and B. In panel A, SDS-PAGE of the extracts with 2% SDS (lane 1), 2% SDS+0.5% of 2-ME (lane 3) and 0.1% NaOH (lane 5) demonstrated that glutelin was composed of two major polypeptides with MWs of 32 and 21 kDa as described

Fig. 1. Accumulation profiles of the storage proteins during the development of seeds of ancient rice cultivars. *Kuro-mochi* (A) and *Tsushimazairai* (B). Numbers at the bottom represent the days after flowering (DAF). M lane, marker proteins. MWs at both ends.

Fig. 2. SDS-PAGE of prolamin fraction extracted from ancient rice cultivars *Akita-kuro* with aqueous alcohols. 55% propanol (lanes 1 and 5); 70% ethanol (lane 2); 60% isopropanol (lane 3); and 55% tert-butanol (lane 4).

Table 2. Efficiency^a of glutelin extraction from ancient and modern rice cultivars with 7 solvents.^b

Solvent ^c	<i>Tsushimazairai</i>	<i>Koshihikari</i> ^d	<i>Hiyoku-mochi</i> ^d
Total protein content (%) ^e	11.4	8.2	7.6
Extractive efficiency (% of total protein)			
2% SDS	88.3	87.0	87.6
2% SDS+0.5% 2-ME ^f	91.6	90.2	88.3
5 M Urea	2.7	3.5	2.5
5 M Urea+0.5% 2-ME ^f	16.1	17.1	16.5
2% SDS+5 M urea+0.5% 2-ME ^f	92.2	92.0	91.2
0.1% NaOH	94.2	92.0	88.3
0.05% CH ₃ COOH	0.5	0.4	0.5

^a Data are the means of triplicate determinations.
^b Insoluble pellets after extraction with 0.5 M NaCl-55% propanol were dissolved in each solvent at a final concentration of g/10 mL by stirring for 60 min at room temperature and treated as described in Materials and Methods.
^c Solvents containing SDS (2%) and/or urea (5 M) were dissolved in 10 mM Tris/HCl buffer of pH 8.5.
^d Modern rice cultivars.
^e On a dry weight basis.
^f 2-Mercaptoethanol.

previously (18–20). In panel B, however, SDS-PAGE of the extracts and pellets with acetic acid (lane 1) and urea (lane 3) displayed not the vary extracts but insoluble pellets gave glutelin components much more (lanes 2 and 4). Protein was more effectively extracted with a

combination of SDS and urea (lanes 5), only reflecting efficiency with SDS. Consequently, the addition of 2% SDS to a 10 mM Tris/HCl buffer of pH 8.5 was considered useful for the extraction of glutelin from ancient rice. Although 0.1% NaOH seemed more effective than 2% SDS, its use brought about degradation of the sample proteins (5).

To confirm whether SDS-PAGE patterns for the 15 kDa prolamin and two glutelin polypeptides hold true to other ancient rice cultivars, 55% propanol-soluble prolamin and 2% SDS-soluble glutelin were sequentially prepared from 14 rice cultivars (8 ancient and 6 modern rice lines) and judged by SDS-PAGE. As shown in Fig. 4A, 10 cultivars contained a major 15 kDa subunit with or without a minor one (lanes 3–7, 9–11, 13–15), while 4 cultivars provided double bands equal in amount (lanes 2, 8, 12, 13). Horikoshi et al. (23) have demonstrated that *Nihonbare* prolamin consists of a single polypeptide (131 residues) with a MW of 14,930. Li and Okita (8) have isolated the gene coding 15 kDa prolamin from rice line M201. These data suggest that the 15 kDa prolamin is a common component occurring in both ancient and modern rice cultivars. Likewise, SDS-PAGE profiles of the 14 samples of glutelin revealed that glutelin was comprised mainly of two subunits, α and β , in common with ancient and modern rice cultivars (Fig. 4B).

Physicochemical properties of the major components of 55% propanol-soluble prolamin and 2% SDS-soluble glutelin

Horikoshi et al. (23) previously demonstrated that *Nihonbare* 15 kDa prolamin gave five bands with PIs of 5.6, 7.1, 7.3, 7.6, and 7.8 on isoelectricfocusing. This profile was exactly like that reported by Padhye and Salunkhe (24). In this connection, *Akita-kuro* prolamin was purified by preparative SDS-PAGE (25) and subjected to 2-DE. As shown in Fig. 5, several bands appeared in the pH range of 5.5 to 6.5, and 2-DE of 2%

Fig. 3. SDS-PAGE of glutelin fraction extracted from ancient rice *Akita-kuro* with various solvents. The crude glutelin pellets, after removal of prolamin, were suspended in 10 volumes and stirred for 60 min at room temperature. Aliquots of the supernatants (odd numbers) were applied to SDS-PAGE plate. The insoluble residues were redissolved for 5 min in boiled buffer and their supernatants were used (even numbers). A: lanes 1 and 2, 2% SDS; lanes 3 and 4, 0.1% NaOH; lanes 5 and 6, 2% SDS+5 M urea+0.5% 2-ME. B: lanes 1 and 2, 0.05% acetate; lanes 3 and 4, 5 M urea; lane 5, 2% SDS+5 M urea+0.5% 2-ME; lane 6, the supernatant of 5 M urea+0.5% 2-ME.

Fig. 4. SDS-PAGE of 55% propanol-soluble prolamin (A) and 2% SDS-soluble glutelin (B) from ancient (8 cv.*) and modern (7 cv.) rice cultivars: 1, *Kinuhikari*; 2, 10, *Koshihikari*; 3, *Takasago-mochi*; 4, *Mangetsu-mochi*; 5, *Aizen-mochi*; 6, *Kuro-mochi**; 7, *Akita-kuro**; 8, *Murasaki-kuro**; 9, *Kuro-73**; 11, *Midorimai**; 12, *Bluebonnet*; 13, *Mitsuyou-23*; 14, *Kaori-eigou**; 15, *Kaori-mochi**; 16, *Kunitsukasa-chukann**.

Fig. 5. Two-dimensional gel electrophoresis of 55% propanol-soluble prolamin (A) and 2% SDS-soluble glutelin (B) from *Akita-kuro*. A sample protein was dissolved (1 mg/100 μ L) in the lysis buffer (18) and 20 μ L of the supernatant was applied to a vertical gel. The first dimension was conducted by isoelectrofocusing with a pH of 3.5–10 ampholine. The second dimension was done by SDS-PAGE in 15% polyacrylamide gel.

Table 3. Comparison of prolamin and glutelin contents in 12 ancient rice cultivars^a.

Rice cultivar	Percentage of total protein		Total protein content (%) ^b
	Prolamin	Glutelin	
<i>Midorimai</i>	5.5	88.4	6.9
<i>Benimiyako</i>	5.8	81.2	7.3
<i>Takaramitsu</i>	4.7	79.0	7.5
<i>Kaori-eigou</i>	4.1	73.9	7.8
<i>Kaori-mochi</i>	4.2	82.9	8.5
<i>Benihikari</i>	5.3	90.1	8.5
<i>Akita-kuro</i>	6.2	87.5	8.8
<i>Kunitsukasa-chukann</i>	7.4	86.7	8.9
<i>Mangetsu-mochi</i>	6.6	82.6	9.6
<i>Kuro-mochi</i>	7.8	88.6	9.6
<i>Kuro-60</i>	5.2	88.7	9.6
<i>Tsushimazairai</i>	4.7	90.6	11.4
<i>Hiyoku-mochi</i> ^c	3.3	81.6	7.6
<i>Nobentadiablanco</i> ^c	4.4	78.7	7.9
<i>Koshihikari</i> ^c	5.5	87.5	8.2
<i>Akitakomachi</i> ^c	3.0	88.6	9.2
<i>Mitsuyou-23</i> ^c	3.9	78.9	10.7

^a Data are the means of triplicate determinations.
^b Total protein content (%) on a dry weight basis.
^c Modern rice cultivars.

Fig. 6. Correlation between total protein and prolamin (above) or glutelin (below) extracted from ancient and modern rice cultivars. Regression lines were calculated by the least squares method barred on plots of prolamin ($Y = -2.93 \times 10^{-2} + 5.43 \times 10^{-2} X$, $R^2 = 0.234$) or glutelin ($Y = -0.907 + 0.947 X$, $R^2 = 0.875$) against the total protein given in Table 3.

SDS-soluble glutelin revealed that two 21 and 32 kDa bands were acidic and basic multicomponents, respectively (Fig. 5B). This heterogeneity coincided with previous observations by other workers (18–20) irrespective of the extraction of glutelin with 0.1% NaOH or 2% SDS. There was a relationship between the total protein and two storage proteins in the Kuromai (black rice) and Akamai (red rice) cultivars (Table 3). The recovery of prolamin also varied 3.0 to 7.8% with total protein contents ranging from 6.9 to 11.4%, but there was no or little correlation between them as shown in Fig. 6A. On the contrary, glutelin accounted roughly for an increase in the total protein content (Fig. 6B). The ratio of prolamin to glutelin was 4.9 : 78.1 for 12 ancient cultivars, being in fair agreement with those reported by Cagampang et al. (26). The protein content in the modern rice cultivars was nearly as much as in the ancient cultivars.

Amino acid compositions of prolamin and glutelin from six ancient rice cultivars

Amino acid compositions of the prolamin and glutelin fractions from 6 ancient rice cultivars including 3 Kuromai and 3 Akamai lines are shown in Tables

Table 4. Amino acid composition of 55% propanol-soluble prolamin^a from 6 ancient rice cultivars.

Amino acid	Kuromai ^b			Akamai ^c		
	K ₁	K ₂	K ₃	A ₁	A ₂	A ₃
	mol %					
Asx ^d	6.3	5.7	4.2	6.5	6.2	5.9
Thr	2.6	3.6	4.1	2.4	2.6	2.2
Ser	5.7	5.9	7.0	5.5	5.6	5.5
Glx ^d	21.1	20.7	24.5	21.9	21.1	26.5
Pro	3.6	4.4	6.0	4.9	5.8	4.4
Gly	6.1	8.3	5.0	5.6	5.7	5.5
Ala	10.7	12.4	9.4	9.9	9.9	9.3
Val	7.1	7.4	6.7	7.3	7.1	6.8
Cys	0.1	0.3	1.5	0.1	0.1	0.1
Met	0.0	0.1	1.0	0.0	0.1	0.1
Ile	5.2	5.6	5.1	5.2	5.2	4.7
Leu	14.2	12.7	10.8	13.9	13.5	12.8
Tyr	5.8	0.9	4.2	5.7	5.9	5.6
Phe	5.3	5.2	5.3	5.3	5.1	4.9
Lys	0.4	1.2	0.2	0.1	0.3	0.2
His	1.2	1.2	1.3	1.1	1.2	1.1
Arg	4.7	4.2	3.8	4.7	4.7	4.6
Protein content ^e	9.64	8.90	9.58	8.66	8.50	8.87

^a Data (in mol%) are the means of duplicate determinations.^b Kuromai (black rice lines), K1-K3: *Akita-kuro*, *Murasaki-kuro* and *Kuro-mochi*.^c Akamai (red rice lines), A1-A3: *Takaramitsu*, *Benimiyako* and *Kunitsukasa-chukam*.^d Aspartate plus asparagine and glutamate plus glutamine, respectively.^e Total protein content (%) on a dry weight basis.

4 and 5. The prolamin fraction contained glutamate plus glutamine, alanine and leucine at high levels, but lysine and sulfur amino acids at low levels. The glutelin fraction was also characteristic of rice in this respect as described previously (6, 23, 24, 26). As a matter of course, there was no difference in amino acid composition between Kuromai and Akamai rice lines. When a comparison was made between the highest and lowest values of a given amino acid concerning the above ancient varieties according to the procedure of Kennedy and Schelstraete (27), the difference was to be much greater in prolamin than in glutelin; for example, in Table 4, the greatest difference was found with respect to Tyr in A₂, where the highest value (5.9 mol%) among the six varieties was 6.56-fold greater than the lowest value (0.9 mol%) in K₂. With respect to Thr in K₃, the highest value (4.1 mol%) among the six varieties was 1.86-fold greater than the lowest (2.2 mol%) in A₃. The same over-rate was found with respect to Pro (the highest 6.0 mol% in K₃, and the lowest 3.6 mol% in K₁). Additionally, the highest value for Gly (8.3 mol% in K₂) was 1.67-fold greater than the lowest value (5.0 mol% in K₃), and the highest value for Asx (6.5 mol% in A₁) was 1.55-fold greater than the lowest value (4.2 mol% in K₃). The differences were found with

Table 5. Amino acid composition of 2% SDS-soluble glutelin^a from 6 ancient rice cultivars.

Amino acid	Kuromai ^b			Akamai ^c		
	K ₁	K ₂	K ₃	A ₁	A ₂	A ₃
	mol %					
Asx ^d	8.2	8.7	8.4	7.7	8.0	8.0
Thr	4.0	4.5	5.8	3.8	4.4	3.9
Ser	6.4	6.2	6.5	7.1	6.6	6.8
Glx ^d	17.9	17.7	17.3	17.4	17.1	18.7
Pro	5.8	4.3	7.0	6.2	5.6	4.6
Gly	8.3	8.8	8.0	7.9	9.0	7.8
Ala	8.4	7.7	7.2	7.0	8.2	8.5
Val	7.2	7.2	7.0	7.0	6.8	7.1
Cys	0.3	0.2	0.2	0.2	0.2	0.1
Met	0.2	0.2	0.3	0.3	0.2	0.1
Ile	4.9	5.3	4.7	5.0	4.8	5.1
Leu	8.7	8.4	7.8	9.1	8.1	9.4
Tyr	2.6	2.6	2.3	3.4	2.7	2.6
Phe	5.1	5.4	5.0	5.2	4.8	5.2
Lys	2.8	3.4	3.5	2.6	3.3	2.5
His	1.8	1.9	2.0	2.1	2.2	2.0
Arg	7.5	7.6	7.1	7.2	8.1	7.5
Protein content ^e	9.64	8.90	9.58	8.66	8.50	8.87

^a Data (in mol%) are the means of duplicate determinations.^b K₁-K₃ are ibidem in Table 4.^c A₁-A₃ are ibidem in Table 4.^d Being ibidem in Table 4.^e Total protein content (%) on a dry weight basis.

respect to Cys and Met, which were minor amino acids of prolamin. Taken altogether, in the prolamin fraction, differences over 30% between the highest and lowest values were observed for 10 amino acids such as Met, Cys, Lys, Tyr, Thr, Pro, Gly, Asx, Ala and Leu. Alternatively, in the glutelin fraction (Table 5), only 4 amino acids including Pro (63%), Thr (53%), Thy (48%) and Lys (40%) exhibited a difference over 30% for the other 11 amino acids, except Met and Cys, where differences averaged 14%. Met and Cys ranged from 0.1 to 0.3 mol% of glutelin, thus it was difficult to calculate the precise value.

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