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PROPERTIES OF PROLAMIN IN MATURE AND DEVELOPING RICE GRAIN*

BERNARDITA E. MANDAC and BIENVENIDO O. JULIANO

Chemistry Department, The International Rice Research Institute, Los Baños, Laguna, Philippines

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Key Word Index—*Oryza sativa*; Gramineae; rice prolamin; amino acid composition; disc electrophoresis.

Abstract—A procedure was developed for preparing lipid- and phenol-free prolamin directly from IR480-5-9 milled rice (*Oryza sativa* L.). The preparation consisted mainly of one protein band on analytical and SDS-polyacrylamide disc gel electrophoresis with subunit MW of 17000 and a minor fraction with subunit MW 23000. The prolamin eluted as a single peak on SDS-Sephadex G-75 gel filtration and on DEAE-cellulose column chromatography. Prolamin was poor in lysine, histidine, cystine, and methionine but rich in glutamic acid, tyrosine and proline. In dehulled developing grain of two different rices, changes in the aminogram of the prolamin fraction coincided with the start of endosperm protein body synthesis and the appearance from 7 days after flowering of a second prolamin subunit with MW 23000.

INTRODUCTION

Prolamin, or alcohol-soluble protein, is a major storage protein in all cereals, except rice and oats [1]. It is the principal protein of endosperm protein bodies in cereals where the matrix protein is mainly glutelin [1]. However, prolamin constitutes only *ca* 3% of milled rice protein and has been shown to have one main subunit with MW 23000 [2]. As part of a study of proteins of mature and developing rice grain [2, 3], we studied the extraction and properties of prolamin from dehulled grain of the high-protein rice IR480-5-9 and the changes in properties of prolamin during grain development. Corresponding changes in properties of prolamin of a low protein rice IR26 during grain development were also examined.

RESULTS

Direct extraction of milled rice with 70% EtOH produced a prolamin preparation with very high UV absorption and high protein values by the Lowry procedure. Its major contaminants are 30.4% lipids and 6% phenols together with 1% carbohydrate. A similar preparation was obtained by 70% EtOH extraction from the residue left after extraction of albumin–globulin from milled rice. Addition of 0.5% NaOAc to the 70% EtOH [4] did not reduce the UV absorption of the extract. Polyclar AT (PVP) addition to the extract [5] only partially reduced the UV absorption of the EtOH solution. However, preextraction of rice flour with 95% EtOH removed most of the UV absorbing contaminants without extracting protein, as shown by the absence of any precipitate on TCA treatment. Crude prolamin was then extracted with 70% EtOH from the rice residue with only 0.97% phenols (Table 1).

Removal of the remaining contaminating lipid and phenols was effected by precipitation of prolamin from 70% EtOH with Me_2CO as reported earlier [6] (Table 1). Stripping of EtOH under reduced pressure and dialysis resulted in coprecipitation of lipid and phenol with prolamin. Lipid and phenols were mainly soluble in organic solvents such as Et_2O and $CHCl_3$–MeOH. The lipids were mainly (88%) neutral, eluted by $CHCl_3$ from a Si gel column, with lesser amounts of glycolipids and phospholipids. Phenols that complexed with prolamin were mainly esters of *p*-coumaric, ferulic and vanillic acids, which are the major phenolic acids of rice straw [7].

Purification of prolamin

DEAE-cellulose chromatography and SDS-Sephadex G-75 gel filtration were not effective in further purifying prolamin. The prolamin was eluted in DEAE cellulose at a NaCl concentration of 0.42 M. Analysis by both analytical and SDS polyacrylamide disc gel electrophoresis of the prolamin gave one major band together with one slower migrating band (Fig. 1). The MW of the prolamin subunit was 17000 (Table 1); a minor subunit was noted with MW 23000. The ratio of the two subunits of prolamin was *ca* 19:1 based on Amido Black staining. The subunit MW of the only prolamin fraction obtained by SDS-Sephadex G-75 chromatography was also 17000. The MW 23000 subunit was also found in lower concentration in the prolamin passed through Sephadex G-75.

Purification of prolamin was achieved by repeated precipitation with Me_2CO from 70% EtOH solution. The prolamin precipitate was redissolved in 0.025 N

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Table 1. Amino acid composition in g per 16.8 g N of rice prolamin preparations from IR480-5-9 milled rice and bran

	Milled rice prolamins*				Bran prolamin			
Protein and amino acid	Crude	PVP treated	Me_2CO ppt.	DEAE cellulose peak	Crude	Me_2CO ppt.	Milled rice	LSD† (5%)
Protein (%)	65.9	69.8	99.6		8.3	57.5	10.2	
Lys	0.30	0.18	0.12	0	0.88	1.52	3.51	0.15
His	1.21	1.08	1.08	0.02	1.46	1.33	2.46	0.17
Amm	3.30	3.44	3.70	4.84	5.39	2.96	2.48	0.51
Arg	5.77	5.82	6.10	5.63	2.74	5.68	8.52	0.30
Asp	8.26	8.29	8.16	7.60	17.2	9.86	10.2	1.09
Thr	2.35	2.36	2.27	2.21	1.94	3.10	3.85	0.45
Ser	4.92	5.55	5.42	5.16	3.96	5.24	6.07	0.81
Glu	31.8	29.4	29.0	25.2	28.8	25.9	20.8	3.71
Pro	6.42	6.26	5.56	6.08	3.28	5.45	4.64	0.68
Cys	0.08	0.50	0.24	0	1.15	2.44	1.84	0.34
Gly	2.91	2.96	3.08	2.90	3.96	7.42	4.60	0 53
Ala	7.25	7.18	7.10	6.66	4.17	7.79	5.85	0.92
Val	6.81	6.54	6.96	6.48	3.14	5.20	6.09	0.51
Met	0.53	0.56	0.15	0	0.42	0.99	1.59	0.49
Ile	5.04	4.74	4.86	4.98	1.72	3.71	4.20	0.49
Leu	13.9	15.0	13.7	12.8	3.06	11.6	6.76	1.21
Tyr	9.42	9.37	10.2	9.62	3.16	8.70	4.94	0.61
Phe	6.40	6.46	6.60	6.42	1.89	6.60	5.73	1.03
Trp	0.50	0.46	0.54	0.61	—	0.69	1.23	NS
Carbohydrate (%)	2.00	0.70	0.46	1.35	30.1			
Phenols (% as vanillin)	0.97	—	0.03	—	—	0.28	—	
MW	17000	17000	17000	17000	17000			

* With preextraction of the rice with 95% EtOH.
† Calculated for prolamin preparations.

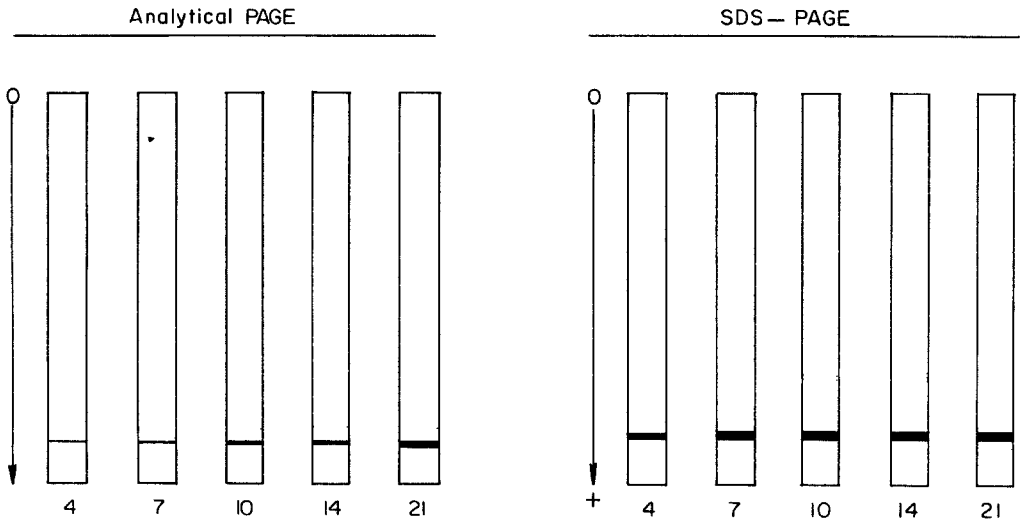

Fig. 1. Changes in analytical and SDS-polyacrylamide gel electrophoresis of crude prolamin from dehulled developing grain of IR480-5-9 rice. Numbers below the figures refer to days after flowering. Similar results were obtained for IR26 rice.

NaOH and made to 70% v/v with respect to EtOH then neutralized to pH 7 with 17 M HOAc.

The aminogram of prolamin showed characteristic low contents of lysine, histidine, arginine, threonine, cystine, methionine and tryptophan and high contents of glutamic acid, leucine and tyrosine in agreement with earlier studies [6] (Table 1). All the preparations had higher proline than was found in milled rice protein.

Purification did not change the electrophoretic pattern of prolamin but reduced the lipid and phenol contents (Table 1). There was also a slight decrease in the amounts of lysine and histidine (Table 1). The lowest lysine, histidine, methionine–cystine and glutamic acid values were observed on the prolamin purified by DEAE cellulose chromatography. A subunit MW of 17000 was obtained for all preparations. Previous results on IR480-5-9 prolamin gave a subunit MW of 23000 [2] and an even higher value using Bio-Gel P-300 [6]. Staining

the gels with Schiff's periodate reagent showed positive staining of crude prolamin for carbohydrate. The purified prolamin (after Me_2CO precipitation) did not stain for carbohydrate. Two protein bands were previously obtained by disc electrophoresis of rice prolamin both of which also stained for carbohydrate [6].

Crude and purified prolamins from rice bran had lower protein content and differed in amino acid composition from those of milled rice (Table 1); they had higher levels of lysine, histidine, aspartic acid, cystine and glycine but lower contents of valine, isoleucine, leucine and tyrosine. Only crude bran prolamin had lower contents of arginine, serine, proline, alanine and phenylalanine than crude milled rice prolamin. An electrophoregram of bran prolamin showed mainly one band, which had a subunit MW of 17000 but the MW 23000 was also present. Their ratio was also *ca* 19:1 based on Amido Black staining.

Prolamin in developing rice grain

Prolamin from dehulled developing grains of IR26 and IR480-5-9 showed only one protein band on analytical and SDS polyacrylamide gel electrophoresis 4 and 7 days after flowering (Fig. 1). A second, faint, slower-moving band (subunit MW 23000) was noted from 10 days after flowering onward for both rices. This band was also present in a prolamin preparation from mature rice endosperm. The ratio of the MW 17000 to MW 23000 subunits remained constant at 9:1 until maturity. In contrast to glutelin [8], prolamin content decreased progressively in the developing grain of both rices (Table 2). However, on a per-grain basis, prolamin increased from 23 to 41 μg in IR26 and from 28 to 115 μg in IR480-5-9.

Amino acid analysis of prolamin from the later grain-development stages showed a decrease in lysine, aspartic acid, threonine, alanine, valine and methionine and an increase in histidine, arginine and leucine in both rices (Table 2). Changes in the aminogram of prolamin coincided with similar changes in the aminogram of glutelin [8].

DISCUSSION

The purification of high nitrogen and low lipid and phenol content prolamin from IR480-5-9 milled rice indicates that the UV-absorbing contaminants and the lipid are not chemically linked to the prolamin. Phenolics are known to complex with protein [5] and the prior extraction of phenolics with 95% EtOH minimizes that interaction; phenolics and lipids are soluble in 70% EtOH. The high UV-absorption and high protein values given by the Lowry method in relation to Kjeldahl N and biuret N were consistent with contamination with phenol esters and with the high tyrosine content of the prolamin itself, which was *ca* twice that of milled rice protein (Table 1).

The phenols and lipid presumably are also more soluble in 70% EtOH than in H_2O and coprecipitate with prolamin on removal of the EtOH. Me_2CO precipitation of the prolamin from the EtOH extract allows efficient purification of the prolamin, because the phenol and lipid remain in solution.

In dehulled developing grain of IR26 and IR480-5-9, changes in the aminogram of prolamin coincided with the start of endosperm protein body synthesis [1] and with the appearance of a second prolamin subunit with MW 23000 from 7 days after flowering. Evidently the prolamin of brown rice and milled rice are electrophoretically identical, with a major subunit MW 17000 and a minor subunit MW 23000. The bran prolamin also had the MW 23000 subunit. In terms of amino acid composition, however, IR480-5-9 milled rice crude prolamin had higher glutamic acid, proline and valine, and less NH_3 than brown rice crude prolamin (Tables 1 and 2).

Table 2. Changes in content and amino acid composition (g/16.8 g N) of brown rice prolamin of IR26 and IR480-5-9 during grain development

Amino acid	IR26					IR480-5-9					LSD (5%)
	days after flowering					days after flowering					
	4	7	10	14	21	4	7	10	14	21	
Prolamin content (%)	1.1	0.7	0.6	0.4	0.3	1.1	0.8	0.7	0.4	0.5	
Lys	2.80	2.07	1.90	1.81	1.28	1.62	1.38	1.30	0.66	0.33	0.15
His	0.09	0	0.06	0.08	0.48	0.10	0.01	0.04	1.83	1.04	0.17
Amm	4.17	4.65	5.64	5.32	5.04	6.30	6.04	6.02	5.19	6.20	0.51
Arg	2.91	2.01	2.08	2.02	3.99	1.68	1.72	2.15	4.82	4.97	0.30
Asp	11.0	9.20	8.44	7.75	7.12	11.2	10.8	9.70	7.17	7.62	1.09
Thr	4.20	3.72	3.50	3.36	3.12	3.09	2.98	2.96	3.00	2.16	0.45
Ser	6.64	5.54	5.48	5.62	5.53	5.58	5.22	5.44	5.60	5.22	0.81
Glu	19.1	18.0	20.3	23.9	26.4	25.5	24.7	23.7	26.7	22.8	3.71
Pro	5.73	5.60	5.28	5.26	4.16	4.96	5.00	5.08	3.86	4.50	0.68
Cys	0.34	0.50	0.54	0.70	0.70	0.30	0.42	0.58	1.23	0	0.34
Gly	5.09	5.38	5.24	4.94	4.72	4.13	4.11	3.95	3.19	3.02	0.53
Ala	13.4	13.8	13.7	13.2	11.0	13.4	13.6	12.2	7.36	6.44	0.92
Val	8.51	7.99	7.40	6.64	6.32	7.15	7.15	6.50	6.50	5.38	0.51
Met	2.57	1.68	1.68	1.52	0.99	1.62	1.65	1.42	1.35	0.39	0.49
Ile	4.43	4.38	4.00	4.06	4.05	3.48	3.61	3.57	4.94	3.90	0.49
Leu	6.26	6.47	6.39	6.94	8.52	4.68	5.43	6.00	9.75	10.8	1.21
Tyr	6.46	7.22	6.61	5.76	6.66	4.00	4.04	4.24	7.74	8.46	0.61
Phe	4.91	4.49	4.14	3.98	4.82	3.47	3.70	3.70	5.32	5.94	1.03

Since the minor protein band started appearing at the same age of the developing grain in the analytical and SDS-polyacrylamide gel electrophoresis, they probably are the same prolamin fraction. Prolamin of wheat is reported to have mainly intramolecular disulfide bonds [9].

EXPERIMENTAL

Samples of IR480-5-9 rice were obtained from the 1976 dry season crop of the Institute. They were dehulled and milled in Satake machines. Developing grains of IR26 and IR480-5-9 were also obtained from the 1976 dry season crop, sorted, freeze-dried and dehulled by hand. The grain samples were ground in a Udy cyclone mill with 60-mesh sieve and passed through a 100-mesh sieve. The material for bran prolamin extraction was the outer 6% milling fraction of IR480-5-9 milled rice.

Extraction of prolamin. Milled rice flour (150 g) was stirred with 600 ml 95% EtOH at 25° for 30 min, allowed to settle for 10 min and the supernatant siphoned through a fine-porosity filter stick. The residue was extracted with 600 ml 70% EtOH for 60 min at 25°, allowed to settle for 10 min and the prolamin extract obtained as above. The 70% EtOH extract was then treated with 12.5 g polyclar AT (PVP)/l. for 90 min and filtered. Protein from the prolamin extract was pptd either by dialysis against H_2O or by the addition of 3 vol. of Me_2CO and collected by centrifuging for 20 min at 15000 *g*.

Identification of impurities. For the study of complexed phenols and lipid, milled rice was extracted directly with 70% EtOH, the prolamin extract was concd under red. pres. and the prolamin suspension dialyzed against H_2O. The prolamin complex was redissolved in 25 mM NaOH, the soln made 70% with EtOH and the pH adjusted to 7 with 17 M HOAc. Prolamin was pptd by the addition of 3 vol. of Me_2CO and the supernatant fluid concd to a small vol. under red. pres. The conc soln was used directly for the lipid and phenol identification. Lipid was extracted from this conc soln with $CHCl_3$–MeOH (2:1), washed with H_2O, made anhydrous with Na_2SO_4 and fractionated on a Si gel column by extracting neutral lipid with $CHCl_3$, glycolipid with Me_2CO and phospholipid with MeOH [10]. The fractions were evapd to dryness and weighed. For phenol extraction [7, 11, 12] the soln was pre-extracted with Et_2O. The aq. phase was acidified with HCl to pH < 2 and re-extracted with Et_2O, and this Et_2O phase was used for phenolic acid characterization by TLC. The first Et_2O extract was evapd to dryness, saponified with 5% $NaHCO_3$, acidified to pH 3 and extracted with Et_2O. The Et_2O extract was also evapd to dryness for TLC

Purification of prolamin. Purification of the prolamin ppt was attempted by Sephadex G-75 gel filtration (3 × 90 cm) using 0.1 M Tris–HCl buffer pH 9.3 with 0.6% SDS as solvent and eluent. A portion of the prolamin prepn was dissolved in 0.025 N NaOH, the pH adjusted to 8.9 with 0.1 N HCl and passed through a Whatman DE-52 DEAE cellulose column (3 × 30 cm) using 0.1 M Tris–HCl buffer pH 9 eluent and a 0 to 1 M NaCl gradient [13]. Purification of prolamin was achieved by redissolving the prolamin ppt. in 25 mM NaOH, the soln made 70% v/v with EtOH and the pH adjusted to 7 with 17 M HOAc before pptn with 3 vol. Me_2CO. Pptn was repeated twice.

Analytical methods. Protein content was determined by *A* at 280 nm, Lowry protein [14] and by the biuret test [15]. Amino acid analysis and analytical and SDS-polyacrylamide disc gel electrophoresis were as previously described [2, 3]. Analytical gel electrophoresis used 7.5% gel and 25 mM Tris–192 mM glycine electrode buffer pH 8.3. SDS-gel electrophoresis used 10% gel, BDH SDS and 25 mM Tris–192 mM glycine–0.1% SDS electrode buffer pH 8.3. Gels were stained with either 0.5% Amido Black in 7% HOAc or 0.0125% Coomassie Brilliant Blue G-250 in 12% TCA. Carbohydrates were detected in the gels with Schiff's–periodate reagent [16]. Phenolic acids were quantitatively assayed by the Folin–Denis reagent with vanillin as standard [17] TLC of phenolic acids on Si gel G coated plates used C_6H_6–MeOH–HOAc (45:8:4) and C_6H_6–dioxane–HOAc (95:25:4) as solvents and 1% diazotyzed sulfanilic acid reagent and 2 M NaOH (UV light) as spray reagents [7, 11, 12]. Carbohydrate content was assayed by the anthrone method [18] with glucose as standard and calculated as glucose × 0.9.

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