

Characterization of Poorly Digested Protein of Cooked Rice Protein Bodies

ADORACION P. RESURRECCION,¹ XINGXIANG LI,² THOMAS W. OKITA,² and BIENVENIDO O. JULIANO¹

ABSTRACT

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Poorly digested protein bodies from cooked IR58 milled rice were prepared by destarching with *Aspergillus oryzae* α -amylase followed by one or two pepsin treatments. The preparation was further purified by gel filtration through a Bio-Gel A-5m column with 0.5% sodium dodecyl sulfate in 0.05M Tris-HCl (pH 8.6) buffer as eluant. The major polypeptide

had a high sulfur amino acid content and a molecular size of about 13 kDa, slightly smaller than that of the 15-kDa rice prolamin. The 13-kDa polypeptide was identified as a prolamin based on Western blot analysis, and it is probably a proteolytic product of the class of 15-kDa rice prolamins that are rich in sulfur-containing amino acids.

Protein bodies of rice endosperm are readily distinguished by their morphology and their distinct proteins. As observed under an electron microscope (Harris and Juliano 1977, Bechtel and Pomeranz 1978, Bechtel and Juliano 1980, Tanaka et al 1980, Ogawa et al 1987, Ogawa et al 1989), the rice endosperm accumulates two types of protein bodies (PB) designated as type I and type II. PB I displays a spherical shape; PB II exhibits an irregularly shaped, electron-opaque crystalline morphology. Biochemical fractionation studies by Tanaka et al (1980) and immunocytochemical studies by Krishnan et al (1986) have shown that PB I is highly enriched with prolamin, whereas PB II contains predominantly glutelin storage proteins.

Although raw rice protein is almost 100% digestible in rat feeding experiments, cooking reduces the digestibility of rice to 85-90% (Eggum et al 1977). Similarly, cooked rice protein is also about 85-90% digestible in humans (Juliano 1985). The poorly digested protein fractions display glutelinlike properties in that they are relatively insoluble. Electron microscopic analysis of the undigested protein fraction revealed that it consists largely of an electron-dense core region from the large spherical PB I (Tanaka et al 1975a, 1978) and may constitute up to 16% of the total dietary rice protein (Tanaka et al 1975b, 1978).

PB I (20% of total protein) contains three different prolamin classes based on molecular size and solubility in alcohol solutions with or without reducing agents. The predominant class is composed of polypeptides with molecular sizes of 13 kDa (Sugimoto et al 1986, Ogawa et al 1987) or 14 kDa (Krishnan and Okita 1986) that are readily solubilized in 70% ethanol or 60% 1-propanol solutions. Analysis of the 13- and 14-kDa prolamin cDNA sequences reveals that the actual molecular size of the encoded polypeptides is 15 kDa (Kim and Okita 1988). We will use this value to designate this major prolamin class. Two other classes, the 10- and 16-kDa prolamins, are also present in PB I preparations, but at much lower levels. These two proteins, particularly the 10-kDa prolamins, have a much higher methionine and cysteine residue content than that of the 15-kDa prolamins (Hibino et al 1989) and require reducing agents for solubilization in alcohol

solutions. The identity of one 15-kDa species, which is also extracted with 1-propanol and 2-mercaptoethanol, was not verified (Ogawa et al 1987). In addition to these three prolamin classes, which account for about 60% of the total protein in PB I, there is also a 15-kDa glutelinlike polypeptide solubilized in 2% sodium dodecyl sulfate (SDS), 8M urea, 5% 2-mercaptoethanol that accounts for about 30% of the total protein in PB I (Ogawa et al 1987).

The nature of the polypeptides inside the protease-resistant inner core of the large spherical PB I is not known. Immunocytochemical data suggest that proteins are not randomly distributed because antibodies raised against the 15-kDa prolamins had a higher reaction (about threefold) in the periphery than in the core region (Krishnan et al 1986). In contrast, the small spherical PB I shows uniform staining with the anti-15-kDa prolamin. This evidence suggests that the bulk of the 15-kDa prolamins are packaged around some unidentified core proteins.

To identify the nature of these core proteins of the large spherical PB I, we have studied the properties of the poorly digested protein bodies of cooked milled rice after one and two pepsin treatments. We used IR58 milled rice because it was previously employed in the study of protein requirements in preschool children (Cabrera-Santiago et al 1986) and in the study of rice-legume diets in rats (Eggum et al 1987).

MATERIALS AND METHODS

Pepsin-treated cooked rice PBs were prepared from cooked IR58 milled rice (11% wb protein; N \times 5.95) after destarching with *Aspergillus oryzae* α -amylase (Mycolase, Wallerstein Co., Deerfield, IL). The resulting PB preparation was incubated once, or twice, in pepsin (ICN, Cleveland, OH 10,000 units/mg) for 5 hr (Tanaka et al 1978, Resurreccion and Juliano 1981). Protein from pepsin-treated PBs was extracted by stirring overnight in 2.5% SDS with 0.6% 2-mercaptoethanol at 25°C and heating at 100°C for 5 min. The protein extract was chromatographed through a Bio-Gel A-5m column (2.6 \times 90 cm, Bio-Rad Laboratories, Richmond, CA) at 25°C with 0.5% SDS in 0.05M Tris-HCl (pH 8.6) as eluant at a flow rate of 3.4 ml/cm² per hour. The major protein fractions, measured by UV absorption at 280 nm, were exhaustively dialyzed against distilled water and freeze-dried.

The purity of the protein fraction samples from gel filtration was assessed by SDS-polyacrylamide gel electrophoresis (PAGE)

¹Plant Breeding, Genetics and Biochemistry Division, International Rice Research Institute, Manila, Philippines.

²Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340.

using 10–15%, a 10–20% gradient, or a 17% acrylamide gel from Wako Pure Chemical Industries Ltd. (Tokyo, Japan), and analytical isoelectrofocusing (IEF) in polyacrylamide gels containing 7.5% acrylamide, 2M urea, and 2% ampholyte, pH 3–10 (du Cros et al 1979). The samples were dissolved in 3M urea and 1% 2-mercaptoethanol in 2% ampholyte. The cathode strip was soaked in 1M NaOH, and the anode strip was soaked in 1M H₃PO₄.

The amino acid composition of the samples was determined after the samples were hydrolyzed in 6N HCl for 23 hr at 110°C, with or without performic acid oxidation. The resulting residues were then resolved and analyzed on LKB 4400 single-column analyzer with Ultropac 8 8- μ m-diameter anion exchange resin column (270 \times 4 mm, Pharmacia LKB, Uppsala, Sweden) and corrected for incomplete recovery of some P-40 amino acids (Houston et al 1969). Tryptophan was also determined by NaOH hydrolysis (Howe et al 1972), followed by amino acid analysis in the LKB 4400 analyzer. Nitrogen recoveries ranged from 85–95% and were recalculated to 100% N recovery.

Western Blot Analysis

Rabbit antibodies were raised against prolamins and glutelins prepared from M-201 brown rice flour according to Krishnan and Okita (1986). PBs of developing M-201 rice grains were obtained by pulverizing frozen 12- to 15-day-old developing M-201 rice seeds in a coffee mill with dry ice. The resultant powder was then suspended in buffer containing 20 mM Tris-HCl (pH 8.5), 50 mM MgCl₂, 100 mM KCl, 1 mM dithiothreitol, and 0.2M sucrose. It was strained through two layers of 80- μ m nylon mesh, and the filtrate was then centrifuged at 500 \times g for 5 min to remove nuclei and starch granules, and recentrifuged at 2,000 \times g for 10 min to obtain an enriched PB fraction pellet. The pellet was washed twice by resuspending the pellet in buffer and centrifuging at 2,000 \times g for 10 min. Electron microscopic examination of the washed pellet revealed numerous intact PBs with very few contaminant starch granules or free membranes (Li and Okita *unpublished observations*). The PB fraction was suspended in SDS sample buffer and analyzed by SDS-PAGE.

Western blot analysis was undertaken at Pullman, WA, on the undigested PB fractions using the methods of Okita et al (1988). Protein was extracted from undigested PBs with 2% SDS in 0.0625M Tris-HCl buffer (pH 6.8) plus 6M urea and boiled for

5 min. Extracts were cooled to room temperature, centrifuged at 12,000 \times g for 10 min, and brought to a 1% final concentration of 2-mercaptoethanol. Protein concentration was measured by absorbance at 214 nm using bovine serum albumin as a standard. Proteins (1–40 μ g) were resolved by SDS-PAGE, transferred to nitrocellulose membranes at 0.5 A for 8 hr using a buffer containing 20 mM Tris, 140 mM glycine, and 20% (v/v) methanol. The nitrocellulose membrane was then incubated with 5% powdered milk in a Tris-saline buffer containing 10 mM Tris-HCl (pH 7.4) and 0.9% NaCl for 30 min with agitation. It was then placed in the same solution with the prolamin or glutelin antibodies (36 μ g/ml) for 30–60 min. The nitrocellulose membrane was extensively washed with Tris-saline containing 1% (v/v) Nonidet P-40 (Gibco BRL, Grand Island, NY) and then incubated for 30 min with 1 μ Ci of ¹²⁵I-protein A (New England Nuclear, Boston, MA) in the same buffer. The filter was then washed as described above and analyzed by autoradiography using a DuPont Lightning Plus intensifying screen (E.I. DuPont de Nemours, Wilmington, DE). In all Western blot analyses, control reactions of the immobilized protein fractions were conducted using preimmune serum.

RESULTS AND DISCUSSION

PB recovery from whole, undigested, milled rice was 12% by weight, with 81% protein content (equivalent to 83% protein recovery from milled rice) (Table I). After one pepsin treatment, the residual protein was 22% of milled rice protein (27% of PB preparation) using a pepsin source other than that employed in the study by Tanaka et al (1978). A second pepsin treatment reduced the residual protein in undigested PBs to 13% of milled rice protein (16% of PB preparation). Fat and carbohydrate content of the residual material increased with the second pepsin treatment. Tanaka et al (1978) and Resurreccion and Juliano (1981) reported 28% recovery of protein in undigested PBs after pepsin digestion using Merck pepsin on IR480-5-9 milled rice. The extent of solubility of the undigested PBs (treated once with pepsin) in different solvents were 8% in 0.7M NaCl, 10% in 55% 1-propanol, and 6% in 55% 1-propanol with 0.6% 2-mercaptoethanol. These values differed from those of the corresponding protein fractions for IR58 raw milled rice, which were 10% in NaCl, 7% in 1-propanol, and 3% in 1-propanol with 2-mercapto-

TABLE I
Composition of IR58 Raw Milled Rice and of Protein Bodies from Whole and Poorly Pepsin-Digested Cooked Rice

Property or Amino Acid	Protein Bodies of Destarched Cooked Milled Rice			
	Raw Milled	Whole	Poorly Digested	
			One Pepsin Treatment	Two Pepsin Treatments
Recovery (g/100 g of milled rice)	100	12.0	4.3	3.0
Protein content, %	11.1 \pm 0.2 ^a	77.4 \pm 0.4	57.4 \pm 0.4	49.1 \pm 0.3
Fat content, % wet basis	2.4 \pm 0.1	7.4 \pm 1.7	18.8 \pm 2.5	21.4 \pm 0.5
Carbohydrate content, % wet basis	75.0 \pm 2.0	5.3 \pm 0.1	8.6 \pm 0.3	21.3 \pm 0.0
Alanine ^b	5.8 \pm 0.0	5.4 \pm 0.1	6.0 \pm 0.0	6.7 \pm 0.0
Arginine	9.5 \pm 0.3	9.0 \pm 0.1	6.5 \pm 0.0	6.1 \pm 0.1
Aspartic acid	9.2 \pm 0.3	9.0 \pm 0.1	7.4 \pm 0.1	7.6 \pm 0.0
Cysteine	3.1 \pm 0.1	3.0 \pm 0.1	4.1 \pm 0.2	3.3 \pm 0.0
Glutamic acid	17.5 \pm 0.2	15.3 \pm 0.1	17.9 \pm 0.1	21.4 \pm 0
Glycine	4.7 \pm 0.0	4.2 \pm 0.0	3.8 \pm 0	3.8 \pm 0.0
Histidine	3.1 \pm 0.1	3.0 \pm 0.0	2.5 \pm 0	2.3 \pm 0.0
Isoleucine	5.2 \pm 0.2	5.3 \pm 0.1	6.3 \pm 0.0	6.6 \pm 0.1
Leucine	9.1 \pm 0.2	8.5 \pm 0.0	10.2 \pm 0	11.3 \pm 0
Lysine	4.4 \pm 0.1	4.0 \pm 0	1.7 \pm 0.1	0.8 \pm 0.0
Methionine	2.3 \pm 0.0	2.2 \pm 0.1	2.6 \pm 0.2	2.2 \pm 0.4
Phenylalanine	7.1 \pm 0.1	6.9 \pm 0.1	7.8 \pm 0.1	7.7 \pm 0.0
Proline	4.3 \pm 0.0	4.2 \pm 0.1	4.2 \pm 0.2	6.4 \pm 0.1
Serine	5.4 \pm 0.1	5.0 \pm 0.1	5.7 \pm 0.0	7.2 \pm 0.0
Threonine	4.4 \pm 0.2	4.8 \pm 0.0	4.5 \pm 0.0	4.6 \pm 0
Tryptophan	1.3 \pm 0.1	1.0 \pm 0.1	0.9 \pm 0.0	0.9 \pm 0.0
Tyrosine	4.7 \pm 1.2	6.7 \pm 0.1	8.4 \pm 0.1	8.8 \pm 0.0
Valine	7.0 \pm 0.0	6.4 \pm 0.0	7.1 \pm 0	7.2 \pm 0.0
Ammonia	2.4 \pm 0.0	2.1 \pm 0.0	2.5 \pm 0.0	2.6 \pm 0.0

^a Mean \pm SD.

^b g/16.8 g of N.

ethanol.

The IR58 raw milled rice protein had 4.4% lysine, the first limiting essential amino acid. Previous amino acid analysis of IR58 raw milled rice revealed 3.6% lysine, 4.6% cysteine plus methionine, 1.4% tryptophan, 4.1% threonine, 5.3% isoleucine, 9.5% leucine, 12.3% phenylalanine plus tyrosine, and 7.5% valine (Cabrera-Santiago et al 1986, Eggum et al 1987).

Whole, undigested PBs (destarched cooked milled rice) had an aminogram similar to that of raw milled rice protein, except that it contained higher amounts of tyrosine and lower amounts of glutamic acid and leucine. The undigested fraction, after one pepsin treatment, had lower amount of lysine but higher amounts of cysteine than that of the starting PB preparation, as previously reported (Tanaka et al 1978, Resurreccion and Juliano 1981). The change in amino acid composition after the second pepsin digestion was particularly dramatic for lysine. The lysine content of residual protein from two pepsin digestions was lower, at 0.8%. Similarly, residual pepsin-digested PBs of IR480-5-9 cooked milled rice (representing 32% of PB protein) had 1.6% lysine, and residual pepsin-digested IR4B0-5-9 raw-rice PBs (representing 13% of PB protein) had 0.5% lysine (Tanaka et al 1978). Evidently, the core 15% of spherical PBs had a different amino acid composition (particularly lysine) than the peripheral or outer proteins.

After the solution of the pepsin-resistant PBs (in 2.5% SDS and 0.6% 2-mercaptoethanol) was passed through a Bio-Gel A-5m column, the major protein peak (with elution vol/void vol = 2.4) consisted mainly of 13-kDa subunits plus the 15-kDa band corresponding to the major prolamin subunits. Prolamins extracted with 1-propanol, with or without 2-mercaptoethanol, have the minor 16-kDa band, but only prolamin extracted with 1-propanol containing 2-mercaptoethanol had the 10-kDa band (data not shown). Only the resistant core protein showed diffused protein staining ahead of the 13-kDa band. These low molecular weight bands were present in both the pepsin-treated PBs and whole, undigested rice PBs. On IEF at pH 3-10, this core protein resolved into at least four bands together with trailing (results not shown). The IEF pattern was not identical to that of the prolamin or the other solubility fractions of milled rice, although the 15-kDa prolamins also resolved into four polypeptides with different pI values (Ogawa et al 1989).

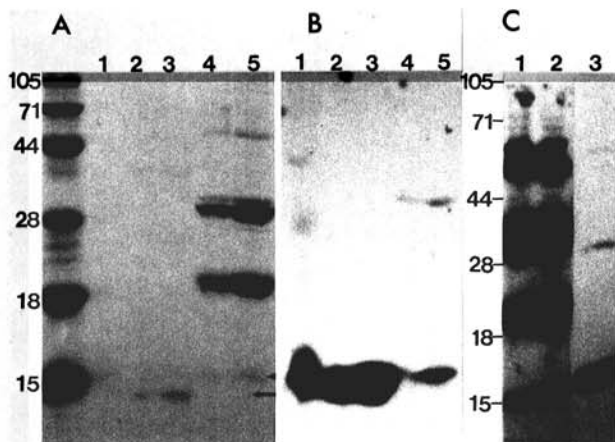


Fig. 1. A, Proteins resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 15% gel. The extreme left lane contains protein standards (obtained from BRL, Inc., Grand Island, N.Y.) with apparent molecular sizes (kDa) as labeled. Lanes 1-3, protein (1, 10, and 20 µg respectively) from pepsin-resistant (treated twice) IR58 protein body fraction. Lanes 4 and 5, protein (20 and 40 µg respectively) prepared from a partially purified protein body fraction of M-201 rice endosperm. Arrowhead: 15-kDa prolamin, arrow: predominant pepsin-resistant 13-kDa band. B, Western blot analysis of A using anti-15-kDa prolamin. C, Western blot analysis of a polyacrylamide gel containing 20 µg of protein from a partially purified M-201 protein body fraction (lanes 1 and 2) and 20 µg protein of pepsin-resistant (treated twice) IR58 protein body fraction (lane 3) probed with a mixture of glutelin and prolamin antibodies.

Western Blot Analysis

The twice-pepsin-treated PBs were only partially soluble in the SDS-urea sample buffer. About 1.5 mg of protein was solubilized from 5 mg (2.5-3.0 mg of protein) of starting material, based on absorbance at 214 nm, even when boiled for 20 min with 2-mercaptoethanol. The amount of Coomassie blue staining on the polyacrylamide gels showed a very low level of protein, indicating that the bulk of the solubilized material was nonproteinaceous or consisted mainly of small peptides that migrated through the gel. Nevertheless, the predominant 13-kDa band, evident on SDS polyacrylamide gels, exhibited its mobility (Fig. 1A). Three other very faint and minor bands of 15, 20, and 40 kDa were also evident on the polyacrylamide gels. The whole, undigested PB preparation showed glutelin subunits at 56, 32, and 22 kDa and a prolamin subunit at 15 kDa.

These proteins were then transferred onto the nitrocellulose membrane and screened with the anti-15-kDa 70% ethanol-soluble, or low-sulfur, prolamin. After autoradiography, the whole, undigested PB preparation exhibited mainly the 15-kDa prolamin band (Fig. 1B). Only a single broad band was evident on the X-ray film for the pepsin-treated PBs. Closer inspection of the autoradiograph showed that the broad band was due to the reactivity of the 15- and 13-kDa bands to the rice anti-15-kDa prolamin. Comparison of the autoradiographed and the Coomassie blue-stained gels showed that the reactivity of the 15- and 13-kDa bands to the anti-15-kDa prolamin were dissimilar. Although the 13-kDa band was present at 10-fold higher levels than the 15-kDa band, based on the extent of Coomassie blue staining (Fig. 1A), these polypeptide bands exhibited similar autoradiographed signals. Despite these differences in antigen recognition, the results indicate that the 13-kDa band is not a glutelinlike protein as implied by its solubility properties; it is actually a prolamin. Moreover, in view of the cross-reactivity of the 13-kDa band to the anti-15-kDa low-sulfur prolamin, it is likely that the 13-kDa band is a proteolytic product of the 15-kDa cysteine-rich prolamins (Kim and Okita 1988). The anti-15-kDa low-sulfur prolamin reacts less with high-sulfur prolamins than with low-sulfur prolamins (Krishnan and Okita 1986).

In attempts to identify the 40- and 20-kDa bands, we also conducted Western blot analysis with the antibodies raised against the glutelin storage proteins. Despite prolonged autoradiographic exposures, the antiglutelins failed to react with any of the bands evident on Coomassie blue-stained polyacrylamide gels (Fig. 1C). Instead, faint autoradiographic signals, consistent with the acidic glutelin subunit and intact glutelin precursor, were evident for polypeptide bands of about 32 and 56 kDa. Although the nature of the 40- and 20-kDa bands remained unidentified, these results support the assertion of earlier studies (Ogawa et al 1987) that glutelins, which are packaged in PB II, are readily digested by pepsin. The whole, undigested PB preparation showed overexposed bands at 59-65, 50-59, 29-42, 19-23, and 15 kDa.

Analysis of recombinant cDNA clones encoding the 15-kDa prolamins indicate that these proteins are heterogeneous with regard to amino acid composition (Kim and Okita 1988). One class of prolamin cDNAs, typified by pProl 14, encodes a mature 15-kDa protein deficient in lysine and sulfur-containing amino acids. The amino acid composition of the deduced protein encoded by pProl 14 is similar to that obtained for authentic prolamins isolated by alcoholic extraction in the absence of reducing agents. A second class, represented by pProl 17, also encodes a lysine-deficient 15-kDa species, but the deduced polypeptide is sulfur-rich and contains about 6% cysteine. In view of the results obtained here (i.e., a pepsin-resistant 13-kDa peptide of high sulfur amino acid content that reacts with antiprolamin), it is likely that polypeptides of this second prolamin class constitute the inner core of the large spherical PB I.

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