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FRACTIONATION OF GLOBULINS OF MILLED RICE

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Abstract—Globulins were prepared by repeated precipitation with 1.3 M $(NH_4)_2SO_4$ from a 0.7 M NaCl extract of milled rice. Isoelectric precipitation at pH 4.5 did not effectively remove the α-globulin from the others. A major fraction that remained in solution during dialysis of the globulin precipitate against water was similar in properties to the globulin soluble at pH 4.5 during the isoelectric precipitation process. Some properties of this water-soluble globulin fraction are reported. Proteins extracted from milled rice at 50° with 0.5 M NaCl and precipitated as 1- to 3-μm particles on cooling were verified to be globulins.

INTRODUCTION

The salt-soluble proteins of milled rice constitute 15% of its total protein, of which 5% is albumin and 10% is globulin [1]. Globulins can be separated from albumins by precipitation from 0.7 M NaCl extracts either by dialysis against water or by addition of $(NH_4)_2SO_4$ to 1.3 M concentration. Little recent work has been done on milled-rice globulins [1–6] in contrast to that on bran globulins [7]. Perdon and Juliano [1] characterized the major globulin of milled rice, called α-globulin, but another major globulin, which is soluble at higher pH, is also present. α-Globulin can be prepared by repeated isoelectric precipitation at pH 4.5. Houston and Mohammad [2] also reported that globulins soluble above pH 4.5 are high in sulfur amino acids. A crystalline globulin has been prepared from milled rice by hot NaCl extraction followed by cooling [6]. This paper describes our attempts to characterize the globulins of milled rice other than α-globulin.

RESULTS

The globulin fraction obtained by repeated precipitation with 1.3 M $(NH_4)_2SO_4$ from a 0.7 M NaCl extract of milled rice showed poor recovery of the insoluble globulin precipitate during dialysis against water. Because of the possible loss of non-α-globulins during this procedure, the method was re-investigated. The redissolved 1.3 M $(NH_4)_2SO_4$ precipitate from the 0.7 M NaCl extract was dialysed either against distilled water, pH 5.5–6, or 0.1 M Tris–HCl buffer, pH 7.5. Globulin precipitation in the latter solution was complete although about 50% of the protein remained soluble in water. The electrophoretic pattern of this soluble globulin fraction was similar to that of the pH-4.5 soluble globulin of the water-insoluble fraction (Fig. 1).

Analytical gel electrophoresis at pH 8.3 showed that the water-insoluble globulin had a major slow migrating band, a slightly faster medium-intensity band, and a fast faint band (Fig. 1). α-Globulin had the slow band as the principal protein whereas the pH-4.5 soluble fraction contained both of the faster moving constituents together with the fainter slow band. The water-soluble proteins obtained after dialysis of crude globulin and the water-insoluble globulin showed a similar pattern in basic gels except that the medium-intensity band was the major one. In acid gels, migration was faster but the major component of the water-insoluble globulin was still the slower moving α-globulin band. There were also three closely spaced faster moving minor bands. The water-soluble globulin had a very faint band, which corresponded to the major band of the crude globulin as well as that of α-globulin. The three minor bands observed in the water-insoluble globulin were the major ones in the water-soluble fraction.

Flat gel isoelectric focusing (IEF) patterns revealed that the α-globulin fraction had a major broad peak with pI 5.3 and intense narrow bands at pH 4.5, 5.4, 5.6, 6.2, 6.5 and 6.9. The globulins soluble at pH 4.5 had broad protein bands at pH 8.0 and 8.5 and narrow intense bands at pH 5.3, 6.0, 6.3, 6.6, 7.7 and 9.0. By comparison, the 3 M urea–1% β-mercaptoethanol extract of IR 36 milled rice had the pH-5.3 protein as the major fraction, followed by those at pH 8.2, 7.8, 6.9 and 4.5 plus many minor bands covering the pH range 4.9–9.1. Polyacrylamide disc gel IEF of the various globulin preparations was also run (Fig. 2). The crude globulin, the water-insoluble globulin, the α-globulin as well as the globulin particles all had a major broad band at around pH 5.5. Several minor bands at pH values less than 5.4, as well as minor bands at pH range of 6.4–8.4, were present. In contrast, the water-soluble globulins and the pH-4.5 soluble globulin had major bands at the basic region, roughly at pH 7.0–8.4. The major α-globulin band at pH 5.5 was also present but less intense. The 2 × $(NH_4)_2SO_4$ precipitated globulin (crude globulin) of IR 36 had an IEF pattern showing both the major bands corresponding to the α-globulin as well as the major bands of the water-soluble and pH-4.5

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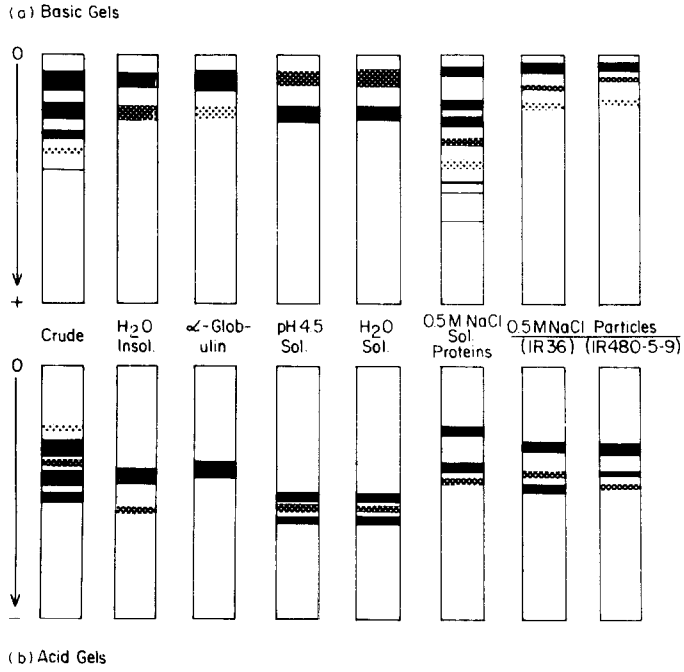

Fig. 1. Analytical disc gel electrophoregrams of various globulin preparations from IR 36 milled rice and 0.5 M NaCl globulin particles of IR 480-5-9 milled rice. (a) pH 8.3, (b) pH 4.5.

soluble globulins. The results on urea–mercaptoethanol extracts of other rice samples were similar [8]. Padhye and Salunkhe [9] reported that isoelectric points (pI) of milled rice globulins range only from 5.85 to 7.27 for 8 bands by isoelectric focusing. The 0.1 M NaCl extract of milled rice showed 21 bands with pI from 4 to 8 [10].

Amino acid analysis of α-globulin and the other globulins showed marked differences (Table 1). The water-soluble globulin was richer than α-globulin in lysine, histidine, aspartic acid, proline, cystine, glycine, alanine and valine, but poorer in arginine, serine and glutamic acid. It contained more total sulfur amino acids (cystine and methionine) than α-globulin in agreement with Houston and Mohammad's [2] results.

Various techniques were tried to separate α-globulin from the other globulins but were ineffective. Gel filtration of crude globulin preparations on Sephadex G-100 at acid pH gave only one major peak with V/V_o of 2.0–2.2 for both IR 36 and IR 480-5-9 milled rice. IR 480-5-9 bran globulin also gave the same peak together with a lesser V/V_o 2.8 fraction. Disc gel electrophoresis of the major peak showed no fractionation relative to the starting crude globulin samples. Iwasaki *et al.* [11] also reported only one major Sephadex G-100 peak at V/V_o 2.0 for the milled-rice globulins they examined.

DEAE-Sephacel chromatography of crude globulin in 0.1 M Tris–HCl buffer, pH 7.5, was ineffective as only 10% of the protein dissolved in the buffer. Of this soluble

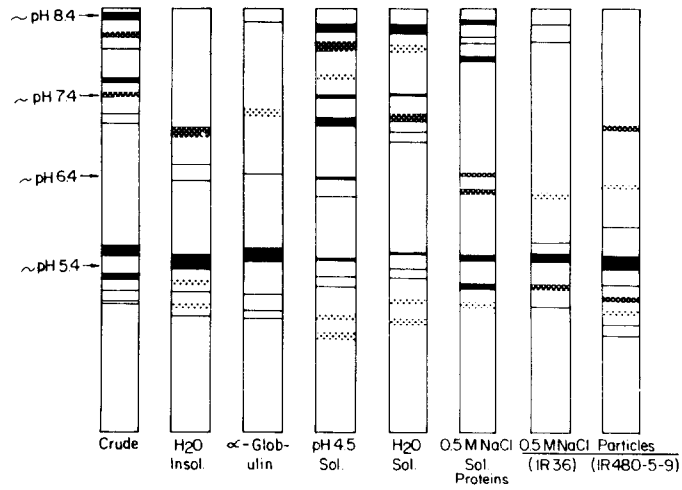

Fig. 2. Isoelectric focusing patterns of various globulin preparations from IR 36 milled rice and 0.5 M NaCl globulin particles of IR 480-5-9 milled rice.

Table 1. Amino acid analysis of α-globulin and water-soluble fraction of IR 36 globulins compared with previous analysis of IR 480-5-9 milled-rice α-globulin and other globulins

Amino acid	α-Globulin		Water-soluble globulins	pH 4.5 soluble globulins	0.5 M NaCl extract at 50°				LSD (5%)
					Particle		Supernatant		
	IR 36	IR 480-5-9	IR 36	IR 480-5-9	IR 36	IR 480-5-9	IR 36	IR 480-5-9	
	g/16 g N								
Lys	0.5	1.1	2.6	1.9	2.4	1.0	2.6	2.2	0.4
His	0.1	0.4	3.0	2.7	1.9	1.0	2.3	2.3	0.3
Amm	3.0	2.3	3.0	1.1	1.9	1.7	1.2	1.5	NS
Arg	16.3	15.2	11.1	12.8	13.1	17.3	14.4	14.5	2.6
Asp	3.5	4.9	8.7	9.5	7.8	5.4	7.7	9.9	1.0
Thr	1.6	2.2	2.4	2.8	2.6	1.9	2.5	2.4	0.2
Ser	7.0	8.8	3.2	3.8	5.1	6.8	4.5	5.2	1.2
Glu	25.3	27.8	9.7	12.5	20.3	25.6	16.9	17.4	3.9
Pro	4.0	5.0	6.6	7.0	4.4	5.2	5.5	6.2	0.8
Cys	2.8	3.0	4.4	5.5	2.6	2.7	4.0	2.7	0.6
Gly	4.4	5.1	6.8	7.9	5.5	6.1	6.8	7.2	1.3
Ala	3.5	4.2	7.2	7.7	5.8	5.2	6.8	6.6	0.6
Val	2.8	4.0	5.8	5.8	5.2	4.3	5.6	4.5	0.2
Met	3.7	4.0	2.5	3.9	2.5	4.6	3.3	1.1	0.8
Ile	1.2	1.8	2.2	2.4	2.9	1.8	2.4	2.0	0.1
Leu	5.6	6.6	6.7	7.3	7.1	6.9	7.3	7.2	0.6
Tyr	6.8	6.7	5.4	6.1	6.3	7.4	6.6	6.4	1.0
Phe	3.0	3.4	2.5	3.0	4.4	3.4	3.4	3.1	0.3
Trp	1.0	0.8	0.4	1.0	1.4	1.3	2.3		0.5
Wt %*	1.9	1.1	3.0	1.3	—	1.2		5.3	

*Of milled-rice protein.

fraction, the major peak eluted at 0.34 M NaCl plus a minor peak at 0.04 M NaCl. DEAE-Sephacel chromatography of the pH-4.5 soluble globulin (from the isoelectric precipitation of α-globulin) at pH 8.5 showed some unadsorbed proteins and broad UV absorbance corresponding to peaks at 0.28 and 0.31 M NaCl and a shoulder at 0.42 M NaCl: however, there was tailing up to 0.69 M NaCl. α-Globulin has been shown to elute at a salt concentration of 0.22 M NaCl [1]. DEAE-Sephacel chromatography of water-soluble globulin at pH 8.5 also showed unadsorbed protein plus peaks at 0.28, 0.43 and 0.52 M NaCl.

Alkylation of the globulin before dialysis gave a better separation of peaks on DEAE-Sephacel chromatography. The alkylated water-soluble fraction had peaks at 0.29 and 0.35 M NaCl and a third broad peak at 0.47 M NaCl. The water-insoluble alkylated fraction had peaks at 0.37 and 0.47 M NaCl. Both had some unadsorbed protein and showed tailing up to 0.8 M NaCl. Disc gel electrophoresis of these various DEAE-Sephacel fractions showed that some separation of the three major bands of water-soluble globulin was possible. Acid gel electrophoresis of the fractions corresponding to the two major peaks at 0.29 and 0.35 M NaCl each gave a single band; their R_f values, however, were very close.

Globulin particles

Extraction of IR 480-5-9 milled rice (10% protein) at 50° followed by cooling resulted in the formation of 1–3 μm spherical protein particles. The size of the particles depended on the extraction temperature, the concentration of the protein, and the rate of cooling. The hot NaCl solvent extracted 1.3% protein particles and 4.4% soluble proteins from the subaleurone-layer protein (11.1% protein) and 1.1% protein particles and 6.2% soluble proteins from the inner-endosperm protein (6.8% protein) of IR 480-5-9 milled rice. The protein particles were 82–83% digested in 5 hr by pepsin, which was the same extent as the soluble protein fraction (81–85% digestion).

The IR 480-5-9 protein particles were identified as globulins based on analytical gel electrophoresis and amino acid analysis. The supernatant proteins were rich in the faster migrating albumins whereas the globulin particles were mainly the three slow migrating globulin bands including α-globulin (Fig. 1). The supernatant proteins contained these three bands plus five faster bands. Subaleurone and inner endosperm preparations had similar electrophoregrams. Amino acid analysis of the globulin particles was closer to that of α-globulin than to that of the soluble globulin (Table 1). By contrast the supernatant proteins were closer in amino acid content to the soluble globulins of IR 480-5-9 rice.

DISCUSSION

The present study of milled-rice globulins demonstrates the unreliability of classifying proteins on the basis of their solubility in water. The non-α-globulins were best recovered, still with some α-globulin contamination, by dialysis of the crude globulin preparation against water. The α-globulin in the insoluble fraction can be further purified by isoelectric precipitation at pH 5.3. These two fractions differed drastically in amino acid composition. Water-soluble globulin actually had an amino acid composition similar to those of the major 2%-lysine albumins of milled rice [12]. It probably contains some basic albumins and albumin I, based on DEAE-Sephacel chromatography, which showed unadsorbed protein (basic albumins) and proteins eluting at low NaCl concentration. The isoelectric focusing data seem to confirm the above observations. The α-globulin fractions could not be further separated [1]. The three major bands of non-α-globulin were partially fractionated by DEAE-Sephacel chromatography: separation, however, was incomplete and the peaks were not completely resolved from one another. The "globulin particles" of Kondo and Ito [6] were shown to be true globulins, with α-globulin predominating, by electrophoresis and amino acid analysis. This was expected because of the greater water solubility of the other globulins of milled rice.

EXPERIMENTAL

High-protein rice IR 480-5-9 (1976 crop) and IR 36 (1978 crop) were obtained from the IRRI farm. The rough rice was dehulled with a Satake THU-dehuller and milled in 150 to 200-g brown-rice lots in a Satake TM-05 grain testing mill with a 36-mesh abrasive cylinder to remove at least 10% by wt as bran-polish. The milled rice was then ground in a Udy cyclone mill with a 60-mesh sieve and the flour was defatted with petrol and then air-dried. The subaleurone layer and inner endosperm of IR 480-5-9 milled rice were collected by overmilling with the Satake TM-05 mill. Subaleurone represented the 12–20% outer milling fraction, and the inner endosperm represented the residual 70% fraction of brown rice. Flours were prepared by passing the rice through a Udy cyclone mill with a 60-mesh sieve, and sifting through a 100-mesh screen.

The procedures used for extracting milled-rice globulins were as described previously [1]. Removal of contaminant albumins was attempted by repeating the $(NH_4)_2SO_4$ precipitation. Globulins extracted by 0.7 M NaCl and precipitated by 1.3 M $(NH_4)_2SO_4$ were redissolved in 0.7 M NaCl and precipitated by the addition of $(NH_4)_2SO_4$ to 1.3 M concn. Removal of α-globulins was by repeated isoelectric precipitation at pH 4.5 [1, 2]. Gel filtration on Sephadex G-100 columns (2.6 × 54.5 cm) utilised 0.1 M sodium acetate buffer, pH 3.6, containing 0.1 M NaCl as eluting buffer. Ion-exchange chromatography was carried out on DEAE-Sephacel columns (1.5 × 36.5 cm) with 0.1 M Tris–HCl buffer, pH 7.5 or 8.5, using a linear NaCl gradient (0–0.6 or 0.8 M). The *in vitro* pepsin digestion test of globulin particles was that described earlier [13, 14].

Rice flour (100-mesh, whole milled rice, inner endosperm or subaleurone) was stirred with 4 v/wt of 0.5 M NaCl at 50° for 2 hr and filtered, and the residue was re-extracted with 4 v/wt of 0.5 M NaCl at 50° for 1 hr. The combined extracts were stored for 15 hr at 4° and centrifuged at 35000 *g* for 10 min. The collected globulin particles were freeze-dried. Protein was precipitated from the supernate by the addition of 5% TCA and aging for 15 hr at 4°. The precipitate was collected by centrifuging for 10 min at 35000 *g* and freeze-dried.

The crude globulin precipitate obtained by 2 × $(NH_4)_2SO_4$ precipitation from 0.7 M NaCl extract was alkylated in 0.1 M Tris–HCl buffer, pH 7.5, by the addition of β-mercaptoethanol to 1.6% concn followed by acrylonitrile to 1.2% concn.

Methods of analyses including analytical polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis have been detailed [1, 14, 15]. The running buffer at pH 4.5 was 0.035 M β-alanine–0.013 M acetic acid [1]. Gels were stained with Coomassie Brilliant Blue G-250.

Flat gel isoelectric focusing of the globulin fractions was on 7.5% acrylamide gels containing 2 M urea and 2% carrier ampholytes, pH 3.5–10 [8]. Proteins were dissolved in 3 M

urea–1% β-mercaptoethanol. Isoelectric focusing on disc gels used 5.5% gels with 4 M urea and 2% ampholine, pH 3–10. Runs used 0.02 M NaOH as anodic buffer and 0.01 M H_3PO_4 as cathodic buffer. Gels were stained according to general procedures [15].

Acid hydrolysates of globulin preparations were analysed using a Beckman Model 120C amino acid analyser using PA-35 and AA-15 or H70 resins [14]. Cysteine was determined as cysteic acid after performic acid oxidation [16] and tryptophan was obtained from $Ba(OH)_2$ hydrolysis before short-column analysis [17].

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