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CHANGES IN SALT-SOLUBLE PROTEINS OF RICE DURING GRAIN DEVELOPMENT

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Key Word Index—*Oryza sativa*; Gramineae; rice; salt-soluble proteins; albumin; globulin.

Abstract—Salt-soluble proteins, albumin and globulin, were prepared from dehulled rice (*Oryza sativa* L., line IR1541-76-3) during grain development. Albumin and globulin progressively increased during grain development up to about 12 days after flowering (DAF) and then decreased slightly during grain desiccation. Free amino N was maximum at 10 DAF. Total protein and glutelin-prolamin (by difference) continued to increase up to 20 DAF. Aminogram of total protein and globulin showed a progressive decrease in lysine and threonine among the essential amino acids. Albumin showed a similar trend except for the lesser change in lysine content. Disc gel electrophoresis showed a maximum of four major and six minor protein bands for albumin and only one major and three minor bands for globulin. Sodium dodecyl sulfate-gel electrophoresis revealed three major polypeptide subunits for albumin with MW of 11000, 8500 and 16000, and two for globulin with MW of 20000 and 12000.

INTRODUCTION

The salt-soluble proteins, albumin and globulin, of rice (*Oryza sativa* L.) constitute less than 20% of brown rice protein [1], but they are of major importance because of their enzyme content. This group of rice proteins has been little studied except for γ-globulins of rice germ [2], a major α-globulin [3], and a hemagglutenin [4]. Rice albumin and globulin differ from glutelin and prolamin in amino acid composition and electrophoretic mobility [5–7]. But portions of glutelin have been extracted in the presence of β-mercaptoethanol which may be considered as denatured globulin and prolamin based on lysine content [5].

Likewise, few studies have been made on the changes in the properties of rice protein during grain development [8, 9]. Endosperm protein is exclusively in the form of protein bodies [10, 11] which begin to appear at 7 days after flowering (DAF) in the developing grain [10]. Isolated rice protein bodies have the same ratio of protein fractions as whole milled rice protein [7, 11]. Albumin-globulin had the highest concentration in the developing grain at about 2 weeks after flowering [10]. Because of advances in techniques of protein characterization since our earlier study on IR8 rice [8] and the discovery that more protein bands are present in electrophoregram of developing grain than in the mature grain [12], we studied some properties of albumin and globulin at regular intervals during maturation in the rice line IR1541-76-3. We also analyzed the aminogram of the various anatomical parts of the mature IR8 grain.

RESULTS AND DISCUSSION

N fractions during grain ripening. Dry wt, total Kjeldahl N, and Lowry protein progressively accumulated during grain (caryopsis) development up to 20 days after flowering (DAF) and increased slightly during grain desiccation until harvest (32 DAF). Values at 20 DAF were 16.4 mg dry wt and 1.48 mg protein per grain. Free amino N progressively increased up to 10 DAF and then continuously decreased in the maturing grain (Fig. 1). Similar results have previously been reported by other workers on other rice samples [8, 9, 13].

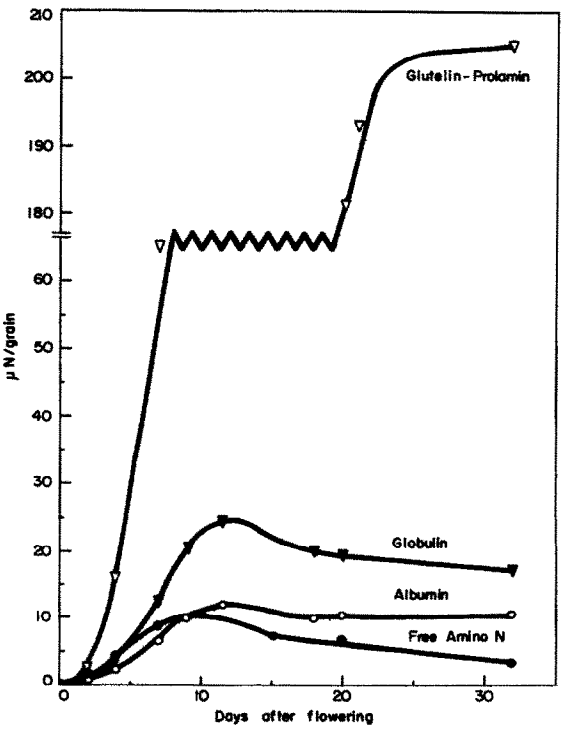

Fig. 1. Changes in the various N fractions of developing rice grain of the line IR1541-76-3.

Table 1. Aminogram of dehulled developing rice grain of the line IR1541-76-3*

Amino acid	Days after flowering 2	4	7	12	20	32	LSD (5%)
Lysine	7.2	6.7	5.8	4.9	4.4	4.0	0.28
Histidine	2.2	2.4	2.4	2.3	2.5	2.4	NS
Ammonia	2.6	2.2	2.7	2.7	2.6	2.6	0.33
Arginine	5.8	6.4	7.3	7.8	8.2	8.6	0.83
Aspartic acid	11.8	10.9	10.5	9.7	9.9	9.7	0.81
Threonine	4.8	4.4	4.3	3.9	3.9	3.8	0.43
Serine	5.7	5.1	5.3	5.2	5.2	5.5	NS
Glutamic acid	12.4	13.5	14.6	14.9	15.6	15.8	1.37
Proline	4.7	4.4	4.5	4.4	4.3	4.4	NS
Cystine	2.0	1.5	2.1	2.0	2.2	2.2	0.43
Glycine	6.2	6.1	5.5	5.2	5.1	5.0	0.72
Alanine	7.6	7.7	7.0	6.2	6.2	6.1	0.53
Valine	7.5	7.4	7.1	6.9	7.0	7.0	NS
Methionine	1.9	2.5	2.4	2.5	2.4	2.2	0.46
Isoleucine	5.5	5.2	5.1	4.9	4.9	4.7	NS
Leucine	8.7	8.6	8.5	8.3	8.8	8.5	NS
Tyrosine	3.7	4.0	4.0	4.5	4.5	4.9	1.08
Phenylalanine	5.0	5.0	5.2	5.2	5.5	5.4	NS
Tryptophan	1.2	1.3	1.3	1.3	1.1	1.2	NS
Total	106.5	105.3	105.6	102.8	104.3	104.0	
N recovery (%)	102.2	100.0	102.6	100.3	101.1	101.1	
Crude protein (% N × 6.25)	13.7	10.2	9.6	9.4	9.4	9.1	

* In g/16 g N.

Both albumin and globulin also progressively increased during grain development up to 12 DAF and then decreased with grain maturity (Fig. 1). The level of globulin was always at least twice that of albumin from 7 DAF onward. Albumin decreased from 16% of total protein in the grain at 2 DAF to 5.6% in the mature grain. The globulin fraction decreased from 17% to 8.5% of total protein. In Japanese rice, albumin and globulin also levelled off earlier than total protein [9].

Glutelin–prolamin (by difference) also increased similarly to total protein (Fig. 1) and constituted from 67% of total protein at 2 DAF to 86% at maturity, in agreement with earlier studies [8, 9]. But dry matter and protein accumulated at a faster rate in tropical rice than in japonica rice.

Amino acid composition of total protein, albumin and globulin. In the total amino acid composition of brown rice, the levels of many of the essential and nonessential acids changed significantly (Table 1). Lysine and threonine, the most limiting essential amino acids [1], progressively decreased during grain protein accumulation as the proportion of salt-soluble protein to total protein decreased and the proportion of glutelin protein increased. Tyrosine also increased during grain development. Methionine increased only from 2 DAF to 4 DAF, while cysteine did not change during grain ripening. Among the nonessential amino acids, arginine and glutamic acid levels increased during grain ripening while aspartic acid, glycine, and alanine decreased. No definite trend was found in the other amino acids during grain development. Similar changes were observed by Palmiano *et al.* [8] for lysine, arginine, glutamic acid, and tyrosine in the developing IR8 rice grain but only between 4 DAF and 14 DAF.

The aminogram of albumin differed from that of total protein; changes in their aminogram during protein accumulation also differed (Table 2). Among the essential amino acids, threonine, valine, isoleucine, leucine, phenylalanine, and tryptophan progressively decreased in albumin during grain development. Lysine increased up to 7 DAF and then decreased progressively during the synthesis of protein bodies [10]. Content of lysine changed relatively less than that of total protein (Table 1). Methionine also decreased slightly during grain development but cysteine values increased. Of the nonessential acids,

Table 2. Aminogram of albumin from dehulled developing rice grain of the line IR1541-76-3*

Amino acid	Days after flowering 2	4	7	12	20	32	LSD (5%)
Lysine	7.2	7.8	8.2	7.3	6.9	6.5	0.36
Histidine	1.9	2.1	2.3	2.2	2.4	2.5	NS
Ammonia	1.7	2.0	2.3	2.6	3.1	2.9	0.46
Arginine	5.1	5.3	5.8	6.3	6.5	7.9	0.49
Aspartic acid	12.8	12.0	11.5	10.5	9.7	9.2	1.00
Threonine	5.4	5.2	4.8	4.4	4.3	4.2	0.17
Serine	6.6	6.8	6.2	6.0	6.0	5.9	NS
Glutamic acid	15.6	15.4	15.4	14.3	13.1	12.9	0.91
Proline	5.5	4.9	5.4	5.1	5.0	4.8	NS
Cystine	1.6	1.8	1.9	2.5	2.3	3.0	0.75
Glycine	5.9	5.5	5.7	5.7	5.8	5.9	NS
Alanine	7.8	7.4	7.4	7.1	7.0	7.0	NS
Valine	7.6	7.4	7.4	6.9	6.4	5.9	0.48
Methionine	2.3	2.0	1.9	2.0	1.4	1.8	0.40
Isoleucine	5.5	5.0	4.8	4.3	3.7	3.0	0.44
Leucine	8.7	8.3	8.1	7.5	6.8	6.1	0.73
Tyrosine	3.9	3.7	3.9	3.6	3.7	3.7	NS
Phenylalanine	4.6	4.1	4.4	4.0	3.3	3.0	0.56
Tryptophan	0.9	0.9	0.6	0.7	0.4	0.3	0.28
Total	110.6	107.6	108.0	103.0	97.8	96.5	
N recovery (%)	99.6	99.5	102.1	100.2	99.3	99.4	

* In g/16 g N.

Table 3. Aminogram of globulin from dehulled developing rice grain of the line IR1541-76-3*

Amino acid	Days after flowering 2	4	7	12	20	32	LSD (5%)
Lysine	7.2	7.0	6.6	5.6	5.2	4.1	0.38
Histidine	2.7	2.7	2.5	2.5	2.5	2.5	NS
Ammonia	2.0	1.9	1.9	1.9	1.9	1.9	NS
Arginine	7.9	8.5	8.6	9.2	11.0	11.4	0.84
Aspartic acid	11.0	10.1	9.9	9.0	8.5	7.5	0.81
Threonine	4.8	4.5	4.2	3.6	3.4	3.0	0.49
Serine	5.3	5.8	5.6	5.8	5.2	6.0	NS
Glutamic acid	12.9	12.6	13.7	15.0	14.9	15.4	0.79
Proline	4.4	4.5	4.4	4.6	4.6	4.6	NS
Cystine	1.6	1.3	3.0	3.4	3.5	4.1	0.62
Glycine	5.3	5.1	5.5	5.5	5.2	5.5	NS
Alanine	6.0	5.8	6.1	5.9	5.6	5.4	0.20
Valine	7.3	6.7	6.9	6.5	6.0	5.8	0.32
Methionine	2.1	2.2	2.5	2.7	2.2	3.0	0.37
Isoleucine	6.0	5.2	5.1	4.5	3.8	3.6	0.31
Leucine	9.8	8.7	8.7	8.0	7.4	7.1	0.37
Tyrosine	4.9	4.6	4.8	5.0	4.9	5.0	0.13
Phenylalanine	5.6	5.0	4.9	4.5	4.1	4.0	0.23
Tryptophan	1.2	1.2	1.4	1.1	1.2	0.9	0.08
Total	107.3	103.4	106.3	104.3	101.1	100.8	
N recovery (%)	102.5	99.6	191.6	100.3	100.1	99.9	

* In g/16 g N.

the level of arginine increased while that of both aspartic and glutamic acids decreased, in contrast to total protein, where glutamic acid level increased during protein accumulation.

Changes in the aminogram of globulin followed more closely the changes in that of total protein (Table 3). Of the essential acids, levels of lysine, threonine, valine, isoleucine, leucine, and phenylalanine decreased progressively during grain development. Both cysteine and methionine increased, while tryptophan decreased. Among the nonessential acids, the levels of arginine and glutamic acid increased, while that of alanine and aspartic acid decreased. Houston *et al.* [15] reported the presence of a rice globulin with 3.7% S.

In the mature grain, globulin had a closer aminogram to total protein than albumin. However, albumin has a better balance of essential amino acids (except tryptophan) than globulin and total protein. Based on the provisional amino acid pattern [14], lysine was the first limiting amino acid of brown rice protein, with an amino acid score of 73%. Lysine, followed by threonine were deficient in globulin, with a score of 75%. By contrast, albumin had an amino acid score based on tryptophan of 30%. Tecson *et al.* [5] also reported differences in aminograms of albumin and globulin in milled rice, but they obtained a tryptophan content of 1.8 g/16 g N in albumin [1].

The changes during grain development of some amino acids in total brown rice protein may be partly explained by the decreasing contribution of salt-soluble protein and the corresponding increase in glutelin–prolamin [8, 9]. Prolamin has less than 1% lysine while glutelin has an aminogram similar to that of total milled rice protein [5, 8]. The aminograms of the salt-soluble proteins themselves also changed. This is to be expected since different enzymes appear in sequence during rice grain development [13, 16, 17]. Another contributing factor is that the nonendosperm parts of brown rice are synthesized earlier than the endosperm in the developing rice grain [1].

Amino acid analysis of the various parts of IR8 brown rice and hull indicated large differences (Table 4). The hull protein was similar to vegetative tissue in aminogram; had high proline content; and was relatively poor

Table 4. Aminogram of defatted parts of IR8 rice grain*

Amino acid	Hull	Brown rice	Milled rice	Aleurone Pericarp	layer	Embryo	LSD (5%)
Lysine	5.4	4.1	3.8	5.4	4.9	6.4	0.54
Histidine	2.0	2.5	2.6	2.8	3.0	3.6	0.37
Ammonia	2.5	2.7	3.1	2.2	2.1	3.2	NS
Arginine	4.0	8.6	8.3	7.4	8.3	9.2	0.88
Aspartic acid	10.4	8.6	8.7	10.7	9.0	8.7	0.95
Threonine	5.0	3.7	3.7	4.4	3.8	4.3	0.29
Serine	5.4	5.5	5.6	5.5	5.4	5.1	NS
Glutamic acid	13.1	16.1	17.5	11.8	13.2	14.4	2.11
Proline	10.3	4.6	4.9	5.5	4.7	4.3	0.37
Cystine	2.0	2.3	2.5	2.8	2.6	1.9	0.58
Glycine	5.5	4.5	4.5	5.8	5.2	5.7	0.57
Alanine	7.0	5.5	5.5	6.4	5.9	6.3	0.42
Valine	7.5	6.3	6.5	6.5	6.0	6.0	NS
Methionine	0.4	2.4	2.4	1.6	1.7	1.8	0.24
Isoleucine	4.0	4.4	4.5	4.3	4.1	3.6	0.98
Leucine	8.2	7.9	8.1	7.8	7.4	6.5	0.43
Tyrosine	1.3	3.8	3.6	2.9	3.4	3.1	NS
Phenylalanine	5.1	5.0	5.2	5.1	4.8	4.0	0.10
Tryptophan	0.6	1.2	1.2	1.0	1.2	1.3	0.09
Total	99.7	99.7	102.2	99.9	96.7	99.4	
N recovery (%)	94.0	98.6	101.6	97.4	95.3	105.0	
Protein (% N × 6.25)	1.6	8.2	7.6	16.8	16.6	26.6	
Wt (%) of brown rice	26.2	100.0	92.2	2.2	3.4	2.3	
Wt (%) of brown rice protein†	4.9	100.0	82.0	4.3	6.5	7.2	

* In g/16 g N. † Based on recovered protein from brown rice parts.

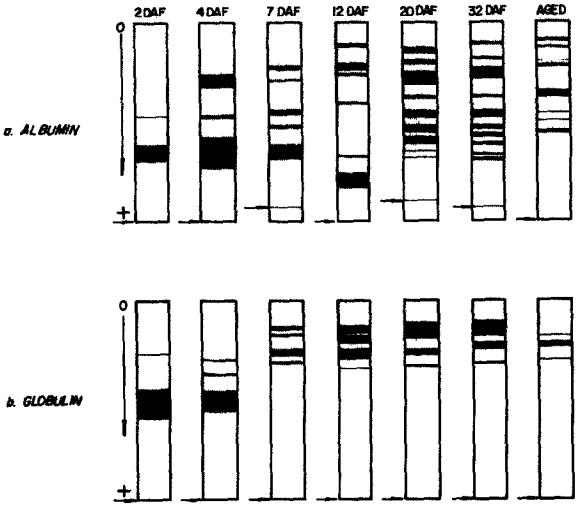

Fig. 2. Polyacrylamide gel electrophoregram of albumin and globulin from developing rice grain of the line IR1541-76-3. Horizontal arrow denotes the position of the tracking dye. Stain: Amido black 10B.

in tryptophan (amino acid score [14] of 60%). Brown rice protein did not have significantly higher levels of any essential amino acid than milled rice protein. But the protein of all nonendosperm tissue, pericarp, aleurone layer, and embryo, was significantly higher in lysine level than that of brown and milled rice. Levels of glutamic acid were also lower. Of the six proteins analyzed, the lysine content of embryo protein was highest. Amino acid score [14] was 75% (lysine) for brown rice; 69% (lysine) for milled rice; 98% (lysine) for pericarp; 89% (lysine) for aleurone layer; and 90% (isoleucine) for bran. The earlier synthesis of these parts may partly explain the higher levels of essential amino acids in the grain at 2 and 4 DAF, since synthesis of endosperm protein bodies commences at 7 DAF [10, 11]. Solubility fractionation of proteins in milling fractions and outer layers of milled rice confirmed the concentration of albumin, and to a lesser extent of globulin, in the outer layers of brown rice [18, 19].

Electrophoregram of albumin and globulin. Analytical disc gel electrophoresis revealed diffuse and rapidly migrating bands of both albumin and globulin in the grain at 2–4 DAF (Fig. 2), in agreement with results in barley [12]. Discrete bands of slow to medium mobility were present in older grains. The results are consistent with Sephadex G-100 chromatography of NaCl–urea extracts of IR8 rice grain which show an increase in mean MW of proteins during grain development [8]. The clearest patterns were in the samples obtained at 20 and 32 DAF. A maximum of 10 protein bands were obtained for albumin, four of which were major bands of intermediate mobility which were distinct principally at 20 and 32 DAF. Other workers obtained similar results on mature grain preparations using polyacrylamide gel electrophoresis [1, 6, 20, 21].

Globulin also generally showed only one major and three minor bands of slower mobility than albumin during grain development (Fig. 2), in agreement with previous reports [1, 6, 21]. It is difficult, however, to assign correspondence of these globulin bands to purified globulins characterized by previous workers [2–4]. Both albumin and globulin did not change appreciably in electrophoregram between 20 DAF and 32 DAF, which corresponded to an increase in cysteine of both protein fractions (Tables 2 and 3). During this period, the changes in the other amino acids were less and no net protein accumulated. Interestingly, the cysteine content of total protein did not change (Table 1). In our previous studies, cysteine, particularly of globulin, decomposed during protein isolation [5, 8].

Ageing of rough rice for several months at ambient temperature reduced the intensity of most of the albumin and globulin bands even though the same amount of protein was introduced to each gel (Figs 1, 2). The faster-moving bands of albumin and the major globulin band were absent in the aged sample. Similar changes in electrophoregrams have been reported in albumin [20, 21] and globulin [21] of milled rice during storage.

SDS–polyacrylamide gel electrophoresis of rice albumin and globulin again showed a diffuse pattern in grains at 2 and 4 DAF (Fig. 3). The electrophoregram of the subunits produced by SDS-β-mercaptoethanol dissociation was different from that in the absence of these reagents (Fig. 2). In albumin, three major subunits with MW 8500, 11000 and 16000 were obtained from 7 DAF onward. Subunit MW ranged between 8500 and 95000 for the 12 bands noted. The most intensely staining band was for the MW 11000 subunit. Subunits of intermediate MW disappeared during grain maturation after 20 DAF.

The major subunit for globulin on SDS–gel electrophoresis had MW 20000, followed by a less intensely staining band corresponding to MW 12000 (Fig. 3). A

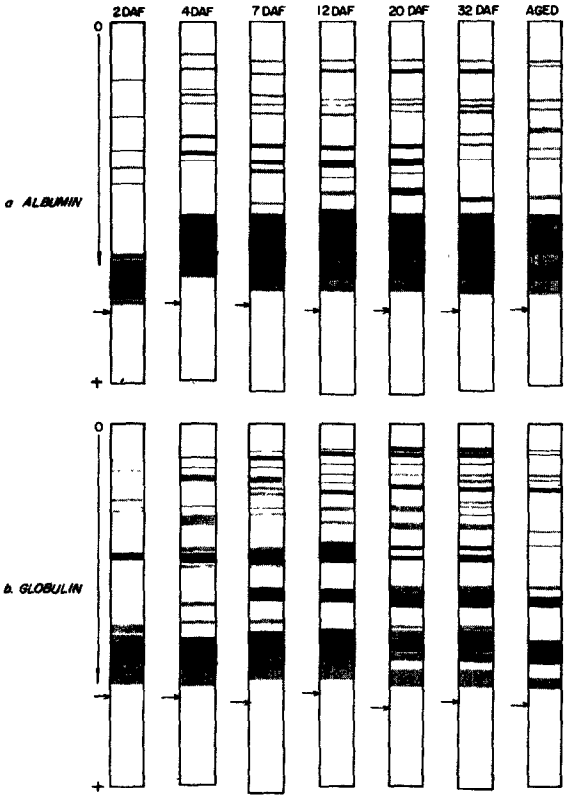

Fig. 3 SDS-polyacrylamide gel electrophoregram of albumin and globulin from developing rice grain of the line IR1541-76-3. Horizontal arrow denotes the position of the tracking dye. Stain: Coomassie blue.

maximum of 20 subunits was obtained with MW ranging from 7900 to 110000. In addition, the electrophoregram of albumin and globulin subunits showed little change between 20 and 32 DAF, in conformity with the results for the undissociated proteins. During ageing, the subunits of albumin decreased less in the intensity and number of stained bands than globulin subunits. But the major subunits of both fractions remained prominent after ageing, in contrast to the results of analytical gel electrophoresis (Fig. 2). A different protein dye was used in each analysis.

EXPERIMENTAL

Materials. Grains of the line IR1541-76-3 (grown with 120 kg N/ha) were collected at different stages of maturation from 2 to 32 (mature) DAF from the 1974 dry season crop of the IRRI agronomy department. The grains were immediately frozen in dry ice and freeze-dried. Preliminary experiments on fresh and freeze-dried material indicated little effect of freeze-drying on the results. The grains were sorted by size, then were manually dehulled and reduced to 100-mesh powder in a dental amalgamator. This powder was the starting material for all subsequent analyses. Samples were aged by storing the dried grain at ambient temp for at least 4 months. For the study of the amino acid composition of the milling fractions of the rice grain, mature IR8 seeds at 14% H_2O were furnished by the IRRI plant breeding department. Seeds were dehulled manually and milled for 60 sec at high speed. The pericarp, bran, polish, and germ were separated by differential sieving, and further cleared the germ of bran and polish contaminants by air flotation.

Extraction of proteins. Portions (1 g) of the powdered samples were weighed in 50-ml centrifuge tubes and were stirred with 10 ml petrol in the cold for 1 hr. This was then centrifuged at 20000 *g* for 15 min, and the residue re-extracted twice with petrol. In the preliminary experiment, defatting was found to improve the yield of proteins extracted by saline soln [18]. The defatted samples were then air-dried for 18 hr. 10 ml of 0.7 M NaCl was added to the tube and the mixture was equilibrated for 18 hr at 2°. The mixture was then gently stirred for 3 hr, and centrifuged at 0° for 20 min at 20000 *g*. The procedure was repeated ×3, except that the stirring was reduced to 1 hr each time. Extracts were pooled, filtered through Whatman No. 1 paper, and dialyzed for 36 hr at 2° against several changes of H_2O. We tried other concentrations of saline soln and found 0.7 M NaCl to give the highest yield of albumin and globulin in our preliminary work. The dialyzed materials were centrifuged at 20000 *g* for 20 min at 2°. The precipitated globulin was washed ×3 with cold H_2O to insure the removal of any albumin contaminant. The supernatant and washings (albumin fraction) and the ppt. (globulin fraction) were lyophilized. The materials were taken up in 3 ml of 0.01 M Na Pi buffer pH 7 and assayed for protein prior to amino acid analysis and gel electrophoresis.

Protein, soluble amino N, and amino acid composition. Total N of the defatted powder was determined by the automatic micro Kjeldahl method of ref [22]. Protein was assayed by the method of ref [23] using crystalline BSA as standard. Free amino N was determined by the manual method of ref [24] with leucine as the standard. Results were expressed on a per grain basis. Protein hydrolysis and analysis of the amino acid composition using an amino acid analyzer with PA-28 and PA-35 resins were as previously described [5, 22]. Cysteine was converted to cysteic acid prior to hydrolysis [25]. Tryptophan was determined by $Ba(OH)_2$ hydrolysis and short column chromatography [26].

Polyacrylamide gel electrophoreses. Anodic disc-electrophoresis was carried out according to the method of ref [27] using a lower gel of 7.5% acrylamide (pH 8.9); an upper gel of 2.5% acrylamide (pH 6.7); and a 25 mM Tris–glycine electrode buffer (pH 8.3). Constant current of 4 mA per gel was used and the samples were applied on equal protein basis. After the run (1–2 hr), the gels were stained with 1% Amido black in 7% HOAc from 2 to 12 hr. Gels were destained with 7% HOAc and visually appraised. The proteins in 10 mM Na Pi buffer was prepared for SDS-polyacrylamide gel electrophoresis by incubation with 2% SDS and 2% β-mercaptoethanol for 2 hr at 37°. The protein-SDS complex was dialyzed against 0.01 M Na Pi buffer which was 0.1% of both SDS and β-mercaptoethanol for 12 hr at room temp. Electrophoresis of samples with equal protein content (25 μg) was conducted at room temp for 4 hr at a constant current of 8 mA per tube according to the method ref [28]. The gels were stained with 0.625% Coomassie blue in MeOH–HOAc–H_2O (6:1:4) for 12 hr at room temp. Gels were destained with MeOH–HOAc–H_2O (3:2:35) and stored in 7.5% HOAc.

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