Purification and Partial Characterization of a Major Globulin from Rice Endosperm[1]

DAVID F. HOUSTON and ALI MOHAMMAD, Western Regional Research Laboratory, Albany, California

ABSTRACT

Albumins and globulins were extracted from rice flour with 5% sodium chloride solution. A major globulin component was purified by ammonium sulfate precipitation, repeated isoelectric precipitations, and gel-filtration chromatography. Progress was monitored by starch-gel electrophoresis. The purified globulin component migrates as a single compound in free-film and starch-gel electrophoresis, in column chromatography, and in ultracentrifugation. In the presence of urea it apparently dissociates into two portions which recombine when urea is removed. It shows no amylase activity, contains 18.1% nitrogen, has high glutamic acid and arginine content, moderately high serine, tyrosine, cystine, and methionine content, about 1% tryptophan, and essentially no lysine or histidine. Molecular weight estimated by Sephadex chromatography is about 25,500, and calculated from amino-acid content is 25,330. It has a sedimentation constant of 1.6S and a calculated specific volume of 0.706.

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Soluble proteins – albumins plus globulins – account for a minor proportion of the total proteins of milled rice; 80% or more of the total is relatively insoluble glutelin. A recent compilation (1) shows considerable variation in the amounts of soluble proteins found by various workers. In general, the globulins are reported as some 7 to 11% of the total proteins and the albumins as 0.5 to 6%. Each of these classes of protein is a mixture of individual components. Both albumins and globulins have been shown to be concentrated in the protein from the outer layers of the endosperm (2); they occur in even higher concentrations in polish and bran (3).

To obtain fundamental data for better evaluation of products made by processing or fractionating rice milling fractions, investigation has been undertaken of the individual components present in the soluble proteins. Available data on globulin components of milled rice are meager. Two major published reports – by Jones and Gersdorff (4) and by Kondo and Ito (5) – were made before the present powerful tools for protein investigation were developed. They are in conflict and in need of confirmation or correction and extension. No additional data are quoted in Bourdet's summary to 1955 on rice proteins (6). Padmoyo and Hogl in 1962 (7) reported electrophoretic mobilities for two globulin components. About the only point of agreement among these reports is the presence of two fractions in the milled-rice globulins.

Morita and Yoshida (8), who isolated a gamma-globulin from rice germ, reported alpha-globulin in all fractions of rice grain and alpha- and beta-globulins concentrated in the endosperm (milled-rice) proteins. Silaev et al. (9) found rice globulins to contain five components by polyacrylamide-gel electrophoresis at pH 8.65 with Tris-acetate buffer.

In a preliminary note Houston et al. (10) have reported separating the milled-rice globulins into two fractions. Segregation, purification, and partial characterization of the major component of the less-soluble fraction is reported herein. The more-soluble fraction, characterized by a high sulfur content, is the subject of separate studies.

MATERIALS AND METHODS

Flour

A commercial flour prepared from California short-grain rice was defatted by percolation with ethyl ether.

Gel Filtration

Globulins were fractionated on a Sephadex G-100 column 2.0 X 93 cm. in a 0.017M aluminum lactate-lactic acid buffer, pH 3.2, at a flow rate of 9 ml. $cm.^{-2}$ $hr.^{-1}$. The molecular weight of the purified component (1% solution) was estimated by the procedure of Whitaker (11) in the same buffer on a 2.0 X 100 cm. calibrated column of Sephadex G-100. Eluate from the columns was monitored for protein content at 280 nm. by a Gilson ultraviolet absorption detector (Gilson Medical Electronics).

Electrophoresis

Smithies' procedure for starch-gel electrophoresis (12) was used with a horizontal water-cooled apparatus (E. C. Apparatus Co.). Acidic gels were prepared

with 18% starch (Connaught Medical Research Laboratories) in 0.017M aluminum lactate-lactic acid (pH 3.2) 7.5M in urea or 12% starch in the same buffer without urea. Alkaline gels were prepared with 0.05M Tris-citrate buffer at pH 8.5. Samples were applied by soaking filter-paper strips in the sample solution and inserting them in a slit cut in the gel. Patterns were developed for 16 or 18 hr. at about 6 v. per cm. Gels were sliced horizontally into three layers and protein in the center layer was stained with Amido Black 10B. Gels were destained in water-glycerol-acetic acid (85:10:5).

Globulins were fractionated in a preparative free-film electrophoresis apparatus (Elphor, Model FF; Brinkman Instruments, Inc.) in 0.05M Tris-citrate buffer, pH 8.5, at about 30 v. per cm., 65 ma., at 10°C.

Ultracentrifugation

A 1% solution of globulin in 0.017M aluminum lactate-lactic acid (pH 3.2), after dialysis against the same buffer, was examined in a Spinco Model E ultracentrifuge at 52,640 r.p.m. at 20°C. Sedimentation and diffusion coefficients were determined from a sedimentation velocity run at one concentration. An apparent molecular weight was determined from the data by use of the Svedberg equation.

Amino Acid Determinations

Samples of protein (10 mg.) were weighed into ampoules and 10 ml. of fresh 6N HCl was added. After the solution was frozen, each ampoule was evacuated with a vacuum pump. The solution was allowed to thaw to remove entrapped air, then refrozen and the ampoule sealed – all under high vacuum. Samples were hydrolyzed at 110°C. for 20 or 70 hr. Hydrolyzed solutions were evaporated in 50-ml. flasks on a rotary evaporator at not over 40°C. Water was twice added to the residue and re-evaporated to remove HCl. The sample was then dissolved in the appropriate buffer solution, and portions were analyzed on a Phoenix automatic amino acid analyzer. Nitrogen was determined in a separate portion. Threonine and serine values were corrected by extrapolation back to zero time; for isoleucine the 70-hr. value was used.

Cystine and methionine were determined on a separate sample after performic acid oxidation (13). Tryptophan was determined by the Spies and Chambers procedure (14).

Other Analyses

Nitrogen was determined by the micro-Kjeldahl method.

Bernfeld's dinitrosalicylic acid procedure (15) was used in testing for alpha- and beta-amylase activity.

PROCEDURES

Preparation of Globulins

Portions of defatted flour were extracted with 4 vols. of 5% sodium chloride solution by stirring for 2 hr. at room temperature. Solids were centrifuged out and the supernatant was decanted and filtered. Globulins were precipitated from the filtrate by 30% saturation with ammonium sulfate, and separated by centrifugation. The supernatant was removed for recovery of albumins. The process is similar to that of Jones and Gersdorff (4).

Crude globulin was redissolved in 2.5% sodium chloride solution, filtered from slight amounts of insoluble matter, and again precipitated by 30% saturation with ammonium sulfate. Globulin recovered from this step after centrifugation was redissolved in 2.5% sodium chloride, dialyzed against distilled water, and freeze-dried.

Isoelectric Precipitation Fractionation

The crude globulins were dissolved in 1% acetic acid at pH 2.5. Gradual addition, with vigorous stirring, of 0.5N NaOH to bring the pH to 4.5 produced a precipitate (G-I). This was separated by centrifuging. The supernatant was decanted and brought to pH 7.0 by further addition of 0.5N NaOH. A second precipitate (G-II) was formed. This was separated by centrifuging and decantation of the supernatant, which still contained protein (G-III). The three fractions were recovered by dialyzing against water and freeze-drying. Further discussion in this report concerns the precipitated fraction (G-I).

Purification of Globulin Component G-I

Component G-I was dissolved in 1% acetic acid and reprecipitated by adjusting the pH to 4.5. After centrifugation and discard of the supernatant, the precipitate was redissolved in 1% acetic acid. The precipitation was made a total of three times. The precipitate was again dissolved, dialyzed against distilled water, and freeze-dried. Finally the component G-I was dissolved in 0.017M aluminum lactate-lactic acid and passed through a Sephadex G-100 column in the same buffer. The purified component was recovered from the appropriate eluate fractions by dialysis and freeze-drying.

RESULTS AND DISCUSSION

Purification

Precipitation of fraction G-I by pH change was highly effective in separating it from more-soluble components of similar molecular weight. This step in purification preferably precedes column separations, as it does not limit amounts that can conveniently be handled, and removes soluble components of about the same molecular size which are present. Separation is not quantitative, and some of the desired material appears in fraction G-II. Repeated precipitation allows removal of traces of soluble material that may be carried down with the precipitate. Fraction G-I is estimated to be about 40% of the total globulins.

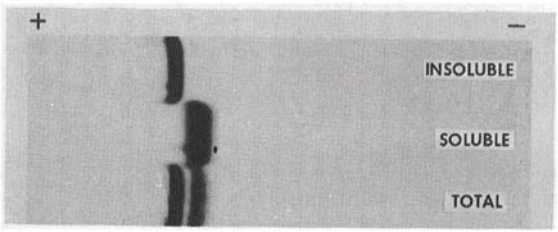

Fig. 1. Electrophoretic patterns in starch gel of milled rice globulins: insoluble at pH 4.5, soluble at pH 7.0, and total. Aluminum lactate-lactic acid, pH 3.2, 0.017M; 16 hr. at 6 v. per cm. Origin at left margin.

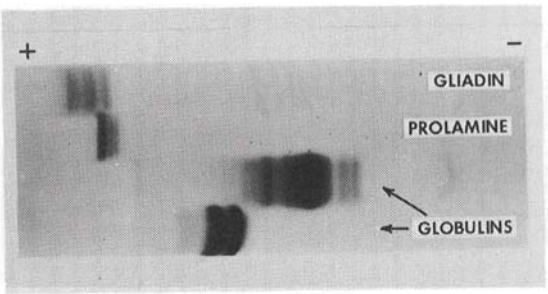

Fig. 2. Electrophoretic patterns in starch gel (7.5M in urea) of globulin insoluble at pH 4.5 and soluble at pH 7.0, milled rice prolamin, and wheat gliadin for comparison. Aluminum lactate-lactic acid, pH 3.2, 0.017M with 7.5M urea; 18 hr. at 6 v. per cm. Origin at left margin.

A starch-gel electrophoregram of the precipitated fraction G-I and the material remaining soluble at pH 7.0 (G-III), shown in Fig. 1 together with the pattern of the total globulins precipitated by ammonium sulfate, illustrates the effectiveness of the combined precipitation and gel-filtration procedures. The wide band for the soluble fraction suggests a complex of several components. This fraction contains the previously noted high-sulfur component (10).

Effect of Urea

When starch-gel electrophoresis was run with aluminum lactate-lactic acid buffer 7.5M in urea, globulin G-I gave two closely spaced bands of nearly equal density. Figure 2 shows the apparent dissociation for slightly impure pH 4.5-precipitated and pH 7-soluble fractions, and demonstrates the greater complexity of the soluble protein. The corresponding single and double band patterns (not shown) of the precipitable component also were found on electrophoresis with Tris-citrate buffer at pH 8.5 in the absence and presence of 7.5M urea, respectively.

When the precipitable component was kept overnight in aluminum lactate-lactic acid buffer, 7.5M in urea, and then recovered by dialysis and freeze-drying, the recovered protein gave a single-band pattern identical with that of the original. The apparent dissociation into two subcomponents caused by 7.5M urea was not found with solutions containing urea at concentrations up to 5M, nor were cyanate ion effects noted.

The suggested dissociation could also result from reversible denaturation by urea, though this seems less likely. The urea could differentially denature two proteins having equal charges and molecular weights. Or there could be an equilibrium between native and urea-denatured protein of a single species. Further study is necessary to resolve this point.

Monocomponent Indications

When the purified protein was re-run on Sephadex G-100, a single component eluted in the same position as the original fraction. When subjected to free-film electrophoresis (Elphor), the collected fractions showed a single

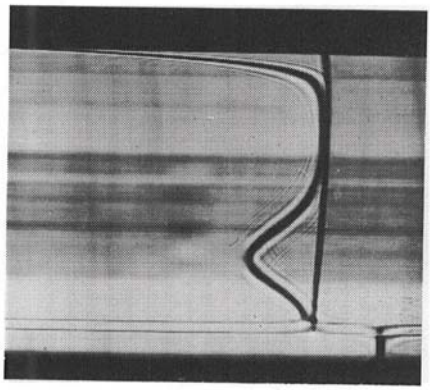

Fig. 3. Sedimentation pattern of purified rice globulin 1.0% in 0.017M aluminum lactate-lactic acid, pH 3.2. Pattern obtained at 20°C., 145 min. after attaining speed of 52,640 r.p.m. Bar angle 60°.

component. Thus, one component was indicated by both molecular size and charge separations. Moreover, ultracentrifugation showed a single peak (Fig. 3).

Analytical

The nitrogen content, determined by the micro-Kjeldahl method, is 18.1% (dry basis). The amino acid composition (Table I) is a little surprising, with almost complete absence of histidine and lysine. A small increase of apparent lysine content with time of hydrolysis suggested some possible formation of ornithine from arginine, which is a major component. A special run to determine ornithine in the presence of lysine indicated that both were present, although the amounts were too small for satisfactory measurement. Ammonia liberated corresponds roughly to about half of the total aspartic and glutamic acids occurring in the amide form.

Glutamic acid and arginine comprise 43% of the total molecule. The sulfur amino acid content is also quite high. Conversely, aspartic acid and tryptophan are rather low.

TABLE I. AMINO ACID COMPOSITION[a]

Amino Acid	Residues g.	mmoles	Amino Acid	Residues g.	mmoles
Lysine	0.13	1.0	Cystine	4.16	37.7
Histidine	trace	trace	Valine	3.14	31.7
Arginine	18.26	116.9	Methionine	5.76	38.8
Aspartic acid	3.15	27.4	Isoleucine	1.28	11.3
Threonine	1.62	16.0	Leucine	5.77	51.0
Serine	7.98	91.6	Tyrosine	7.42	45.5
Glutamic acid	24.89	192.8	Phenylalanine	3.02	20.5
Proline	3.99	41.1	Tryptophan	0.98	5.3
Glycine	4.12	72.2	Total	99.02	847.9
Alanine	3.35	47.1	Ammonia	1.73	107.8

[a]Per 100 g. protein containing 18.1% N.

Tests for alpha- and beta-amylase activity were negative under conditions showing moderate positive values for the pH 7-soluble fraction and more strongly positive ones for rice albumins.

Molecular Weight

Gel filtration on a calibrated Sephadex G-100 column provided an estimated molecular weight of 25,500. The ultracentrifuge data gave a preliminary value of 20,000 to 25,000, and a sedimentation coefficient of 1.6S, uncorrected. Based on the amino acid composition, the molecular weight would be 12,665 for one tryptophan or 25,330 for two. Calculated specific volume (16) is 0.706. From these data the molecular weight is estimated at about 25,400.

General

The solubility of the protein is that of a globulin. The high arginine and low tryptophan agree with Pence and Elder's (17) distinction of globulins from albumins. The sedimentation coefficient would place this protein among the alpha-globulins (18,19), or with what Djurtoft (20) calls "< alpha-components" – those having S-values of 2.1 or less.

However, this globulin has a rather unusual composition in two respects. The arginine content (18.26%) is somewhat high for cereal globulins as a class (19), though Taira (21) has reported 16.9% in rice globulins and Waldschmidt-Leitz (22) has reported 21.6% in barley globulins and 15.4% in oat globulins. Also, the essential absence of both histidine and lysine appears unique among cereal proteins. It will be of considerable interest to learn if the gamma-globulin from rice germ (8) shows any similarity of composition to the present component.

Additional studies are required to discover whether other protein components from rice have unusual amino acid patterns and how and to what extent rice proteins may differ from proteins of other cereals. Information of this type will improve means of evaluating rice and its milling fractions for specific food and feed uses.

Acknowledgment

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Literature Cited

1. JULIANO, B. O. Physicochemical data on the rice grain. Intern. Rice Res. Inst. Tech. Bull. 6, 150 pp. (1966).
2. HOUSTON, D. F., IWASAKI, T., MOHAMMAD, A., and CHEN, LORA. Radial distribution of protein by solubility classes in the milled rice kernel. J. Agr. Food Chem. 16: 720 (1968).
3. CAGAMPANG, GLORIA B., CRUZ, LOURDES J., ESPIRITU, S. G., SANTIAGO, R. G., and JULIANO, B. O. Studies on the extraction and composition of rice proteins. Cereal Chem. 43: 145 (1966).
4. JONES, D. B., and GERSDORFF, C. E. F. The globulins of rice, *Oryza sativa*. J. Biol. Chem. 74: 415 (1927).
5. KONDO, K., and ITO, T. Proteins. XII. Globulins of polished rice. J. Chem. Soc. Japan 51: 67 (1930); Chem. Abstr. 24: 3522 (1930).
6. BOURDET, A. Les protides des céréales. Ann. Technol. Agr. 2: 181 (1956). [English translation by Majel M. MacMasters, March 1960]

7. PADMOYO, M., and HÖGL, O. Beiträge zu der Papierelektrophorese des Getreide-Eiweisses. Mitt. Geb. Lebensm. Hyg. 53: 290 (1962).
8. MORITA, Y., and YOSHIDA, C. Studies on γ-globulin of rice embryo. Part I. Isolation and purification of γ-globulin. Agr. Biol. Chem. (Tokyo) 32: 664 (1968).
9. SILAEV, A. B., LIEH, S-H., and SAFONOV, V. I. Investigation of proteins of Vietnam rice by polyacrylamide gel electrophoresis. Prikl. Biokhim. Microbiol. 1: 250 (1965); Chem. Abstr. 63: 8958 (1965).
10. HOUSTON, D. F., MOHAMMAD, A., and ALFONSO-HERNANDEZ, NORMA E. High-sulfur seed proteins. Cereal Chem. 41: 427 (1964).
11. WHITAKER, J. R. Determination of molecular weights of proteins by gel filtration on Sephadex. Anal. Chem. 35: 1950 (1963).
12. SMITHIES, O. Zone electrophoresis in starch gels and its application to studies of serum proteins. Advan. Protein Chem. 14: 65 (1959).
13. MOORE, S. On the determination of cystine as cysteic acid. J. Biol. Chem. 238: 235 (1963).
14. SPIES, J. R., and CHAMBERS, D. C. Chemical determination of tryptophan in proteins. Anal. Chem. 21: 1249 (1949).
15. BERNFELD, P. Assay method for α- and β-amylases. In: Methods of enzymology, S. P. Colowick and N. O. Kaplan, eds.; vol. I, p. 149. Academic Press: New York (1955).
16. COHN, E. J., and EDSALL, J. T. Proteins, amino acids and peptides as ions and dipolar ions, p..375. Reinhold: New York, N.Y. (1943).
17. PENCE, J. W., and ELDER, A. H. The albumin and globulin proteins of wheat. Cereal Chem. 30: 275 (1953).
18. DANIELSSON, C. E. Seed globulins of the *Gramineae* and *Leguminosae*. Biochem. J. 44: 387 (1949).
19. FEILLET, P. Les proteines soluble des ceréales. Ann. Technol. Agr. 16: 135 (1967).
20. DJURTOFT, R. Modern techniques of protein investigation and their use in brewing. Wallerstein Labs. Communs. 26: 83 (1963).
21. TAIRA, H. Studies on amino acid contents in plant seed. II. Amino acid pattern of seed protein fractions of *Gramineae*. Botan. Mag. (Tokyo) 75: 273 (1962). [English translation by H. Ueno]
22. WALDSCHMIDT-LEITZ, E. Zur chemischen Systematik der Getreideeiweisskörper. Proc. 9th European Brewery Conv., p. 78 (1963; publ. 1964).

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