Isolation and Characterization of Rice α-Globulin[1]

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ABSTRACT

A new, mild isolation scheme for rice α-globulin is reported. The α-globulin is separated from low molecular weight components of the globulin fraction by chromatography on hydroxyapatite. A major difference between this approach and a previous purification procedure for the protein is that in our method the protein is not exposed to low pH. Our method of purification should allow conformational studies to be conducted on rice α-globulin. An initial physicochemical characterization of the purified protein is reported.

Cereal Chem. 65(4):316–319

The globulin fraction is the second most abundant Osborne solubility group in rice (Juliano 1985). Nonetheless, limited information is available on individual components of the globulin fraction, which has been divided into two subfractions (Houston and Mohammad 1970, Cagampang et al 1976). One of these is a protein that has been called α-globulin (a name that we will use in this paper). The other subfraction contains polypeptides that have lower molecular weights than does α-globulin (Houston et al 1964).

The method that has been used previously for purifying this α-globulin involves repeated precipitation at pH 4.5 and dissolution at pH 2.5 (Houston and Mohammad 1970, Perdon and Juliano 1978). We are concerned that exposure to these low pH values could denature the protein. Here we report a new, mild isolation scheme for rice α-globulin. This procedure is designed to minimize the possibility of denaturating the protein. The α-globulin prepared by this method is suitable for determining the protein's physicochemical properties. We report some initial studies of that sort in this paper.

MATERIALS AND METHODS

Sample Preraration

Rice seeds (Newbonnet variety) were supplied by Robert Dilday of the USDA Rice Research Center in Stuttgart, AR. Seeds were milled and ground to 68-mesh. The milled rice flour was sequentially extracted with deionized water, 0.5*M* NaCl, 70% ethanol, and 0.5% sodium dodecyl sulfate (SDS) with 0.6% β-mercaptoethanol. The sample was stirred with two volumes of solvent at room temperature for 20 min. Extracts were separated from residues by centrifugation at 3,500 × *g* for 10 min. The procedure was repeated three times. The extracts were stored in the freezer. A direct extraction with 0.5% SDS/0.6% β-mercaptoethanol was also performed. Milled rice flour was extracted with two volumes of this solvent three times, for 20 min each time, at room temperature. The residue was removed by centrifugation at 3,500 × *g* for 10 min.

Gel Electrophoresis

In two-dimensional gel electrophoresis, the first dimension was nonequilibrium pH gradient electrophoresis (O'Farrell et al 1977). The second dimension was discontinuous SDS slab gel (210 × 150 × 0.8 mm) electrophoresis. First dimension electrophoresis was carried out at an initial voltage of 300; the voltage was then increased in several steps over a 1-hr period to 1,000 until a total of 3,000 Vhr was reached. An ampholyte mixture (LKB) ranging from pH 3.5 to pH 10 was used in the first dimension.

One-dimensional SDS polyacrylamide gel electrophoresis (PAGE) was carried out following the same procedure used for the second dimension of the two-dimensional electrophoresis.

Proteins used as molecular weight standards were: lysozyme (14,000), soybean trypsin inhibitor (Kunitz) (21,000), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,000), and phosphorylase B (92,000). All were purchased from Sigma.

Purification of α-Globulin

An NaCl extract was subjected to $(NH_4)_2SO_4$ precipitation at 30% saturation. The precipitate was collected by centrifugation at 10,000 × *g*, dissolved in 0.5*M* NaCl/0.05*M* Tris-Cl (pH 8.2), and subjected to chromatography on a hydroxyapatite column (2.8 × 18 cm) equilibrated with the same buffer. Hydroxyapatite (Bio-gel HTP) was obtained from Bio-Rad Laboratory. The flow rate was 120 ml/hr. After the flow through had been completely eluted, 0.02*M* sodium phosphate (pH 7.0) was applied to the column. This resulted in the elution of α-globulin in a rather broad peak. More recently we eluted the protein by applying 0.02*M* phosphate buffer (pH 7.0)/0.5*M* NaCl.

Gel Permeation Chromatography

A Sephadex G-75 column (1.4 × 82 cm) was equilibrated and eluted with 0.05*M* Tris-Cl (pH 8.2)/0.5*M* NaCl. The flow (downward) was at 23 ml/hr. Proteins used as molecular weight standards were: cytochrome C, lysozyme, myoglobin, soybean trypsin inhibitor (Kunitz), α-chymotrypsinogen, ovalbumin, and bovine serum albumin. All were obtained from Sigma.

Ultracentrifugal Analyses

Ultracentrifugation was carried out in a Beckman model E analytical ultracentrifuge equipped with Schlieren and interference optics and a rotor temperature indicator and control unit.

The molecular weight of α-globulin was determined by meniscus depletion sedimentation equilibrium using interference optics (Yphantis 1964). Purified α-globulin (2.4 mg/ml) in 0.5*M* NaCl/0.02*M* phosphate buffer (pH 7.0) was examined after dialysis against the same buffer overnight at 4° C. Centrifugation was carried out at 60,000 rpm at 20° C. After the run, the baseline-corrected fringe displacement was used to plot ln(concentration) versus the square of the radial position. The molecular weight of α-globulin was computed from the slope of this plot. A partial specific volume of 0.699 was calculated (McKeekin and Marshall 1952) from the amino acid composition of α-globulin as determined in this study.

The extinction coefficient of α-globulin was obtained by counting the fringes across a boundary formed by low speed centrifugation of a sample of known absorbance at 280 nm. A solution of 1 mg of protein per milliliter corresponds to 4.1 fringes (Babul and Stellwagen 1969). α-Globulin was dissolved in 0.02*M* phosphate buffer (pH 7.0)/0.5*M* NaCl and dialyzed overnight against the same buffer. The solution was then subjected to ultracentrifugation.

Amino Acid Composition

The amino acid composition of purified α-globulin was determined after hydrolysis with 6*M* HCl at 110° C for 24, 48, and

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This manuscript was prepared for electronic processing.

72 hr. Threonine and serine values were obtained by extrapolation to zero time of hydrolysis; 72-hr values were used for isoleucine and valine. Half-cystine was determined as cysteic acid after performic acid oxidation and hydrolysis with 6*M* HCl for 24 hr. Amino acid analysis was performed using the Pico Tag method (Bidlingmeyer et al 1984) of the Waters Chromatography division of Millipore Corporation. All reported values are averages of two or more individual determinations.

Circular Dichroism Measurements

Circular dichroism spectra were obtained on a Cary 60 spectropolarimeter with a model 6001 CD attachment. A 1-mm cell and a protein concentration of 0.37 mg/ml were used. Ellipticities are reported in deg-cm^2/dmol of amino acid residues. The mean molecular weight of the amino acid residues was taken as 110.

RESULTS

PAGE

Figure 1 shows one-dimensional SDS-PAGE patterns of the four Osborne solubility fractions of rice flour and that of the total rice protein. A similar analysis was published by Robert et al (1985). In the gel for Figure 1, however, we applied amounts of each of the four solubility fractions that were derived from equivalent portions of rice flour. This gives a visual impression of the relative amounts of protein in the four fractions.

In rice, up to 80% of the endosperm protein is in the glutelin fraction (Juliano 1985). Therefore, the gel pattern of the glutelin fraction is expected to be rather similar to that of total rice protein (lanes 4 and 5, Fig. 1). The most abundant groups of polypeptides in these lanes have apparent molecular weights of 37,000 and 22,000, which is consistent with the results of others (Yamagata et al 1982, Zhao et al 1983).

α-Globulin was previously reported to have an apparent molecular weight of about 25,000 (Houston and Mohammad 1970, Perdon and Juliano 1978). It was therefore tentatively identified as being the most intense band in lane 2, and this identification was verified in work reported below. The lower molecular weight components of the globulin fraction are likely to be the "more soluble globulins" with high sulfur content reported by Houston et al (1964).

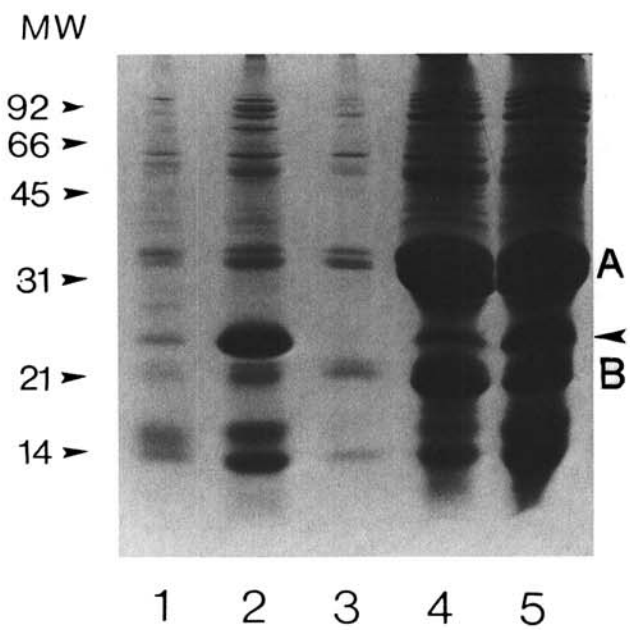

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of total rice protein and the four Osborne solubility fractions. Lane 1, water-extracted fraction; lane 2, NaCl-extracted fraction; Lane 3, ethanol-extracted fraction; lane 4, sodium dodecyl sulfate/β-mercaptoethanol extracted fraction; lane 5, total rice endosperm proteins. **A** and **B** are the rice glutelin polypeptides (M_r 37,000 and 22,000, respectively). Arrow head indicates α-globulin.

Rice prolamin has been reported to contain mainly polypeptides with a molecular weight of 13,000 (Yamagata et al 1982). We obtained little protein in the prolamin fraction (lane 3, Fig. 1). This is consistent with published values concerning rice prolamin content (Robert et al 1985).

Total proteins extracted from rice flour with SDS were subjected to two-dimensional gel electrophoresis (Fig. 2). Considerable heterogeneity was observed in the acidic and basic polypeptides of rice glutelin. This is consistent with the published two-dimensional gels of Wen and Luthe (1985). In contrast, α-globulin consists very largely of one major spot. After purification, either by previously published methods or the approach described in this paper, α-globulin preparations consist of the major spot indicated by an arrowhead in Figure 2 and the single, much less intense spot indicated by a second arrowhead (Pan 1984). Of course, one cannot rule out the possibility that the major spot contains several closely related proteins, but at the current level of analysis, α-globulin appears to be much less heterogeneous than rice glutelin.

Two-dimensional gels were subjected to a sensitive fluorescence stain for carbohydrate (Eckhardt et al 1976). We observed no fluorescence associated with α-globulin (result not shown). As a verification of the method, we did observe strong fluorescence for soybean glycinin, as reported by Lei and Reeck (1987).

Isolation and Characterization of α-Globulin

The use of ion-exchangers for column chromatography was ruled out by the considerable NaCl concentration required for maintaining solubility of proteins in the globulin fraction. We obtained no useful separation of the globulin proteins by gel permeation chromatography. We did find, however, that α-globulin could be effectively separated from low molecular weight components of the globulin fraction by chromatography on hydroxyapatite. This was possible because α-globulin was retained by the column even in 0.5*M* NaCl. Elution of the α-globulin (Fig. 3) was achieved with sodium phosphate (see Methods). We have observed a limited degradation of α-globulin during this chromatography step. The extent of degradation is somewhat erratic, with a band of electrophoretic mobility between that of α-globulin and the low molecular weight globulins frequently being observed. Fortunately, the degraded material is partially separated from intact α-globulin in the elution process (Fig. 3).

In the further studies reported below, we used undegraded material from the initial portion of the α-globulin peak.

Amino Acid Composition of α-Globulin

Overall, the composition reported here (Table I) is similar to

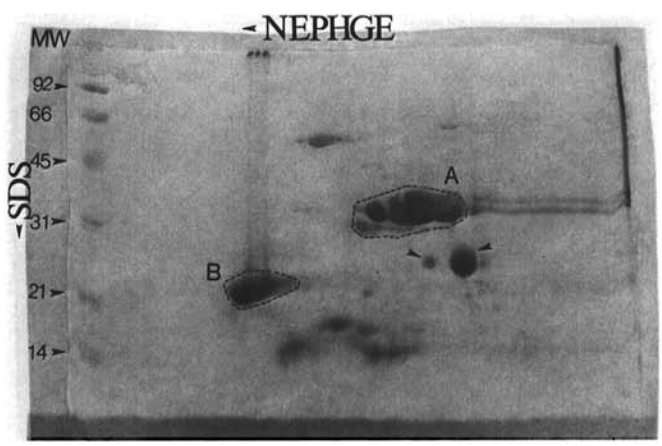

Fig. 2. Two-dimensional gel electrophoresis of rice total proteins (nonequilibrium pH gradient electrophoresis **[NEPHGE]** and discontinuous sodium dodecyl sulfate **[SDS]** slab gel electrophoresis). **A** denotes acidic polypeptides and **B** denotes basic polypeptides of glutelin. Arrow heads indicate major and minor components of α-globulin.

those reported by other researchers (Houston and Mohammed 1970, Perdon and Juliano 1978, Pascual et al 1981), but we obtained lower tyrosine and methionine values and higher values for serine, threonine, and half-cystine. Hydrolysis of α-globulin in methane sulfonic acid produced no detectable tryptophan.

Physical Properties of the Purified α-Globulin

Two methods were used to obtain an initial estimate of the protein's molecular weight. Gel filtration on a calibrated Sephadex G-75 column provided an apparent molecular weight for α-globulin of 17,400 (Fig. 4). The molecular weight determined by SDS-PAGE was 26,000 (Fig. 1).

Because these methods were in disagreement, we used sedimentation equilibrium in the analytical ultracentrifuge to obtain a rigorous determination of molecular weight. A plot of the logarithm of concentration as a function of the square of the radial distance (Fig. 5) showed no indication of heterogeneity and yielded a molecular weight of 16,700. Evidently, α-globulin migrates aberrantly (more slowly than expected) on SDS-PAGE, thus giving an estimated molecular weight somewhat too high.

The far-ultraviolet circular dichroism spectrum of our preparation of rice α-globulin is shown in Figure 6. The spectrum is consistent with a content of about 49% helix, as determined with the algorithm of Siegel et al (1980).

The extinction coefficient of α-globulin ($A_{280}^{0.1\%}$) was found to be 1.07.

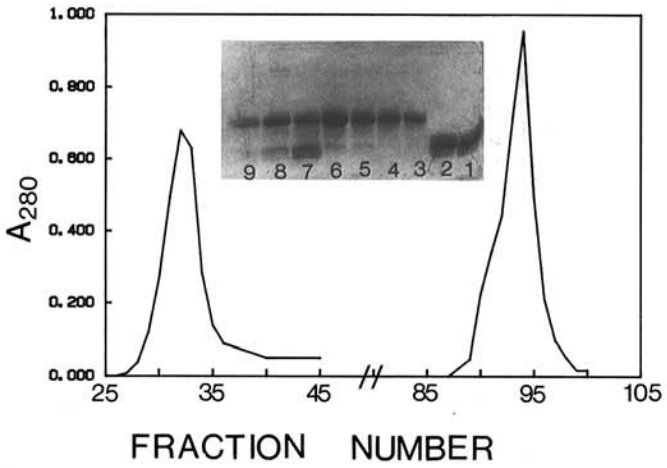

Fig. 3. Elution profile for hydroxyapatite chromatography of rice α-globulin. The lower molecular weight globulins were in the flow through. The leading shoulder (fraction 90–93) of the elution peak contains largely intact α-globulin. Samples for gel inset: Lanes 1 and 2, flow-through fractions 32 and 33; lanes 3–9, fractions 90–96, respectively.

TABLE I
Amino Acid Composition of Rice α-Globulin[a]

Amino acid	This Work	A[b]	B[b]	C[b]
Aspartic acid	3.1	3.3	4.7	3.8
Glutamic acid	22	23	24	25
Serine	14	11	11	10
Glycine	10	8.6	8.5	8.5
Histidine	0.0	trace	0.3	0.1
Arginine	12	14	11	14
Threonine	3.5	1.9	2.3	2.0
Alanine	6.1	5.6	5.0	5.7
Proline	5.1	4.9	5.4	5.1
Tyrosine	2.9	5.4	4.7	5.5
Valine	4.2	3.8	4.4	3.5
Methionine	1.7	4.6	3.4	3.6
Half-cystine	6.7	4.5	3.1	3.4
Isoleucine	1.5	1.3	1.8	1.3
Leucine	4.9	6.1	6.3	6.2
Phenylalanine	2.4	2.4	2.6	2.6
Lysine	0.1	0.1	0.9	0.5

[a] Data are given in mol %.
[b] A, B, and C are the amino acid compositions of α-globulin as reported by Houston and Mohammad (1970), Perdon and Juliano (1978), and Pascual et al (1981), respectively.

DISCUSSION

The main emphasis in the work reported here was to develop an isolation procedure for rice α-globulin that did not expose the protein to low pH. It is our concern that repeated exposure to pH 2.5 in the earlier purification procedure for the protein (Houston and Mohammad 1970, Perdon and Juliano 1978) might result in changes in protein conformation that would not be easily reversed. More fundamentally, one would have no reference point with which to judge whether a reversal (i.e., renaturation) had occurred. The purification procedure we have developed should provide that needed reference point since, in it, we have worked at neutral pH and not exposed the protein to temperatures or solvents that are likely to cause denaturation.

A problem in our purification procedure, as it currently stands, is a persistent degradation of α-globulin during the hydroxyapatite chromatography. We assume this is caused by a contaminating

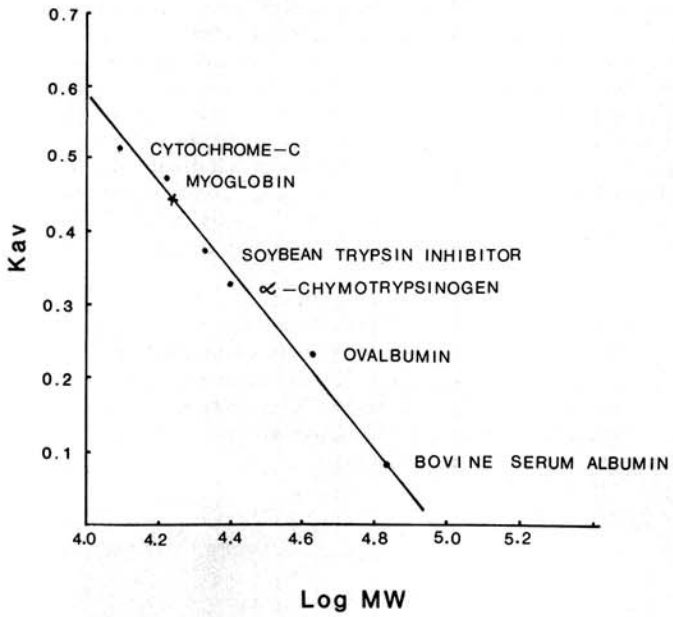

Fig. 4. Estimation of molecular weight of rice α-globulin by gel permeation chromatography. **Kav,** the fraction of the stationary gel volume that is accessible to a protein. **Log MW,** log molecular weight.

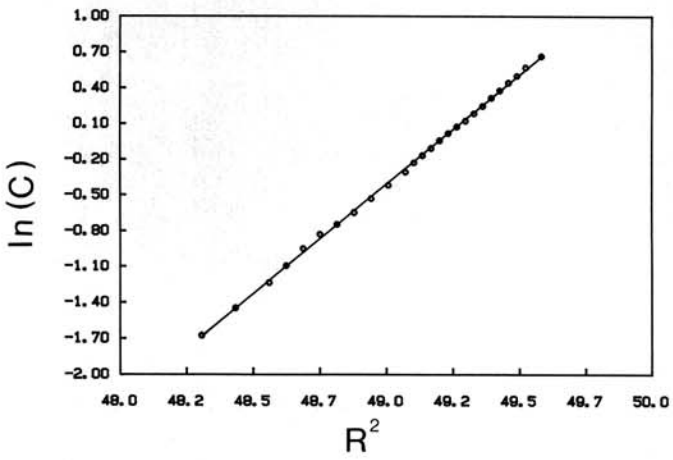

Fig. 5. Sedimentation equilibrium of rice α-globulin. Abscissa value is the square of the distance from the meniscus to the center of the sample solution. Ordinate value is the corresponding concentration of the sample solution.

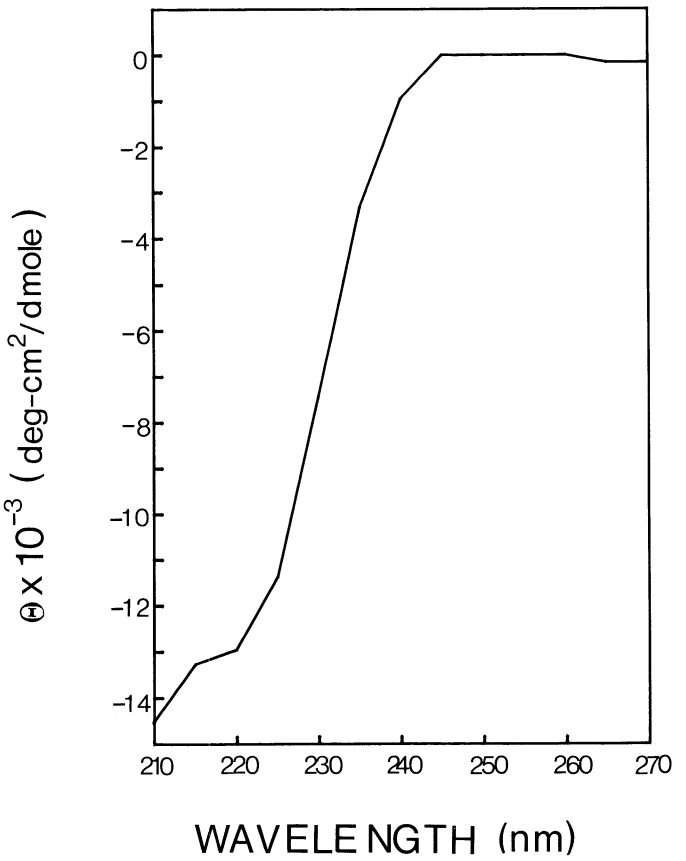

Fig. 6. Circular dichroism spectrum of rice α-globulin in 0.05*M* NaCl/0.02*M* sodium phosphate (pH 7.0).

protease, but we have not been able to inactivate the proteolytic activity with commonly used chemical or protein inhibitors of proteases. The partial separation of degraded and nondegraded α-globulin on the hydroxyapatite column does, however, provide a preparation of intact protein that can be used for physicochemical characterization.

None of the studies we have reported here prove that our preparation of α-globulin is in fact in a different conformational state than is α-globulin prepared by earlier methods. It is interesting, however, that our preparation shows no tendency to aggregate, whereas Perdon and Juliano (1978) reported that α-globulin underwent aggregation between pH 6 and pH 9. This, however, could be due to differences in solvent conditions.

In summary, the purification procedure we developed will allow conformational studies on rice α-globulin that has not been exposed to overt denaturing conditions. We are currently undertaking such studies, including the investigation of pH-dependent conformational changes.

ACKNOWLEDGMENTS

We thank David Manning for his help in the ultracentrifuge study. This work was supported by the Kansas Agricultural Experiment Station and by a grant from the Rockefeller Foundation.

LITERATURE CITED

BABUL, J., and STELLWAGEN, E. 1969. Measurement of protein concentration with interferences optics. Anal. Biochem. 28:216.

BIDLINGMEYER, B. A., COHEN, S. A., and TARVIN, T. L. 1984. Rapid analysis of amino acids using pre-column derivatization. J. Chromatogr. 336:93.

CAGAMPANG, G. B., PERDON, A. A., and JULIANO, B. O. 1976. Changes in salt soluble proteins of rice during grain development. Phytochemistry 15:1425.

ECKHARDT, A. E., HAYER, C. E., and GOLDSTEIN, I. J. 1976. A sensitive fluorescent method for the detection of glycoproteins in polyacrylamide gels. Anal. Biochem. 73:192.

HOUSTON, D. F., MOHAMMAD, A., and HERNANDEZ, N. E. A. 1964. High-sulfur seed proteins. Cereal Chem. 41:427.

HOUSTON, D. F., and MOHAMMAD, A. 1970. Purification and partial characterization of a major globulin from rice endosperm. Cereal Chem. 47:5.

JULIANO, B. O. 1985. Polysaccharides, proteins, and lipids of rice. Page 59 in: Rice: Chemistry and Technology, 2nd ed. B. O. Juliano, ed. Am. Assoc. Cereal Chem.: St. Paul, MN.

LEI, M. G., and REECK, G. R. 1987. Two-dimensional analysis of soybean protein bodies. J. Agric. Food Chem. 35:296.

MCKEEKIN, T. L., and MARSHALL, K. 1952. Specific volumes of proteins and the relationships to their amino acid contents. Science 116:142.

O'FARRELL, P. Z., GOODMAN, H. M., and O'FARRELL, P. H. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell 12:1133.

PAN, S. J. 1984 Electrophoretic studies of rice proteins and characterization of rice endosperm α-globulin. M.S. thesis. Kansas State University, Manhattan.

PASCUAL, C. G., JULIANO, B. O., and TANAKA, Y. 1981. Fractionation of globulins of milled rice. Phytochemistry 20:2471.

PERDON, A. A., and JULIANO, B. O. 1978. Properties of a major α-globulin of rice endosperm. Phytochemistry 17:351.

ROBERT, L. S., NOZZOLILLO, C., and ALTOSAAR, I. 1985. Homology between rice glutelin and oat 12S globulin. Biochim. Biophys. Acta 829:19.

SIEGEL, J. B., STEINMETZ, W. E., and LONG, G. L. 1980. A computer-assisted model for estimating protein secondary structure from circular dichroic spectra: Comparison of animal lactate dehydrogenases. Anal. Biochem. 104:160.

WEN, T.-N., and LUTHE, D. S. 1985. Biochemical characterization of rice glutelin. Plant Physiol. 78:172.

YAMAGATA, H., SUGIMOTO, T., TANAKA, K., and KASAI, Z. 1982. Biosynthesis of storage proteins in developing rice seeds. Plant Physiol. 70:1094.

YPHANTIS, D. A. 1964. Equilibrium ultracentrifugation of dilute solutions. Biochemistry 3:297.

ZHAO, W.-M., GATEHOUSE, J. A., and BOULTER, D. 1983. The purification and partial amino acid sequence of a polypeptide from the glutelin fraction of rice grains; homology to pea legumin. FEBS Lett. 162:96.

[Received July 8, 1987. Revision received January 25, 1988. Accepted January 26, 1988.]