Studies on γ Globulin of Rice Embryo

Part II. Separation of Three Components of γ Globulin by Ion Exchange Chromatography

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An improved method has been described for the isolation and purification of γ globulin from rice embryo. The method involves the extraction with phosphate buffer, pH 7.0 and ionic strength 0.1, the fractionation in saline solution of ionic strength 0.31, the removal of nucleic acids by precipitation with ammonium sulfate and the gel filtration chromatography on a Sephadex G-200 column. Although the preparations exhibited homogeneous patterns in sedimentation analysis, the electrophoretic patterns on polyacrylamide gels at pH 8.35 and ionic strength 0.11 exhibited at least two components. Three major components, γ_1 , γ_2 and γ_3 globulins, were isolated by ion exchange chromatography on a DEAE Sephadex A-50 column. These components were revealed to be homogeneous in electrophoresis as well as sedimentation. N-Terminal amino acid compositions have also been described.

In the first paper of this series of investigation, Morita and Yoshida¹⁾ first demonstrated the composition of globulin of rice kernel, consisting of α , β , γ and δ components, by means of ultracentrifugal analysis and gel filtration chromatography. They found that γ globulin was associated predominantly with rice embryo as in the cases of the seed proteins of Gramineae and Leguminosae^{2,3)} and accounted for about 20% of the total protein of rice embryo. The rice γ globulin had the sedimentation coefficient of 7 S suggesting the molecular weight of about 150,000, and it was soluble in saline solution of high concentration at neutral pH and was precipitated at low temperature. These characteristics are very

similar to those of the major globulins of Leguminosae such as glycinin of soybean seed and edestin of hemp seed. Morita and Yoshida presented a simple method of isolation and purification of γ globulin of rice embryo by gel filtration chromatography, giving a homogeneous preparation by sedimentation analysis, although the electrophoretic homogeneity could not be examined at neutral pH owing to the least solubility at low ionic strength. However, we have experienced that the simple method of isolation had a disadvantage of contaminating often some other protein components and especially nucleic acids in the γ globulin preparation. Furthermore, we have found that the γ globulin preparation seemed to be heterogeneous by the solubility test in saline solution of lower concentrations. A preliminary electrophoretic experiment of the γ globulin preparation also supported the heterogeneity, showing the presence of at least two components at pH 10.5, although

1) Y. Morita and C. Yoshida, *Agr. Biol. Chem.*, **32**, 664 (1968).

2) O. Quensel, "Dissertation," University of Uppsala, Sweden, p. 96 (1942). See J. W. Pence and A. H. Elder, *Cereal Chem.*, **30**, 275 (1953).

3) C. E. Danielsson, *Biochem. J.*, **44**, 387 (1949).

the sedimentation coefficient, 7S, did not decrease at this pH (Yoshida and Morita: unpublished).

In order to elucidate the chemical nature of γ globulin of rice embryo, it is indispensable to isolate the homogeneous components of γ globulin. The present paper describes an improved method for preparing γ globulin and a moderate method for isolating at least three components by means of ion exchange chromatography.

MATERIALS AND METHODS

Preparation of crude globulin fraction. Rice embryos were prepared by sieving the bran fraction from grains of "Takanenishiki," one of *japonica* varieties, harvested in Kyoto prefecture in 1967. The crude globulin fraction was prepared by the procedures reported previously¹⁾ with a slight modification in extracting conditions; that is, rice embryo was extracted with four times weight of the buffer solution at 5°C for 120 min.

Gel filtration chromatography on Sephadex G-200. In this paper, unless otherwise stated, 0.04 M phosphate buffer containing 1 M sodium chloride, pH 6.5, was used because of the exclusion of the possibility that γ globulin of rice embryo may undergo aggregation or dissociation in the low pH range. The protein sample dissolved in the buffer was chromatographed at 20°C on a Sephadex G-200 column, 5×85 cm, equilibrated with the same buffer.

Ion exchange chromatography on DEAE Sephadex A-50. The protein sample was dissolved in 0.03 M Tris-hydrochloric acid buffer containing 0.1 M sodium chloride, pH 8.75, and then applied on a DEAE Sephadex A-50 column, 2.5×35 cm, equilibrated with the same buffer. The elution was carried out at 20°C with a linear increase of sodium chloride concentration from 0.1 to 0.5 M in the same buffer.

Polyacrylamide gel electrophoresis. The buffers used in both the electrode vessels and the gels were 0.03 M Tris-hydrochloric acid buffer containing 0.1 M sodium chloride, pH 8.35 and 8.50, and 0.03 M glycine-sodium hydroxide buffer containing 0.05 M sodium chloride, pH 9.80. Polyacrylamide gels contained 4.75% acrylamide monomer and 0.25% N, N'-methylenebisacryl-

amide. Runs were conducted in a horizontal gel apparatus for 3 hr at 5°C. Over-all current was 13.7 mA per cm² of gel cross section. After completion of the run, the gels were stained with amide black 10 B for 1 hr. Destaining was carried out first with 10% aqueous acetic acid and then with water.

Ultracentrifugal analysis. Ultracentrifugal analysis was carried out with a Spinco model E analytical ultracentrifuge at 20°C.

N-Terminal amino acid analysis. The protein sample was treated with 1-fluoro-2, 4-dinitrobenzene (FDNB) for 2 hr under the conditions recommended by Sanger.⁴⁾ The dinitrophenyl (DNP)-protein was washed with water, ethanol and ether successively and dried *in vacuo*. Hydrolysis was performed at 104±1°C for 6 hr in a sealed glass tube under argon gas phase, containing approximately 40 mg of DNP-protein and 7 ml of constant boiling hydrochloric acid redistilled in a glassware. The ether-soluble fraction of the hydrolyzate was dried *in vacuo* and then identified and determined by means of two-dimensional paper chromatography according to the Levy's method,⁵⁾ using *tert* amylalcohol-phthalate and 1.5 M phosphate buffer as the solvent system.⁶⁾ The yellow spot on the chromatogram was cut off, and the paper piece was extracted with 4.0 ml of 1% sodium bicarbonate for 15 min at 60°C. The optical density of the extract at 360 m μ was measured to determine the quantity of DNP-amino acids. The water-soluble DNP-amino acids were once adsorbed on a talc column and eluted with ethanol-1 N hydrochloric acid.⁷⁾ The eluate was dried *in vacuo*, dissolved in methanol and then applied to the paper. Development was carried out by using *tert* amylalcohol-phthalate as the solvent system. Since the separation of DNP-arginine from ϵ -DNP-lysine was incomplete, the spot of DNP-arginine was confirmed by spraying the Sakaguchi reagent⁸⁾ on the guide chromatogram and then cut out. The little pieces of paper were extracted with 6.0 ml of 1% sodium bicarbonate for 15 min at 60°C. Five ml of the

4) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

5) A. L. Levy, *Nature*, **174**, 126 (1954); H. Fraenkel-Conrat, J. I. Harris and A. L. Levy, "Methods of Biochemical Analysis," Vol. II, ed. by D. Glick, Academic Press Inc., 1955, p. 395.

6) S. Blackburn and A. G. Lowther, *Biochem. J.*, **48**, 126 (1951).

7) F. Sanger, *ibid.*, **45**, 563 (1949).

8) R. Archer and C. Crocker, *Biochim. Biophys. Acta*, **9**, 704 (1952).

extract was reacted with the Sakaguchi reagent⁹⁾ and the optical density at $534\text{ m}\mu$ was measured. The standard curve of the Sakaguchi reaction obtained by using authentic DNP-arginine was linear and ϵ -DNP-lysine did not influence on the optical density at this wavelength. The decomposition ratios of DNP-amino acids were determined as follows. The authentic DNP-amino acid mixture of the expected molar ratios, found on the DNP- γ_1 globulin in the manner described above, was added to the corresponding amount of γ_1 globulin in the molar ratio. The mixture was hydrolyzed and then the remained DNP-amino acids were determined in a similar fashion as described above. The ratios of the amount of the detected DNP-amino acids to that of the added one were referred to the decomposition ratios of these DNP-amino acids. The determined decomposition ratios were 0.75 for DNP-glutamic acid, 0.80 for DNP-phenylalanine, 0.70 for di-DNP-lysine, 0.60 for DNP-glycine and 0.75 for DNP-arginine.

RESULTS AND DISCUSSION

1. Preparation of γ globulin

According to our experience, the Morita and Yoshida's method of purifying γ globulin has the following disadvantages and noticeable points: (1) the procedures of the preparation were performed in saline solutions without controlling the pH of the protein solutions, (2) the precipitation method of globulins by decreasing the ionic strength by means of dialysis or dilution often caused the loss of a γ globulin component having the higher solubility when the decrease of the ionic strength was insufficient, and (3) the simple method often caused the contamination with impurities, especially nucleic acids, which could not easily be removed by the gel filtration chromatography and the cold precipitation in saline solutions. The present method involves the improvements of these points as well as a preliminary separation of the component proteins of γ globulin.

First, the crude globulin fraction could be divided into two kinds of globulin fraction,

evidently different with respect to the solubility, through the selective precipitation with sodium chloride. The crude globulin fraction was dissolved in 0.04 M phosphate buffer containing 1 M sodium chloride, pH 6.5, centrifuged at 0°C to remove insoluble substances and then diluted with 0.01 M phosphate buffer, pH 6.5, to give 0.27 M sodium chloride concentration. This solution was chilled to 0°C and centrifuged. The precipitate fraction is hereafter referred to "0.27 M sodium chloride-insoluble globulin fraction." When the supernatant fraction was further diluted with the same volume of water, characteristic precipitation appeared. This latter precipitate fraction is henceforth called "0.27 M sodium chloride-soluble globulin fraction." Although these two fractions contain γ globulin as the major protein, the component proteins of γ globulin were different in electrophoretic analysis, as will be described later. At this step, the yields of the 0.27 M sodium chloride-insoluble and -soluble globulin fractions were about 32 and 19% of the crude globulin fraction respectively. Since 1 M sodium chloride-insoluble substances and albumin fraction accounted for about 12 and 23% of the crude globulin fraction respectively, these two globulin fractions were considered to occupy the most parts of globulins of rice embryo. Residual 14% of the crude globulin fraction was 0.14 M sodium chloride-soluble proteins, in which γ globulin was no more the major component.

Second, these two globulin fractions were chromatographed individually on a Sephadex G-200 column, typical chromatograms being shown in Figs. 1 and 2. The major protein component was eluted as a peak appeared at fraction number 49 in either case. This peak corresponds to the γ globulin fraction reported previously.¹⁾ However, the chromatograms show that nucleic acids having the absorption maximum at $260\text{ m}\mu$ contaminate throughout the fractions more intensely than in the case of the previous report.¹⁾ These impurities

9) C. J. Weber, *J. Biol. Chem.*, **86**, 217 (1930).

FIG. 1. Chromatography of 0.27 M Sodium Chloride-Soluble Globulin Fraction on a Sephadex G-200 Column (5×85 cm).

Solid line: optical density at $280\text{ m}\mu$, and broken line: optical density at $260\text{ m}\mu$.

FIG. 2. Chromatography of 0.27 M Sodium Chloride-Insoluble Globulin Fraction on a Sephadex G-200 Column (5×85 cm).

Solid line: optical density at $280\text{ m}\mu$, and broken line: optical density at $260\text{ m}\mu$.

would be derived probably by more exhaustive extracting conditions as compared to the previous report, and they could not completely be eliminated by means of gel filtration chromatography. Therefore, the additional procedure using the selective precipitation with ammonium sulfate was adopted. The major protein fractions indicated by the horizontal arrows in Figs. 1 and 2 were collected sepa-

FIG. 3. Rechromatography of 0.27 M Sodium Chloride-Soluble γ Globulin Fraction on a Sephadex G-200 Column (5×85 cm).

Solid line: optical density at $280\text{ m}\mu$, and broken line: optical density at $260\text{ m}\mu$.

FIG. 4. Effect of pH on Solubility of γ Globulin.

Solid line: at the protein concentration of 1 mg/ml in 0.03 M Tris-hydrochloric acid buffer containing 0.10 M sodium chloride, and broken line: at the protein concentration of 0.5 mg/ml in 0.03 M Tris-hydrochloric acid buffer containing 0.05 M sodium chloride.

ately and added with ammonium sulfate until slight turbidity appeared at 25°C , at this point the concentration of ammonium sulfate being 54% saturation. The resultant insoluble matter was removed by centrifugation at 25°C . The supernatant solution was then brought to

FIG. 5. Polyacrylamide Gel Electropherograms of 0.27 M Sodium Chloride-Soluble and -Insoluble γ Globulin Fractions at pH 8.35.

A: 0.27 M sodium chloride-soluble γ globulin fraction.

B: 0.27 M sodium chloride-insoluble γ globulin fraction.

60% ammonium sulfate saturation, allowed to stand at 0°C overnight, and then centrifuged at 0°C to obtain γ globulin as the precipitate. By these procedures most parts of nucleic acids could be eliminated efficiently with little loss of γ globulin.

The two γ globulin fractions purified through ammonium sulfate fractionation were rechromatographed on a Sephadex G-200 column. The elution pattern on the purified γ globulin from 0.27 M sodium chloride-soluble globulin fraction gave a symmetrical peak as shown in Fig. 3, and the preparation from the other fraction also gave the similar chromatogram. The gel filtration chromatography made no distinctions between the two γ globulin fractions. But polyacrylamide gel electrophoresis gave an evidence for dissimilarity of the two. As noted in the previous report, γ globulin has least solubility in neutral solution of low ionic strength suitable for electrophoretic experiments. However, the increase in pH of the solution results in the increase in solubility of γ globulin. The curves in Fig. 4 shows an increase in solubility even at 0.11 ionic strength at alkaline region above pH 8.0, under which condition the sedimentation

coefficient of the protein remained to be about 7S indicating that the protein did not dissociate into its subunits. It enables us to perform the electrophoretic analysis on the γ globulin fraction. The typical electropherograms of the two γ globulin fractions at pH 8.35 are shown in Fig. 5. The 0.27 M sodium chloride-soluble γ globulin fraction was composed of two major components stained to about same intensities, anodic and cathodic ones, while the 0.27 M sodium chloride-insoluble γ globulin fraction had a major cathodic component but almost no anodic one.

2. Isolation of component proteins of γ globulin by ion exchange chromatography

As described in the previous section, γ globulin is composed of at least two component proteins. Since ion exchange chromatography was found preferable to separate these electrophoretic components, the two γ globulin fractions were chromatographed individually on a DEAE Sephadex A-50 column. The recovery of the chromatography was estimated at about 100% in either case on the basis of the optical density at 280 m μ . The elution pattern of the 0.27 M sodium chloride-soluble γ globulin fraction was composed of two peaks as shown in Fig. 6. One had a maximum at fraction number 84 and the other at fraction number 60. The former was estimated to be about twice as much as the latter. On the other hand, the chromatogram of the 0.27 M sodium chloride-insoluble γ globulin fraction indicated a large peak at fraction number 54 and a small peak at fraction number 60 as shown in Fig. 7. The protein eluted at fraction number 54 was presumed to be almost the same in quantities as the major protein of the 0.27 M sodium chloride-soluble γ globulin fraction. As it can be assumed that the component proteins emerged at fraction number 60 in both chromatograms were the same protein, it seems reasonable to consider that the so-called γ globulin was a mixture of at least three kinds of γ globulin, which were

FIG. 6. Chromatography of 0.27 M Sodium Chloride-Soluble γ Globulin Fraction on a DEAE Sephadex A-50 Column (2.5×35 cm).

Solid line: optical density at 280 m μ , and broken line: sodium chloride concentration.

FIG. 7. Chromatography of 0.27 M Sodium Chloride-Insoluble γ Globulin Fraction on a DEAE Sephadex A-50 Column (2.5×35 cm).

Solid line: optical density at 280 m μ , and broken line: sodium chloride concentration.

designated as γ_1 , γ_2 and γ_3 globulins in order of increasing basicity. Thus the 0.27 M sodium chloride-soluble globulin fraction composed of γ_1 and γ_2 globulins, and the other fraction composed of γ_2 and γ_3 globulins as the major components.

These three γ globulin fractions indicated by the horizontal arrows in Figs. 6 and 7 were rechromatographed on a DEAE Sephadex A-50 column. As shown in Fig. 8, they were eluted

at the different positions on the chromatogram. Although in the case of γ_3 globulin the shape of the elution peak got out of symmetry to a slight degree, the chromatograms of γ_1 and γ_2 globulins both gave single symmetrical peaks. The polyacrylamide gel electropherograms of γ_1 , γ_2 and γ_3 globulins at pH 8.50 and 9.80 are shown in Fig. 9. These three γ globulins migrated as single bands with slightly different mobilities at both pH.

FIG. 8. Rechromatography of γ_1 , γ_2 and γ_3 Globulin Fractions on a DEAE Sephadex A-50 Column (2.5×35 cm).

Bold line: γ_1 globulin, chain: γ_2 globulin, fine line: γ_3 globulin, and broken line: sodium chloride concentration.

FIG. 9. Polyacrylamide Gel Electropherograms of γ_1 , γ_2 and γ_3 Globulins at pH 8.50 and 9.80.

A: γ_1 globulin, B: γ_2 globulin, C: γ_3 globulin.

They also gave single symmetrical peaks with the sedimentation coefficients of 7S all in the sedimentation analysis.

TABLE I. N-TERMINAL AMINO ACID COMPOSITIONS OF γ_1 , γ_2 AND γ_3 GLOBULINS

Amino acid	Moles per 10^5 g of protein		
	γ_1 Globulin	γ_2 Globulin	γ_3 Globulin
Arginine	1.81	3.24	4.00
Glutamic acid	0.83	0.31	0.19
Phenylalanine	0.77	Trace	
Lysine	0.44	Trace	
Glycine	0.34	0.25	0.26
Serine	Trace	Trace	Trace

Besides the chromatographic and electrophoretic properties, the N-terminal amino acid analysis of the three γ globulins indicated that they were dissimilar proteins with each other. As shown in Table I, γ_1 globulin contained arginine, glutamic acid, phenylalanine, lysine and glycine as N-termini, whereas γ_2 and γ_3 globulins had arginine, glutamic acid and glycine. Although γ_2 and γ_3 globulins were the same in the kinds of N-terminus, the former was different in the lower content of arginine termini from the latter. The difference in these two γ globulins, however, might be small.

Morita and Yoshida¹¹ reported that the majority of rice endosperm proteins were glutelin, a reserve protein of rice, while embryo

contained a considerable amount of globulin, especially γ globulin, together with glutelin. In this connection, soybean cotyledon contains a large quantity of 7S globulin, and the heterogeneity of 7S globulin has been reported also on the soybean proteins.^{10~12)} However, the physiological functions of these 7S globulins have not yet been known, but the fol-

lowing two possibilities will be assumed: (1) each of the globulin components will be the active protein such as an enzyme or its latent form, and (2) all of the components will be the reserve proteins in scutellum or cotyledon.

The details of chemical and physicochemical properties and gross-structures of these component proteins will be reported in succeeding papers, together with the discussion on their physiological roles.

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11) W. J. Wolf and D. A. Sly, *Arch. Biochem. Biophys.*, **110**, 47 (1965).

12) I. Koshiyama, *Agr. Biol. Chem.*, **33**, 281 (1969).

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