Phytochemistry, Vol. 23, No. 8, pp. 1523–1526, 1984.
Printed in Great Britain.

0031–9422/84 $3.00 + 0.00

IMPROVED ISOLATION METHOD AND SOME PROPERTIES OF SOYBEAN GAMMA-CONGLYCININ

W. SATO, Y. KAMATA, M. FUKUDA and F. YAMAUCHI

Department of Food Chemistry, Faculty of Agriculture, Tohoku University, Sendai 980, Japan

(*Revised received* 30 *January* 1984)

Key Word Index—*Glycine max*; Leguminosae; soybean protein; electrophoresis; MW; gamma-conglycinin.

Abstract—Soybean gamma-conglycinin was isolated by isoelectric precipitation and ammonium sulphate fractionation. The crude protein was purified by ion-exchange chromatography on DEAE-Sepharose CL-6B and gel filtration on Sepharose CL-6B. The purified gamma-conglycinin was homogeneous on two kinds of gel electrophoresis and an ultracentrifugal analysis. A subunit band, distinguishable from other subunit bands of beta-conglycinin and glycinin, was detected by sodium dodecyl sulphate electrophoresis. Amino acid composition was similar to those of the other storage proteins of soybean. Some physical properties were also studied.

INTRODUCTION

Gamma-conglycinin, a 7S globulin, is one of the components of soybean storage protein. Catsimpoolas and Ekenstam [1] have isolated four major antigenically different components from soybean reserve protein. These components were given the names glycinin, alpha-, beta- and gamma-conglycinin. These conglycinins were separated by gel filtrations on Biogel A-1.5 m and Sephadex G-100, and by ion exchange chromatography with DEAE-Sephadex A-50. In these experiments, gamma-conglycinin and beta-conglycinin were obtained from 7S globulin isolated by the procedures described by Koshiyama [2] and 7S globulin isolated by the methods of Roberts and Briggs [3], respectively. Thereafter, the major 7S ultracentrifugal component of soybean protein was proven to be identical with beta-conglycinin [4], not gamma-conglycinin, from the comparison of immunological and other properties. It was reported that beta-conglycinin and gamma-conglycinin were the 7S ultracentrifugal components and that the former had the ability of dimerization with a change of ionic strength from 0.5 to 0.1 but the latter lacked this ability [5].

Since gamma-conglycinin is a minor protein, special devices are needed to remove the major proteins. Koshiyama and Fukushima [6] isolated pure gamma-conglycinin by using the following methods: (i) affinity chromatography on concanavalin A (Con A) Sepharose, (ii) gel filtration on Sepharose 6B, (iii) preparative scale disc electrophoresis and (iv) affinity chromatography on beta-conglycinin-antibody Sepharose. Among these procedures, method (iii) is difficult for obtaining a moderate amount of gamma-conglycinin, and method (iv), although a good procedure to remove a special protein, is complicated and tedious. Furthermore, the electrophoretic behaviour and the subunit composition of the protein have not been studied.

The present paper describes improved separation methods including isoelectric precipitation and ammonium sulphate fractionation. The purified protein was analysed by electrophoresis. Some chemical and physical properties of the protein were also studied.

RESULTS

The gamma-conglycinin prepared by a partially modified procedure of Koshiyama and Fukushima [6] gave a single band on polyacrylamide gel electrophoresis by the Ornstein–Davis system [7, 8] and the protein was used as a standard. Precipitation behaviour at various pH levels of a buffer-extracted protein was examined by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis as shown in Fig. 1. The precipitate at pH 6.6 contained a large amount of glycinin but little gamma-conglycinin. Another precipitate was obtained at pH 6.3 by precipitation of the supernatant at pH 6.6. The precipitate at each pH was obtained in a similar manner. Glycinin contents decreased with reduced pH; conversely, those of gamma-conglycinin and beta-conglycinin increased. At pH 5.7 most of the gamma-conglycinin was precipitated, but beta-conglycinin was precipitated at even lower pH's. From these results, the precipitate at

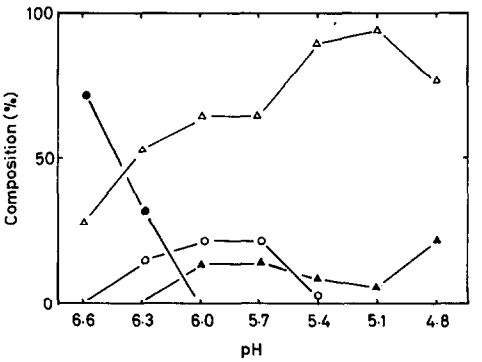

Fig. 1. Composition of the isolated fractions by isoelectric precipitations. The precipitated fractions were analysed by SDS gel electrophoresis followed by scanning with a densitometer. Gamma-conglycinin (○), glycinin (●), beta-conglycinin (△) and others (▲).

pH 5.7 of the supernatant at pH 6.4 was chosen for the preparation of gamma-conglycinin.

To remove most of the beta-conglycinin, precipitation at 60% saturation of ammonium sulphate was used. A large portion of gamma-conglycinin was precipitated at 60% saturation of ammonium sulphate, while beta-conglycinin remained in the supernatant. After fractionation at pH 6.4–5.7 followed by precipitation at 60% saturation with ammonium sulphate, 2–3 g of the crude gamma-conglycinin was obtained from 100 g of the defatted meal. The sample was further purified by ion-exchange chromatography on a column of DEAE-Sepharose CL-6B. The first eluted fraction, which was unadsorbed and/or weakly adsorbed proteins, contained proteins similar to those of the starting material but with less gamma-conglycinin on the SDS polyacrylamide gel electrophoresis. The next peak contained gamma-conglycinin. After elution of the peak, glycinin and nucleic acids were eluted. The gamma-conglycinin-containing fraction gave a yield of 140–150 mg from 100 g of defatted meal and still contained the bands which had lower MWs than that of the subunit of gamma-conglycinin on the SDS gel electrophoresis as shown in Fig. 2b. The sample was further purified on Sepharose CL-6B. Three peaks were obtained and only the second peak was a protein peak which corresponded to gamma-conglycinin and a single band was shown by SDS gel electrophoresis (Fig. 2c). The polyacrylamide gel electrophoresis by the Ornstein–Davis system also showed a single band (Fig. 3a) and the mobility agreed with that of the standard gamma-conglycinin (Fig. 3d). The yield of pure gamma-conglycinin was 50–60 mg from 100 g of defatted meal.

The isolated gamma-conglycinin fraction was analysed by ultracentrifugation at ionic strengths of 0.1 and 0.5. At ionic strengths of both 0.5 and 0.1, the protein showed a single peak of 6.8 S and 8.3 S, respectively, which is different from that of beta-conglycinin which showed 7.3–10.0 S at the same region [9]. Double gel immunodiffusion showed a single precipitin band between the isolated gamma-conglycinin and the antibody of the standard gamma-conglycinin, which did not react with beta-conglycinin.

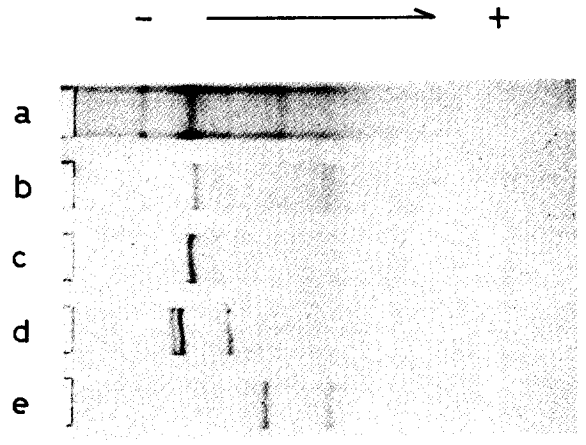

Fig. 2. SDS gel electrophoretic patterns of the fractions from the purification processes. The sample fractionated by ammonium sulphate (a) was purified by ion exchange chromatography (b) and gel filtration (c). The electrophoresis of beta-conglycinin (d) and glycinin (e) are also shown.

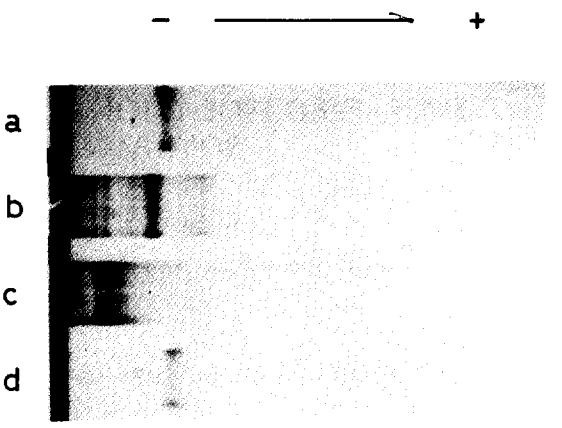

Fig. 3. Gel electrophoretic patterns of purified gamma-conglycinin. Samples were analysed by Ornstein–Davis electrophoresis [7, 8]. The band of gamma-conglycinin (a) moved faster than those of glycinin (b) and beta-conglycinin (c). The gamma-conglycinin (d) purified by the modified procedure of Koshiyama and Fukushima [6] was also shown.

Amino acid composition and physical properties of the protein are shown in Tables 1 and 2, respectively. Sugar components of the protein were mannose (1.5%) and glucosamine (0.45%).

DISCUSSION

The purified gamma-conglycinin was shown to be homogeneous on ultracentrifugation, the electrophoresis and immunochemical methods. Most of the glycinin can be removed in the first step of the preparation procedures by precipitation at a higher pH, which agrees with results reported by Thanh and Shibasaki [10]. Most of the gamma-conglycinin was precipitated at pH 5.7, which is nearly in accord with the isoelectric point (5.80) reported by Koshiyama and Fukushima [6]. The precipitated proteins at pH 5.7 contained some beta-conglycinin. Ammonium sulphate fractionation is useful for the removal of beta-conglycinin. This procedure has been reported for the elimination of beta-conglycinin from glycinin by Wolf *et al.* [11]. Further separation on DEAE-Sepharose CL-6B yielded gamma-conglycinin at the beginning of the elution of the adsorbed protein. This agrees with results on the DEAE-Sephadex A-50 column reported by Catsimpoolas and Ekenstam [1]. Before gel filtration on Sepharose CL-6B, the sample was suspended in a buffer at low ionic strength and the precipitated material was removed. This effectively removes some beta-conglycinin, especially B0-conglycinin, which polymerizes and precipitates at lower ionic strengths [12].

Finally, gel filtration on Sepharose CL-6B removed contaminants of larger and smaller molecules. The isolation procedures of (i) fractionation at pH 6.4–5.7, (ii) precipitation at 60% saturation with ammonium sulphate, (iii) DEAE-Sepharose chromatography and (iv) Sepharose CL-6B chromatography are simpler and easier than those reported by Koshiyama and Fukushima [6]. The purified gamma-conglycinin gave a yield of

Table 1. Amino acid composition of gamma-conglycinin

Amino acid	Amino acid residues /100 amino acid residues	g amino acid /100 g protein
Lys	6.82	8.78
His	2.76	3.81
Arg	6.27	9.83
Asp	9.97	11.55
Thr	4.19	4.26
Ser	6.49	5.68
Glu	17.37	22.56
Pro	5.86	5.73
Gly	6.09	3.49
Ala	4.72	3.38
1/2 Cys*	1.06	1.34
Val	6.36	6.35
Met	1.39	1.83
Ileu	4.73	5.39
Leu	7.60	8.65
Tyr	2.12	3.49
Phe	5.46	8.03
Trp	0.71	1.39
Total	99.97	115.62

*As cysteic acid.

Table 2. Physico-chemical properties of the gamma-conglycinin

Properties	Values
Sedimentation coefficient, $S^0_{20,w}$	7.35
Diffusion coefficient, $D_{20,w}$	3.92×10^{-7} cm²/sec
Intrinsic viscosity	0.062 dl/g
Stokes' radius	52Å
Partial specific volume	0.728 ml/g
Absorption maximum	278 nm
Absorption minimum	255 nm
Extinction coefficient, $E^{1\%}_{1cm,280nm}$	6.8
MW* A	175 000
B	177 000
C	176 000
D	163 000

*MWs by sedimentation—Stokes' radius (A), by sedimentation—viscosity (B), by diffusion—viscosity (C), and by sedimentation—diffusion (D).

50–60 mg from 100 g of defatted meal. However, the defatted meal would contain more gamma-conglycinin because some portion of gamma-conglycinin must be removed during purification.

During protein purification, SDS gel electrophoresis enables purity to be quickly checked. The gamma-conglycinin subunit and the subunits of the other components, that is, beta-conglycinin and glycinin, were clearly distinguished by electrophoresis. In the case of the Ornstein–Davis system [7, 8], gamma-conglycinin moved slightly faster than glycinin. However, these bands could not be completely distinguished from each other. The dissociating system of the SDS gel electrophoresis also showed a single band (Fig. 3b) which reveals that gamma-conglycinin consists of subunits of identical MW. This differs from the result of Catsimpoolas [13] who found nine subunit bands of the protein. The multiple bands may arise from impurities. Amino acid composition of gamma-conglycinin has not been reported. Our results showed that the amino acid composition resembles that of beta-conglycinin and glycinin in respect to a higher content of acidic amino acids and lysine, and a lower content of the sulphur-containing amino acids. The gamma-conglycinin contains more sulphur-containing amino acids than beta-conglycinin but less than glycinin. Sugar components of mannose and glucosamine in gamma-conglycinin were the same sort of sugars as those in beta-conglycinin. However, gamma-conglycinin contained smaller amounts of both mannose and glucosamine than those of beta-conglycinin. Koshiyama and Fukushima [6] have also reported the carbohydrate content, 5.49%, but the sugar components and contents were not reported.

The sedimentation coefficient was higher than that reported by Koshiyama and Fukushima [6]. However, the diffusion coefficient was lower than in their results. The difference of the values resulted in a higher MW than those (102 000 and 104 000) reported by them. The MW estimated by four methods gave the values from 163 000 to 177 000. Intrinsic viscosity was similar to that (0.0638) of beta-conglycinin [14] and the Stokes' radius was smaller than that (59 Å) of beta-conglycinin [14].

EXPERIMENTAL

Protein samples. Defatted soybean meal was prepared from soybeans (var. Raiden) harvested in 1977 at Motoyoshi, Miyagi. Soybean seeds were dehulled, flaked and defatted with hexane, and ground in a sample mill. Beta-conglycinin and glycinin were isolated by the methods of ref. [10]. A pure gamma-conglycinin was prepared by partially modified methods of ref. [6]. These methods include: (i) precipitation of a buffer extract at pH 5.8, (ii) affinity chromatography on Con A Sepharose, (iii) ion-exchange chromatography on DEAE-Sephadex A-50, (iv) gel filtration on Sepharose 6B and (v) affinity chromatography on beta-conglycinin-antibody Sepharose.

Extraction and isolation at various pH's. The defatted soybean meal was agitated with a 20-fold excess of 0.03 M Tris–HCl buffer (pH 8) containing 0.01 M 2-mercaptoethanol (2-ME) for 1 hr at room temp. The resulting suspension was centrifuged (10 000 rpm, 30 min, 20°). The supernatant was adjusted to pH 6.6 with 2 M HCl and stirred for 30 min at 5°. The suspension was centrifuged (10 000 rpm, 20 min, 5°). The supernatant was adjusted to pH 6.3 with 2 M HCl and centrifuged. In this way, ppts at pH 6.6, 6.3, 6.0, 5.7, 5.4, 5.1 and 4.8 were obtained.

Recommended procedure for preparation of crude gamma-conglycinin. Buffer extraction from the defatted meal was carried out with a similar procedure except 15 times the amount of the Tris–HCl buffer was used. The extract was passed through gauze and centrifuged (8000 rpm, 30 min, 20°). The supernatant was passed through gauze, adjusted to pH 6.4 with 2 M HCl, and stirred for 30 min at 5°. The suspension was centrifuged (8000 rpm, 20 min, 5°). The supernatant was adjusted to pH 5.7, stirred for 30 min and centrifuged under similar conditions. The ppt was washed by suspending it in 0.03 M Tris–HCl (pH 5) followed by centrifugation (8000 rpm, 15 min, 5°). The ppt was suspended with standard KPi buffer [2.6 mM KH_2PO_4,

32.5 mM K_2HPO_4, 0.4 M NaCl, 10 mM 2-ME, pH 7.6, ionic strength (I) = 0.5], and salted-out at 60% with $(NH_4)_2SO_4$ (780 g to 1 l.). After stirring for 30 min at room temp, the suspension was centrifuged (8500 rpm, 10 min, 20°) to yield a ppt, the crude gamma-conglycinin.

Electrophoreses. The disc electrophoresis [7, 8] with 7.5% separating gel and 5.0% spacer gel was carried out by using a slab gel apparatus. SDS gel electrophoresis in 7.4% acrylamide gel using a slab gel apparatus was carried out according to the method of ref. [15]. The gels were stained with Coomassie Blue G-250. The destained gels were scanned at 570 nm (reference 720 nm) with a Shimadzu Dual-Wavelength TLC Scanner CS-900.

Ultracentrifugal analysis. The sample in standard KPi buffer (I = 0.5) or KPi buffer (2.6 mM KH_2PO_4, 32.5 mM K_2HPO_4, pH 7.6, I = 0.1) was analysed with a Hitachi UCA-1 ultracentrifuge at 55 430 rpm.

Immunochemical methods. Antisera to gamma-conglycinin were prepared by the procedure of ref. [1]. Double gel immunodiffusion in agar was carried out according to the method of ref. [16]. The gel medium consisted of 1% agar in standard KPi buffer. Protein samples were dissolved in the same buffer and reactants were allowed to diffuse at room temp for 24 hr.

Analyses of physical properties. Ultracentrifugation was carried out at room temp at 55 430 rpm in the standard KPi buffer containing 0.02% NaN_3. The diffusion coefficient of the protein was determined by immunological method of ref. [17] in the same conditions described above. Partial specific vol. was calculated from the amino acid composition. Viscosities of the protein solns were measured with a Ubbelhode viscometer at 20° in the buffer. The Stokes' radius was estimated by gel filtration according to the procedure of ref. [18]. A column (2.6 × 113 cm) on Sepharose 6B was eluted with the standard buffer at the rate of 30 ml/hr with the reference of bovine serum fibrinogen (107 Å), bovine liver catalase (52 Å), bovine gamma-globulin (55.5 Å), bovine serum albumin (35.5 Å), and bovine hemoglobin (31.3 Å). The MW of gamma-conglycinin was estimated by the four equations using the following values; (1) sedimentation coefficient—Stokes' radius [19], (2) sedimentation coefficient—intrinsic viscosity [20], (3) diffusion coefficient—intrinsic viscosity [20] and (4) sedimentation coefficient—diffusion coefficient [20].

Chemical compositions. Amino acid composition was estimated with a Hitachi KLA 3B amino acid analyser according to the method of ref. [21]. Cystine and cysteine were measured as cysteic acid after performic acid oxidation. Hexose was analysed quantitatively by a phenol–sulphuric acid method [22]. Glucosamine was analysed by a modified procedure of ref. [23]. Hexose and hexosamine was analysed on a plate (10 × 10 cm) of Avicel with solvent A (EtOAc–*iso*-PrOH–H_2O, 16:6:3) or B (80% PhOH–H_2O, 5:1, by vol).

REFERENCES

1. Catsimpoolas, N. and Ekenstam, C. (1969) *Arch. Biochem. Biophys.* **129**, 490.
2. Koshiyama, I. (1965) *Agric. Biol. Chem.* **29**, 885.
3. Roberts, R. E. and Briggs, D. R. (1965) *Cereal Chem.* **42**, 71.
4. Koshiyama, I. and Fukushima, D. (1976) *Phytochemistry* **15**, 157.
5. Koshiyama, I. (1969) *Agric. Biol. Chem.* **33**, 281.
6. Koshiyama, I. and Fukushima, D. (1976) *Phytochemistry* **15**, 161.
7. Ornstein, L. (1964) *Ann. N. Y. Acad. Sci.* **121**, 321.
8. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121**, 404.
9. Iibuchi, C. and Imahori, K. (1978) *Agric. Biol. Chem.* **42**, 25.
10. Thanh, V. H. and Shibasaki, K. (1976) *J. Agric. Food Chem.* **24**, 1117.
11. Wolf, W. J., Babcock, G. E. and Smith, A. K. (1962) *Arch. Biochem. Biophys.* **99**, 265.
12. Yamauchi, F., Sato, M., Sato, W., Kamata, Y. and Shibasaki, K. (1981) *Agric. Biol. Chem.* **45**, 2863.
13. Catsimpoolas, N. (1970) *Cereal Chem.* **47**, 70.
14. Koshiyama, I. (1968) *Cereal Chem.* **45**, 394.
15. Kamata, Y., Otsuka, S., Sato, M. and Shibasaki, K. (1982) *Agric. Biol. Chem.* **46**, 2829.
16. Ouchterlony, O. (1949) *Acta Pathol. Microbiol. Scand.* **26**, 507.
17. Allison, A. C. and Humphery, J. H. (1960) *Immunology* **3**, 95.
18. Laurent, T. C. and Killander, J. (1964) *J. Chromatogr.* **14**, 317.
19. Koshiyama, I. (1972) *Int. J. Peptide Protein Res.* **4**, 167.
20. Schachman, H. K. (1957) *Methods Enzymol.* **4**, 32.
21. Pusztai, A. and Morgan, J. T. (1963) *Biochem. J.* **88**, 546.
22. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Analyt. Chem.* **28**, 350.
23. Gardell, S. (1953) *Acta Chem. Scand.* **7**, 207.