Studies on Soybean Protein

Part I. A Ribonucleoprotein and Ribonucleic Acids in Soybean Casein Fraction

By Ikunori KOSHIYAMA and Nobuyoshi IGUCHI

Noda Institute for Scientific Research, Noda City, Chiba-ken.

Received September 7, 1964

Soybean casein fraction was fractionated by gel filtration with Sephadex G100. From the effluent diagram of soybean casein fraction, several minor components were obtained with a main component.

One of them showed a single pattern by ultracentrifugal analysis, but it was found to be contaminated with RNA by column chromatography on DEAE-cellulose. From the result of purification by the repeated rechromatography, the purified component contained about 7% of RNA per protein. Therefore, it was considered to be a ribonucleoprotein.

Among other minor components, free ribonucleic acids, which seemed to have different molecular weights were also present, but their ratios of purine to pyrimidine and base compositions coincided well each other.

INTRODUCTION

Soybean protein, especially soybean casein fraction, has been much used for foods and other industrial purposes. However, it has been reported to be heterogeneous by many investigators. Ultracentrifugal studies by Naismith¹⁾ showed at least four components corresponding to the four fractions observed in whole soybean protein, and Smith et al.²⁾ reported the presence of one major and four minor components by electrophoresis.

Moreover, soybean casein fraction is contaminated by non-protein impurities,³⁾ ash,³⁾ phytate^{4,5)} nucleic acid⁵⁾ and possibly phosphatides.³⁾

Recently, the techniques of protein separa-

tion have much progressed. Wolf,⁶⁾ Hasegawa,⁷⁾ Soejima⁸⁾ and Terada⁹⁾ et al. reported some methods by which they tried separation and purification of soybean protein components.

But, in regard to the properties of soybean casein fraction, there remained many obscure points because of the difficult separation and purification of its components.

This paper describes the separation of soybean casein fraction by gel filtration, the purification of a minor component which contains RNA and also the existence of free ribonucleic acids in soybean casein fraction.

MATERIALS AND METHODS

Preparation of Soybean Casein Fraction.

1) W. E. F. Naismith, *Biochim. Biophys. Acta*, **16**, 203 (1955).

2) A. K. Smith, E. N. Schubert and P. A. Belter, *J. Am. Oil Chemists' Soc.*, **32**, 274 (1955).

3) W. G. Smiley and A. K. Smith, *Cereal Chem.*, **23**, 288 (1946).

4) L. L. McKinney, W. F. Sollard and E. A. Setzkorn, *J. Biol. Chem.*, **178**, 117 (1949).

5) A. K. Smith and J. J. Rackis, *J. Am. Chem. Soc.*, **79**, 633 (1957).

6) W. J. Wolf, N. W. Babcock and A. K. Smith, *Arch. Biochem. & Biophys.*, **99**, 265 (1962).

7) K. Hasegawa, T. Kusano and H. Mitsuda, *This Journal*, **27**, 878 (1963).

8) M. Soejima, T. Hayashi, I. Suda and T. Aoyama, Reported at the general meeting of the Kanto Branch of the Agricultural Chemical Society of Japan, Tokyo, April 1, 1964.

9) U. Terada, Reported at the general meeting of the Agricultural Chemical Society of Japan, Hokkaido, July 19, 1964.

A sample of soybean (var. tsurunoko), 1961 crop, in Hokkaido was crushed, ground and screened through a sieve (20 mesh). After it had been extracted with ethyl ether for twenty-four hours at 35°C, the residue was air-dried.

Defatted soybean meal was extracted twice with the distilled water which was previously adjusted to

pH 7.6~7.8 with 2N NaOH, first ten times and second five times of water. The extraction was carried out by stirring for one hour at room temperature. After all water extracts had been dialysed against water for thirty-six hours at 2~4°C, it was acidified to pH 4.5 with 2N HCl. The precipitate was washed twice by suspending in the distilled water which was

FIG. 1. The Preparation of Soybean Casein RNA.

(1): 0.01M sodium citrate containing 0.9% NaCl.

(2): Five per cent solution of 45% ethanol.

*: Centrifugation at 7,500r.p.m. for 5 min.

FIG. 2. Gel Filtration of Soybean Casein Fraction on Sephadex G100 (3.0×70 cm column).

Sample: 200 mg of soybean casein fraction in 5 ml of the buffer (0.02M glycine-NaOH, pH 9.50). Flow rate: 30~40 ml per hour. Fraction volume: 9 ml. Dashed area: samples of each peak collected for analytical assays.

●—●: O. D. at 280 mμ

○- - -○: O. D. at 260 mμ

previously adjusted to pH 4.5. Then, the washed precipitate was dissolved in distilled water to a final pH of 7.6~7.8 with 2N NaOH and lyophilized to the stock sample.

Preparation of Soybean Casein RNA.

The preparation process of the crude soybean casein RNA is illustrated in Fig. 1. This procedure is a modified the Kay-Dounce method.¹⁰⁾ Briefly speaking, the crude soybean casein RNA was separated from the protein by treatment with sodium dodecyl sulfate and bentonite. Then, it was precipitated in ethanol solution at pH 1.0 and gathered. The sample was purified by the Schmidt-Thannhauser-Schneider method and the Sevag's method.^{11,12)} The purified sample was dried on P₂O₅ in a desiccator.

Preparation of Sephadex.

10) E. A. Kay and A. L. Dounce, *J. Am. Chem. Soc.*, **75**, 4041 (1953).

11) E. Volkin and W. E. Cohn, "Method of Biochemical Analysis," Vol. I, 1954, p. 287.

12) M. G. Sevag, *Biochem. Z.*, **273**, 419 (1934).

The dried Sephadex G100 powder (Pharmacia Upsala Sweden) was suspended in excess distilled water overnight at 2~4°C to allow it to swell. After elimination of fine particles by decantation, the water swollen Sephadex was packed into a 3.0×70cm. column and thoroughly equilibrated with a 0.02M glycine-NaOH buffer solution, pH 9.50.

Preparation of DEAE-Cellulose.

DEAE-cellulose (Eastman Organic Chemicals U.S.A.) was cycled with 0.5N HCl and 0.5N NaOH, and washed with distilled water, then it was packed into a 2.0×30cm. column and two 2.0×10cm. columns prepared for rechromatography and thoroughly equilibrated with the same buffer solution (the starting buffer) as used for Sephadex G100.

Ultracentrifugal Analysis.

Sedimentation analysis was carried out at room temperature with a Hitachi UCA-I centrifuger at 55430 r.p.m. The solvent was 0.01M K₂HPO₄-KH₂HO₄

buffer containing 0.4M NaCl and 0.01M mercaptoethanol, pH 7.60.

Paper Chromatography.

n-Butanol-acetic acid-water (4:1:1 by vol.) was employed for the detection of amino acids and phosphorus by ascending type chromatography using Tōyō Rōshi No. 51 paper.

The next two solvents were used for detection of the acid-hydrolysates of RNA in soybean casein fraction and in each peak.

- (1) isopropanol-conc. HCl-water. (85:20.5:19.5 by vol.)¹³⁾
- (2) sat. $(\text{NH}_4)_2\text{SO}_4$ -water-isopropanol (79:19:2 by vol.)¹⁴⁾

Determination of Base Composition of RNA.

The molar proportions of bases in soybean casein RNA preparations were determined by the methods of Wyatt,¹³⁾ Markham¹⁴⁾ and DiCarlo et al.¹⁵⁾ RNA samples weighing 1~5mg were hydrolysed with 0.2ml of 1N HCl at 100°C for one hour. Then, they were chromatographed for forty hours at room temperature with the solvent 1 on a Tōyō Rōshi No. 51 paper. The chromatograms were air-dried. The spots located with a Manasuru lamp were eluted with 3.5ml of 0.1N HCl for three hours and quantitatively estimated with a spectrometer using the ultraviolet absorption data.^{13,16)}

Determination of Protein Content.

Protein content of each peak was measured by the Lowry's method.¹⁷⁾

Determination of RNA Content.

RNA content of each peak was measured by the spectrophotometric procedure described by Webb¹⁸⁾ and by orcinol reaction¹⁹⁾ with a Klett-Summerson photoelectric colorimeter. A sample for spectrophotometric analysis was prepared by the treating it five times with the same volume of ethyl ether to remove trichloroacetic acid as used to extract RNA.

RESULTS

(1) Fractionation of Soybean Casein Fraction by Gel Filtration.

- 13) G. R. Wyatt, *Biochem. J.*, **48**, 584 (1951).
- 14) R. Markham and J. D. Smith, *ibid.*, **47**, 401 (1951).
- 15) F. J. DiCarlo, A. S. Schultz and A. K. Kent, *Arch. Biochem. & Biophys.*, **55**, 253 (1955).
- 16) E. Chagaff and J. N. Davidson, "The Nucleic Acid", Vol. I, Academic Press, 1955, p. 502, 503, 508, 509, 513.
- 17) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 18) J. M. Webb, *ibid.*, **230**, 1023 (1958).
- 19) A. H. Brown, *Arch. Biochem.*, **11**, 269 (1946).

In this experiment, as shown in Fig. 2, six peaks appeared by column chromatography on Sephadex G100. The first peak, designated as SPI, was the main component of soybean casein fraction and contained 90% of the protein which was applied to the column.

SP2, SP3 and SP4 were materials having absorption maximum at about 260 m μ . In order to estimate the properties of the peaks obtained, ultraviolet absorption spectrum was measured and biuret²⁰⁾ and the Lowry's reactions for protein, orcinol reaction for ribose of RNA and diphenylamine reaction²¹⁾ for deoxyribose of DNA were tested. The existence of protein and phosphorus in those peaks, after they had been hydrolysed with 6N HCl at 100°C for twenty hours was confirmed by testing amino acids by ninhydrin reaction and phosphorus by the Hanes-Isherwood reaction.²²⁾

FIG. 3. Ultraviolet Absorption Spectra of Each Peak Fractionated on Sephadex G100 in 0.02M Glycine-NaOH Buffer, pH 9.50.

The samples of each peak applied to this experiment were the parts of oblique line shown in Fig. 2.

- 20) M. Holden and N. W. Pirie, *Biochem. J.*, **60**, 46 (1955).
- 21) Z. Dische and K. Schwarz, *Microchem. Acta.*, **2**, 13 (1937).
- 22) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

As shown in Fig. 3, SP1 has a absorption maximum at 277~278 m μ , whereas SP2 263 m μ . Both SP3 and SP4 have them at 260 m μ . By estimating the properties of each peak no DNA was found in all peaks. Protein existed in SP1 and SP2, but did not in the other peaks. SP2 contained about 10 % of protein which was applied to the column. Phosphorus reaction was positive in the peaks except SP5 and SP6.

(2) Estimation of RNA in Each Peak.

In order to confirm the existence of RNA in each peak shown in Fig. 2, it was hydrolysed and chromatographed with two kinds of solvents. The resulting spots on paper chromatograms were observed to be guanine, adenine, cytidine, 3'-CMP, uridine and 3'-UMP. As there were no free bases, peaks from SP1 to SP4 were found to contain RNA.

Soybean RNA was measured by Smith,⁵⁾ DiCarlo¹⁵⁾ and Matsushita.²³⁾ In this experiment, RNA was determined by the spectro-

photometric method described by Webb and by orcinol reaction. In our analysis, carbohydrate in soybean casein fraction was about 3.5% and 1.5% as total carbohydrate and as pentose respectively. There were, however, no spots by paper chromatography except ribose from RNA in SP2, SP3 and SP4. So, RNA in SP2, SP3 and SP4 were measured by orcinol reaction.

As shown in Table I, soybean RNA was mostly contained in soybean casein fraction and protein free RNA existed in SP3 and SP4 was no more than 25% of RNA contained in soybean casein fraction. About 50% of RNA contained in soybean casein fraction existed in SP1. It was, however, unconfirmed whether RNA in SP1 was combined to the protein or not.

The molecular weights of RNA in SP3 and SP4 were considered to be different each other, because they could be separated by the gel filtration.

TABLE I. THE DISTRIBUTION OF RNA IN SOYBEAN AND IN EACH FRACTION

	Total dry weight	RNA content/dry weight*	Distribution of RNA in each sample
Defatted soybean meal	103.0mg	1.76%	100.0%
Water-extractable fraction***	42.2	3.01	70.1
Soybean casein fraction	37.5	3.06	63.3
Soybean whey fraction	4.7	2.00	5.2
	Fraction number	RNA content	Distribution of RNA in each fraction
Soybean casein fraction		6.12 mg* (/200 mg)	100.0%
SP1	18~31	3.18*	51.9
SP2	32~51	1.43**	24.2
SP3	52~66	0.62**	10.5
SP4	67~96	0.80**	13.4

* Measured by the Webb's method.

** Measured by orcinol reaction.

*** 1:15 water extraction of room temperature (pH 7.80).

FIG. 4. Ultracentrifugal Patterns of Soybean Water-Extractable Protein, Soybean Casein Fraction, SP1 and SP2 Fractions.

	$S_{20,w}$ values of components*			
	2S	7S	11S	15S
A: Water-extractable soybean protein	1.8	6.5	10.8	15.9
B: Soybean casein fraction	1.8	6.3	9.8	13.6
C: SP1	1.7	6.3	10.4	—
D: SP2	1.9	—	—	—

* Sedimentation constants, $S_{20,w}$ are expressed in Svedberg units.

Photographs were taken after 45 min. of centrifugation at 55430 r.p.m. Direction of sedimentation is from right to left.

(3) A Minor Component SP2.

As shown in Fig. 4, SP2 exhibits a single pattern in ultracentrifugal analysis and has $S_{20,w}$ value of 1.9S. SP2 fraction was divided into DP1 and DP2 peaks by column chromatography on DEAE-cellulose as shown in Fig.

23) S. Matsushita, *Mem. Res. Inst. Food. Sci. Kyoto Univ.*, **14**, 14 (1958).

FIG. 5. Chromatograms on DEAE-Cellulose of A, SP2 Fraction, B, DPI Fraction and C, R₁DPI Fraction.

The dotted lines indicate the sodium chloride gradient as measured in the eluate. The samples applied on the columns were the dashed areas of SP2, DP1 and R₁DPI peaks. Fractions, 5 ml each, were collected with a flow rate of 30 ml per hour at 2~4°C.

●—●: O. D. at 280 mμ. ○—○: O. D. at 260 mμ.
×—×: Amount of RNA by orcinol reaction

5A. Moreover, after purifying by repeating rechromatography, DP1 showed nearly a single peak as shown in Fig. 5C.

RNA and protein tests showed that the purified DP1 (R₂DP1) contained both protein and RNA. DP2 contained only RNA and its amount was about 25% of RNA in SP2. The content of RNA per protein and the ratio of 280 mμ to 260 mμ of each purifying step of SP2 on DEAE-cellulose chromatography are shown in Table II.

The molar proportion of bases in R₂DPI and the ratio of purine to pyrimidine coin-

cided well with those of DP2, SP3, SP4 and soybean casein fraction as shown in Table III.

DISCUSSIONS

Hasegawa et al.⁷⁾ isolated two 2S proteins from water-extractable soybean protein by gel filtration with Sephadex G 200. In this experiment, the isolated 2S protein might be one of two 2S proteins isolated by them.

The reagents which prevented formation of

TABLE II. RNA CONTENT AND 280mμ/260mμ RATIO OF EACH PURIFYING STEP OF SP2 ON DEAE-CELLULOSE CHROMATOGRAPHY

	RNA content (per protein)	280mμ/260mμ ratio
SP2	8.83%	0.75
DPI	6.91	1.03
R ₁ DPI	7.17	1.02
R ₂ DPI	6.97	1.03

TABLE III. BASE MOLAR RATIO OF RIBONUCLEIC ACIDS IN R₂DPI, DP2, SP3, SP4 AND SOYBEAN CASEIN FRACTION (Adenine=1.00)

	Soybean casein RNA	R ₂ DPI	DP2	SP3	SP4
Guanine	1.16	1.17	1.18	1.12	1.12
Cytidine	0.09	0.07	0.06	0.08	0.09
3'-CMP	0.89	0.92	0.95	0.90	0.90
Uridine	0.04	0.03	0.04	0.05	0.04
3'-UMP	0.65	0.79	0.79	0.62	0.73
Purine/ Pyrimidine	1.29	1.20	1.18	1.28	1.20

a disulfide polymer, such as mercaptoethanol, and cysteine, were not used in present gel filtration. But, as shown in Fig. 4, 2S peak in the ultracentrifugal pattern of soybean casein fraction was considered to be identical with a single peak in the pattern of SP2, considering the decrease of 2S peak in the pattern of SP1. So, R₂DPI may be a protein having little relation to disulfide bond.

Belozerskii²⁴⁾ obtained a proteinlike ribonucleoprotein from soybean seedlings. DiCarlo et al.¹⁵⁾ suggested that soybean RNA might combine the protein and exist as a so-called ribonucleoprotein.

Because R₂DPI obtained from SP2 which showed a single pattern ultracentrifugally was homogeneous chromatographically on DEAE-cellulose. Moreover, little change was found in the ratio of 280 m μ to 260 m μ and also in the content of RNA per protein, in each step of purification by chromatography as shown in Table II. Therefore, RNA in R₂DPI was considered to be combined with the protein.

The obtained molar proportions of bases in soybean casein RNA differed slightly from those of DiCarlo's data¹⁵⁾. The ratio of purine to pyrimidine was lower than that of his data, because soybean casein RNA showed more cytosine and less guanine than those found by him. However, the ratio of purine to pyrimidine in each peak closely resembles each other as shown in Table III.

Acknowledgement. The authors wish to express their sincerest thanks to Prof. Y. Sakurai and Assist. Prof. M. Fujimaki of University of Tokyo for their kind guidance and encouragement throughout this work. Great indebtedness is also acknowledged to Prof. K. Arima and Prof. Y. Ikeda of University of Tokyo for their valuable suggestions. Thanks are also due to the Department of Fermentation, Institute of Physical and Chemical Research, for ultracentrifugal analysis. The authors wish to thank Dr. M. Mogi, Director of Noda Institute for Scientific Research, Dr. F. Yoshida and Dr. S. Nakamura for their encouragement.

24) A. N. Belozerskii, *Biokhimiya*, **1**, 255 (1936).