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Abundant proteins associated with lecithin in soy protein isolate

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Abstract

A new method for fractionating acid-precipitated soy proteins, so called soy protein isolate, involving three step acidification of a water extract of defatted soy flour was developed. Soy protein isolate was shown to be mostly composed of not only β-conglycinin and glycinin, but also a group of lipophilic proteins associated with lecithin (phospholipids). The proportions of three major proteins were 23%, 46%, and 31%, respectively. The proportions changed as the nitrogen solubility index (NSI) of defatted soy flour changed. The yield of lipophilic proteins depended on the NSI of defatted soy flour, different from those of β-conglycinin and glycinin. The variation in the proportions of the three major proteins may be due to the yield of lipophilic proteins.

Keywords: Soy proteins; Fractionation; Phospholipids; Lipoproteins; Solubility

1. Introduction

Commercial soy protein isolate (SPI) is manufactured from proteins precipitated by acidifying a water extract of defatted soybeans to pH 4.3–4.8. These proteins are found to be mostly composed of β-conglycinin (7S) and glycinin (11S) on electrophoretic analysis.

Soybeans are known as a source of phospholipids, known as lecithin. Phospholipids are the main component of cell membranes, the envelopes of oil and protein bodies, and organella membranes. The occurrence of a large amount of phospholipids in soybean is supported by the occurrence of abundant oil and protein bodies. This suggests the existence of large amounts of membrane proteins simultaneously. Generally, membrane proteins are hydrophobic and lipophilic. The known oil-body associated proteins (OBAPs) (Herman, 1987; Kalinski, Weisemann, Matthew, & Herman, 1990) appear to be a kind of membrane protein. Hence, it is necessary to follow the fate of membrane proteins during the precipitation of acid-precipitated proteins.

Iwabuchi and Yamauchi (1987) carried out immunoassaying, showing that 7S and 11S globulins comprise about 50% of the total proteins. In addition, it was reported from this laboratory that a poor-tasting soy protein isolate contained many lipid-binding proteins. Furthermore, it was reported previously that poor-tasting SPI was rich in lipids and contained OBAPs (Samoto, Miyazaki, Kanamori, Akasaka, & Kawamura, 1998). Hence, it is necessary to reevaluate the distribution of major proteins in acid-precipitated proteins.

In this paper, we describe a new method for fractionating 7S, 11S, and a group of lipophilic proteins associated with phospholipids, which are considered to be membrane proteins, and present the proportions of these proteins in SPI. In addition, the proportions varied in acid-precipitated proteins depending on the heat history of the defatted soy flour.

Abbreviations: CBB, Coomassie Brilliant Blue; NSI, nitrogen solubility index; OBAP, oil-body associated proteins; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SPI, soy protein isolate.

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2. Materials and methods

2.1. Materials

US-produced non-genetically modified soybeans comprising a variety of species were crushed and then hexane-defatted at 40 °C. After filtration, the defatted soybeans were dried by evaporation at 70 °C to remove hexane and then further crushed into particles that passed through a 60-mesh sieve in order to prepare low-denatured defatted soy flour. The nitrogen content of the defatted soy flour was 8.35%, and the value for the oil component determined using ether extraction was 0.9% and the nitrogen solubility index (NSI) was 85%.

2.2. Heating of defatted soy flour

Defatted soy flour was added to a kneader with controlled humidity and then heated and stirred with stirring plates at 20 rpm. The heated defatted soy flour was prepared by controlling the product temperature as desired for 30 min. The NSI was used as an indicator of the heat history.

2.3. Preparation of acid-precipitated proteins

Fifty grams of defatted soy flour (NSI 85) were added to 400 ml of water, followed by adjustment to pH 8.0 with 5 N NaOH. The mixture was stirred for 1 h at 20 °C, and then centrifuged at 3000*g* for 10 min to obtain a supernatant and precipitate. A further 250 ml of water (20 °C) were added to the resulting precipitate, followed by stirring for 30 min before centrifugation at 3000*g* for 10 min in order to collect the remaining soluble components. The supernatant was then mixed to obtain the water extract, which was adjusted to pH 4.5 with 3.5 N H_2SO_4 to precipitate soy proteins. This mixture was centrifuged at 3000*g* for 10 min to yield the acid-precipitated soy proteins from the supernatant (soybean whey).

2.4. Fractionation of acid-precipitated proteins

The 7S, 11S and LP (lipophilic proteins) fractions were prepared by the following procedure. A water extract of defatted soy flour (NSI 76.5, 73.0 and 70.5) was prepared under the same conditions as for the preparation of acid-precipitated proteins. Then, a reducing agent, Na_2SO_3, was added to a final concentration of 1 mM to the water extract and the pH was adjusted to 5.8 with 3.5 N H_2SO_4. The fraction precipitated on centrifugation (3000*g*, 10 min) was designated as the 11S fraction. The supernatant was adjusted to pH 5.0 with 3.5 N H_2SO_4 and then heated for 15 min at 55 °C. It was then adjusted to pH 5.5 with 5 N NaOH and centrifuged at 3000*g* for 10 min, the resulting precipitate being designated as the LP fraction. The supernatant was adjusted to pH 4.5 with 3.5 N H_2SO_4. After centrifugation the precipitate was designated as the 7S fraction. The supernatant was removed as the soybean whey fraction.

2.5. Amino acid analysis

The amino acid compositions of the protein fractions were analyzed after acid hydrolysis, in 3 mol/l methanesulphonic acid at 110 °C for 24 h in vacuo with a JEOL Amino Acid Analyzer (JLC500/V, Japan Electronic Co., Ltd., Tokyo, Japan).

2.6. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis

After applying the given amounts of the respective fractions, electrophoresis was carried out according to the method of Laemmli (1970). A 10–20% Gradient Gel PAGE-mini was used (Daiichi Kagaku Yakuhin Co., Ltd., Tokyo, Japan). The electrophoresed proteins were stained with Coomassie Brilliant Blue (CBB) for 15–20 h, and then decolorization with decolorizing solvent for 5 h. A densitometer (GS-710 Calibrated Imaging Densitometer/Quantity One Software Version 4.2.3; scanning width, 5.3 mm; sensitivity, 30–50; Bio Rad Japan Co., Ltd., Tokyo, Japan) was used to determine the stained peak areas.

2.7. Determination of the nitrogen solubility index (NSI)

Initially, a 3.5-g sample was added to 100 ml of water. The mixture was then stirred for 1 h at 25 °C, and centrifuged at 1000*g* for 10 min to obtain a supernatant and precipitate. The latter was extracted with 100 ml water for 1 h at 25 °C, and then centrifuged at 1000*g* for 10 min to obtain a supernatant and precipitate. The supernatants were combined to bring the bottom of the meniscus to the 250 mark. The nitrogen levels were determined after filtration by the Kjeldahl method. The NSI was calculated as the percentage of the nitrogen extracted as to the total amount of nitrogen in the sample.

2.8. Determination of the nitrogen-distribution ratio

The nitrogen contents of the fractions were determined by the Kjeldahl method.The ratio of nitrogen-distribution to the respective fractions were calculated as a percentage against the total nitrogen in the defatted soy flour, which was regarded as 100%, or against the total nitrogen in the acid-precipitated proteins, which was regarded as 100%.

2.9. Lipid analysis of the 7S, 11S and LP fractions

Each fraction was freeze-dried and then added to a 10-fold weight of chloroform/methanol (2:1 by volume), followed by stirring for 2 h at 50 °C. The extract was then evaporated and solidified, and then the weight was determined.

2.10. Thin-layer chromatography (TLC) analysis

TLC analysis was performed using an established method (Yamaoka, Tanaka, Rahayu, Hernandez, & Jamilah, 1988) for developing polar lipids. Lipids that had been extracted with 10 ml of chloroform /methanol (2:1 by volume) from 1.0 g of each sample (7S, 11S and LP fractions) for 2h at 50 °C and then dried were dissolved in 1.0 ml of chloroform/methanol (2:1 by volume). A 10-μl sample was then spotted on a 60F254 silica gel plate (Merk Co., Darmstadt, Germany) and developed with a solvent system, chloroform–methanol–acetic acid–water (85:15:10:3, by volume). The plate was sprayed with another solvent system (methanol:water, 1:1 by volume) containing 0.5% 1-naphthol, and then sprayed with a 10% H_2SO_4 solution to detect lecithin. The plate was heated at 105 °C for 15 min to visualize the phospholipids spots.

3. Results and discussion

3.1. Separation of β-conglycinin (7S), glycinin (11S), and lipophilic proteins associated with phospholipids (LP)

Substantial amounts of lecithin (membrane phospholipids) are removed with *n*-hexane during oil extraction. The rest of the phospholipids as well as the membrane proteins remain in defatted soybeans. *n*-Hexane can not extract a large amount of membrane proteins. Therefore, membrane-originating proteins may be recovered as lipophilic proteins associated with phospholipids in the acid-precipitated proteins, which include β-conglycinin and glycinin. We developed a new procedure for separating 7S, 11S and LP involving three step acidification of a water extract of defatted soy flour. This method did not require a large amount of salt or/and reducing agents, or cool-precipitation to separate 7S and 11S, differing from the methods reported previously (Nagano, Hirotsuka, Mori, Kohyama, & Nishinari, 1992; Samoto et al., 1994; Thanh & Shibasaki, 1976). The new procedure is shown in Fig. 1. After the addition of sodium sulphite to a final concentration of 1 mM to the water extract of defatted soy flour, the pH was adjusted to 5.8 with 3.5 N sulphuric acid. The insoluble 11S was then collected by centrifugation. After separation of the 11S, LP remained together with 7S in the supernatant. Separation of LP from 7S required a method that only precipitates LP from the supernatant. It was considered that LP could be easily salted out due to its highly hydrophobic properties, like those of membrane proteins. Hence, we designed a method for making LP insoluble under conditions which kept 7S soluble. Heating for 15 min at 55 °C was applied after which the supernatant was adjusted to pH 5.0 with 3.5 N sulphuric acid, and the protein became insoluble. The pH was gradually neutralized with 5N sodium hydroxide. After the pH had returned to 5.5, 7S was dissolved. LP remained insoluble even when the pH reached 5.5. However, it became partially soluble

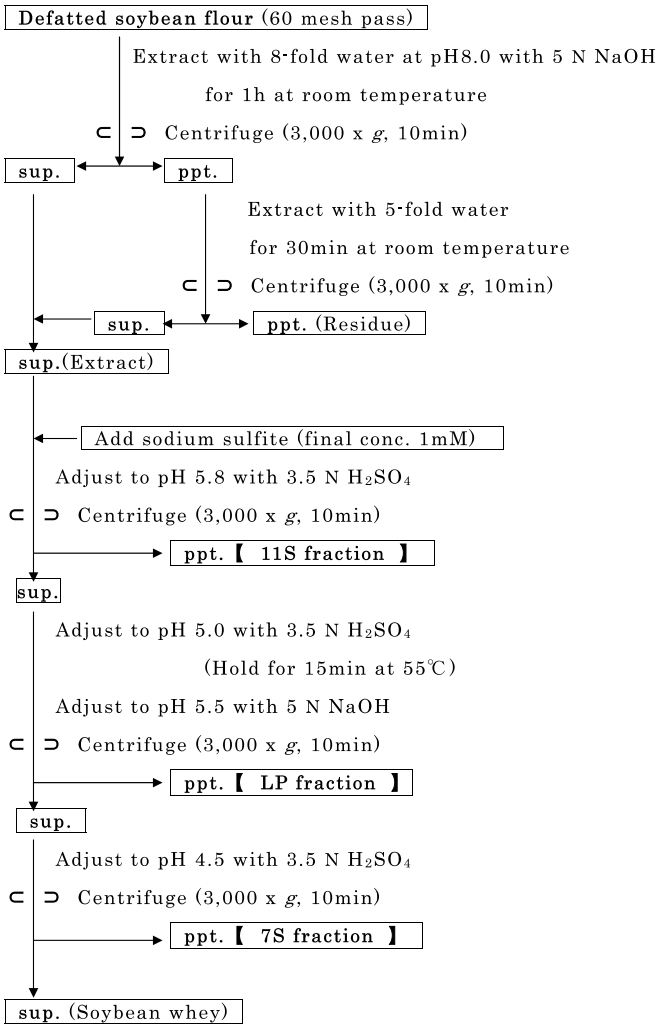

Fig. 1. Schematic diagram of fractionation of β-conglycinin (7S), glycinin (11S), and lipophilic proteins (LP).

when the pH approached about 6. Therefore, the proteins that precipitated at pH 5.5 were collected as LP. Subsequently, the supernatant was adjusted to pH 4.5, and 7S was precipitated and collected by centrifugation. The yield of the LP fraction with this procedure was much higher than the yield estimated on SDS–PAGE. LP was not detected on SDS–PAGE, due to its lower sensitivity to Coomassie Brilliant Blue (CBB) compared with 7S and 11S. Thus, on conventional separation of 7S and 11S, contamination by LP was hardly detected. LP tended to show relative weak CBB-staining sensitivity on SDS–PAGE. Therefore, this analysis is not suitable for true determination of LP in SPI, unless the sensitivity of LP to CBB is improved. Therefore, it was important to determine the true yield of LP by measuring the nitrogen contents of the protein fractions obtained with this procedure. However, there was a problem. In the case of the non-heated defatted soy flour used as the starting material, the LP fraction was partly contaminated by 11S (data not shown). Then, an attempt was made to improve the separation of LP and 11S. The defatted

soy flour had a heat history of around 70 °C for the removal of *n*-hexane. It was necessary for clear separation to heat the defatted soy flour at around 75–80 °C. This treatment of the crude defatted soy flour was shown to prevent the co-precipitation of LP and 11S. Most 11S was precipitated at pH 5.8. Contamination by 11S of the LP fraction markedly decreased. Based on this finding, fractionation was performed using defatted soy flour of which the NSI value had been decreased from 85 to 76.5 by pre-heating (75 °C). The SDS–PAGE profile of each fraction was obtained (Fig. 2). This indicated that LP comprises a group of several proteins. The amino acid composition of LP is shown in Table 1. The densitometry results indicated that 11S and 7S were about 90% pure, respectively (Fig. 2, lanes 3 and 5). There was little 11S contamination in the LP fraction (Fig. 2, lane 4). Thus, it was possible to separate 7S, 11S and LP by means of this procedure.

3.2. Characteristics of 7S, 11S, and LP

Table 2 shows the characteristics of the 7S, 11S and LP obtained with the method described above. The LP contained more than 10% lipids as to the total weight of dry matter, the high lipid content of LP thus being revealed. TLC analysis of the lipid components of the LP fraction indicated the presence of phospholipids such as phosphatidylethanolamine, phospatidylcholine and phosphatidylinositol (Fig. 3). This may imply that a certain amount of LP was derived from membrane proteins. However, lipids were mostly absent in the 7S and 11S fractions. The nitrogen-distribution ratios for 7S, 11S and LP as to the total nitrogen in defatted soy flour (NSI 76.5) were 14%, 28%, and 19%, respectively, as shown in Table 3.

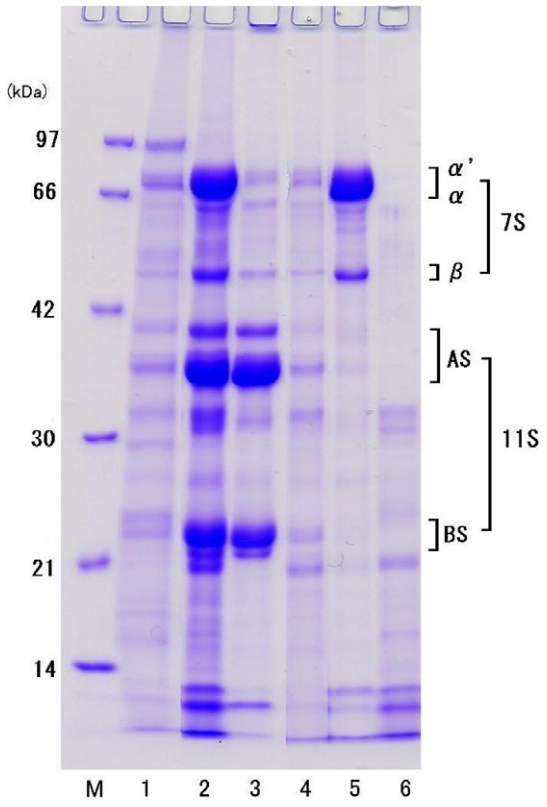

Fig. 2. SDS–PAGE of the protein fractions prepared from heated defatted soy flour. Soymilk, as a standard, containing 2 ng of nitrogen was applied to a well. The amount that corresponded to the yield of each fraction was applied. Lane 1, residue; lane 2, soymilk; lane 3, 11S; lane 4, LP; lane 5, 7S; lane 6, soy whey; AS, acidic subunit; BS, basic subunit.

Table 1
Amino acid compositions of β-conglycinin (7S), glycinin (11S), and lipophilic proteins (LP)

Amino acids (mg/100 g)	Proteins			
	APP[a]	7S	11S	LP
Arginine	7.7	8.7	8.0	7.1
Aspartic acid	11.1	11.6	12.2	10.6
Glutamic acid	19.7	24.0	21.8	16.2
Glycine	3.6	2.6	4.2	4.6
Lysine	6.4	6.9	5.2	6.6
Serine	4.5	4.2	5.1	5.2
Proline	5.4	5.3	5.7	5.3
Threonine	3.5	2.4	3.6	4.7
Alanine	3.8	2.8	3.5	5.1
Histidine	3.0	3.0	2.5	2.8
Cystine	1.3	0.8	1.4	1.3
Methionine	1.3	0.9	1.2	1.6
Valine	4.9	4.0	4.3	5.3
Isoleucine	4.9	4.8	4.2	4.7
Leucine	7.7	7.7	7.3	8.3
Tyrosine	3.7	3.1	3.6	4.0
Phenylalanine	6.2	6.6	4.9	5.1
Tryptophan	1.3	0.6	1.3	1.5
Total	100	100	100	100

[a] APP, acid-precipitated protein.

Table 2
Characteristics of β-conglycinin (7S), glycinin (11S), and lipophilic proteins (LP)

Fraction	Nitrogen (%)	Proteins[a] (%)	Lipids[b] (%)	Ash (%)
APP[c]	14.6	83.4	5.2	4.5
7S	15.2	87.0	0.8	6.0
11S	16.3	93.1	3.3	2.4
LP	13.3	75.9	11.7	4.0

[a] Calculated as nitrogen × 5.71.
[b] Materials extracted with a solvent system of chloroform:methanol (2:1, by volume).
[c] APP, acid-precipitated protein.

3.3. Effect of heating of defatted soy flour on the yields of 7S 11S and LP

By heating defatted soy flour, it was possible to separate 7S, 11S and LP. However, heating may affect the yields of these proteins. We prepared some defatted soy flours with different heat histories. Then, 7S, 11S

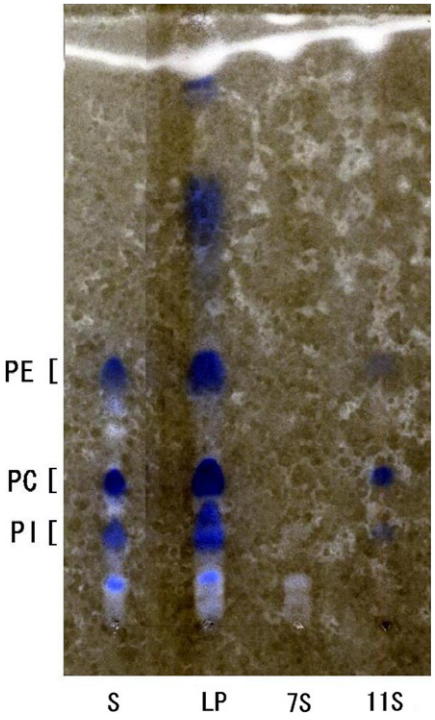

Fig. 3. TLC of lipids from protein fractions. Chloroform/methanol (2:1) was added to each fraction (7S, 11S, and LP), and the extracted materials were used. A 10 μl sample was then spotted to a silica gel plate and developed as described under Section 2. S, commercial soy lecithin; PE, phosphatidylethanolamine; PC, phospatidylcholine; PI, phosphatidylinositol.

Table 3
Effect of heat treatment on defatted soy flour as to yields of proteins

Heating temperature[a]	NSI	Protein distribution[b]				
		Soy whey	7S	11S	LP	Residue
Unheated	85.4	14	68	(7S, 11S and LP)		18
75 °C	76.5	14	14	28	19	25
80 °C	73.0	12	13	28	15	32
85 °C	70.5	13	14	29	11	33

[a] Heated under the conditions of 100% humidity for 30 min.
[b] Calculated as the nitrogen-distribution ratio.

and LP were separated from the respective acid-precipitated proteins. The nitrogen-distribution ratios for the respective fractions were determined. The results are shown in Table 3. As the temperature was elevated, the LP yield decreased, and simultaneously the residue yield increased. Probably, a higher temperature may accelerate aggregation of lipophilic proteins due to their hydrophobic properties. In addition, SDS–PAGE analysis suggested that hardly any 7S and 11S remained in the residue (Fig. 2, lane 1). Thus, the heating of defatted soy flour of which the NSI was in the range of 70.5–76.5 could cause a reduction in the LP yield, but not the 7S and 11S yields.

3.4. Proportions of 7S, 11S and LP in the acid-precipitated proteins

The LP content of the acid-precipitated proteins prepared as described above varied. The yield of LP tended to vary due to the heat history of the defatted soy flour, different from those of 7S and 11S. Hence, the proportions of 7S, 11S and LP in the acid-precipitated proteins were expected to change depending on the yield of LP. The nitrogen-distribution ratios for the three proteins in acid-precipitated proteins prepared from mildly heated defatted soy flour (NSI 76.5) were 23%, 46% and 31% for 7S, 11S and LP, respectively. On the other hand, those for the soy flour (NSI 70.5) were 26%, 54% and 20% for 7S, 11S and LP, respectively. These findings suggested that the proportions of 7S, 11S and LP in the acid-precipitated proteins varied due to the yield of LP. Separation of LP from 11S was difficult when they were prepared from non-treated defatted soy flour. This may be due to the association between LP and 11S (data not shown). LP and 11S could be separated by mild heat treatment. However, the yields of 7S and 11S from the water extract of defatted soy flour of NSI 70.5, 73.0 and 76.5 did not change, whereas the yield of LP decreased as the NSI decreased. This suggests that heat treatment may influence the structure and properties of LP.

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