

Short Communication

Simple and Efficient Procedure for Removing the 34 kDa Allergenic Soybean Protein, *Gly m I*, from Defatted Soy Milk

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The 34 kDa protein in the salted-out fraction of soy milk is shown to be identical to the allergenic soybean protein, *Gly m I*, by N-terminal amino acid sequencing and immunoblotting, using the monoclonal antibody against *Gly m I*. Treatment of soy milk with 1 M Na₂SO₄, acidifying to pH 4.5 and centrifugation removed more than 90% of the 34 kDa protein with an overall yield of 70% of total soy protein.

Cases of food allergy have recently shown a sharp increase. Ogawa *et al.* has found the allergenic soybean protein, *Gly m I*, to be readily recognized by IgE antibodies in the sera of soybean-sensitive patients with atopic dermatitis.¹⁾ This protein has been isolated from the crude 7S-globulin fraction in its oligomeric form with a molecular weight of exceeding 300,000 by gel-filtration and identified as a 34 kDa oil-body-associated protein (P34).^{2,4)} It would thus appear to easily aggregate in defatted soy milk.

A spectroscopic examination of the protein particle-size distribution in defatted soy milk indicated a bimodal distribution; *i.e.*, both large and small particles well dispersed in defatted soy milk. The large-particle fraction was rich in 34 kDa protein, considered possibly to be *Gly m I*, and the other oil-body-associated proteins^{3,4)} were similar to ASF (acid-sensitive fraction),⁵⁻⁷⁾ being insoluble at high ionic strength and pH 4.5. A simple and efficient procedure was thus developed by the authors to remove *Gly m I* from defatted soymilk to obtain a low allergenic soybean protein.

Defatted soybean meal was prepared from ground soybean IOM (Indiana, Ohio, Michigan; 1993 crop) by solvent extraction with *n*-hexane. Defatted soy milk was prepared by dispersing the meal in distilled water (1:15, w/w), whose pH was adjusted to 7.5 with 1 N NaOH, and then by mixing at room temperature for 3 h. The mixture was centrifuged at 10,000 × *g* for 10 min to remove the insoluble material, the supernatant being used as defatted soy milk. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted by using a 12% gel (Tefco-Co., U.S.A.) and the Laemmli system.⁸⁾ After western blotting, the allergenic protein, *Gly m I*, was detected by using a monoclonal antibody specific to *Gly m I* (provided by Dr. Ogawa, University of Tokushima) and ECL immunodetection reagents (Amersham Institute, England). The particle-size distribution of the soy protein was determined by the ELS-800 laser light scattering system (Ootsuka Electronics Co., Japan). The N-terminal amino acid sequence of the

western-blotted protein on a PVDF membrane was determined with a gas-phase protein sequencer (477A, ABI, CA, U.S.A.). The percentage removal of *Gly m I* was calculated according to a densitometric determination of the ECL immunofluorescent intensity on X-ray film.

The analysis of particle-size distribution of the defatted soy milk by laser light scattering indicated an average particle-size peak of 10 nm and a 40 nm–2 μm peak as shown in Fig. 1a. It would thus appear possible to separate the two different sizes of particles by ultracentrifugation. The particle-size distribution of the supernatant and redissolved precipitates after ultracentrifugation (200,000 × *g* for 50 min) of the defatted soy milk indicates good resolution of the two peaks (Figs. 1b and c). The protein compositions of the two fractions were determined by SDS-PAGE as shown in Fig. 2.

The supernatant (10 nm particle size) consisted primarily of the major soybean storage proteins, 7S and 11S, and some 34 kDa protein, apparently the allergenic protein. The SDS-PAGE patterns of the precipitates were similar to those of the so-called acid-sensitive fraction (ASF),⁶⁾ the acid-insoluble fraction high in ionic strength and rich in 34 kDa protein. An attempt was thus made to remove the 34 kDa protein by salting-out through changing the concentration of ammonium sulfate.

As shown in Fig. 3, the 34 kDa protein was effectively removed by 40% saturated ammonium sulfate precipitation without significant loss of the storage proteins. To confirm the 34 kDa band (lane 2 in Fig. 3) was identical to *Gly m I*, an allergenic protein, the N-terminal amino acid sequence of the 34 kDa protein electroblotted on a PVDF membrane was determined as shown in the Table. The 18 N-terminal amino acid sequence of the 34 kDa protein was identical to that of the *Gly m I* protein. Immunostaining of the gel indicated only the 34 kDa protein to be stained with anti-*Gly m I* (Fig. 4, lane 1). All these results indicate that the 34 kDa protein to be the allergenic protein, *Gly m I*, and that the 34 kDa allergenic protein can be removed by salting-out at acidic pH and then by conventional centrifugation.

With the present method, 0.3–1 M Na₂SO₄ at pH 4.5 and centrifuging at 10,000 × *g* for 10 min were the conditions found to provide optimal removal. Figure 4 shows the efficient removal of the 34 kDa allergenic protein by 0.3 M (lanes 2 and 3) and 1.0 M (lanes 4 and 5) Na₂SO₄, which is applied as a food additive. The 34 kDa protein from defatted soy milk could be removed by more than 80% (lane 2) and

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Abbreviations: SDS, sodium dodecyl sulfate; PVDF, polyvinylidene fluoride.

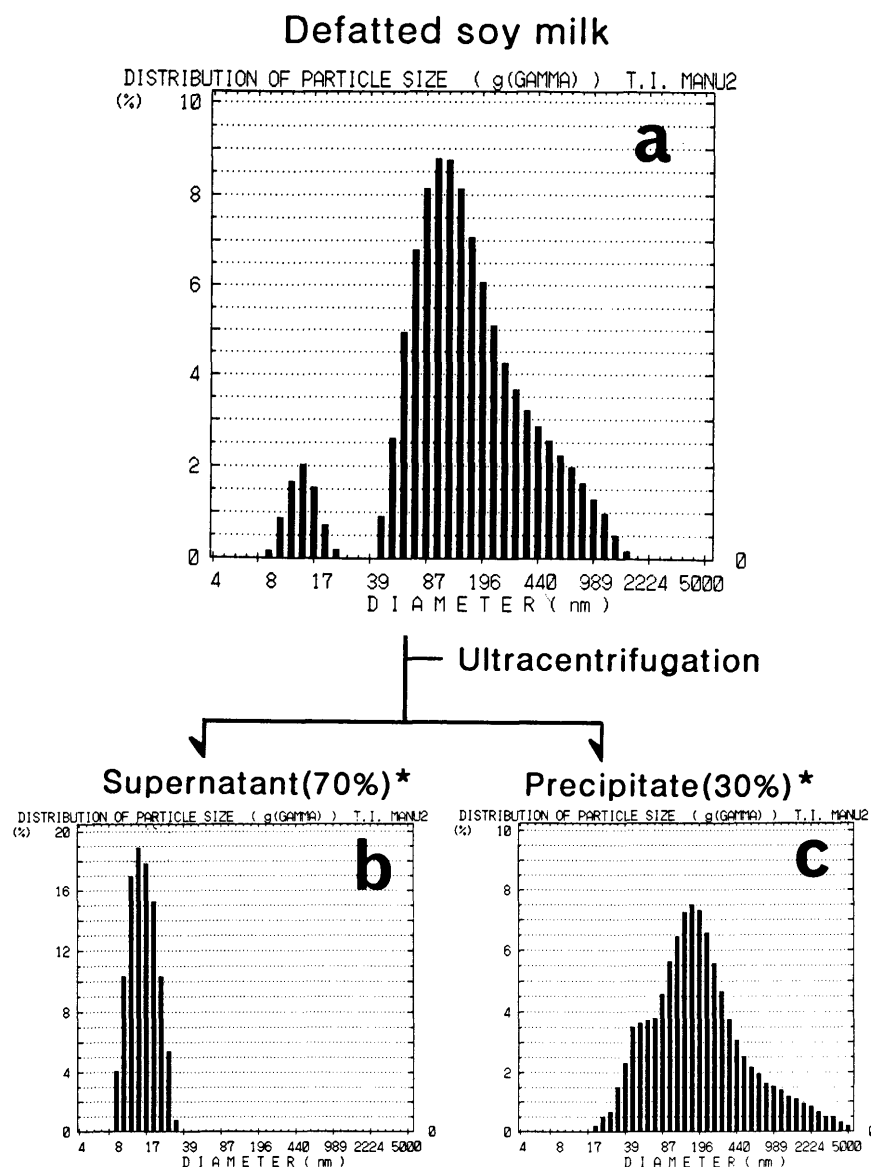


Fig. 1. Particle-size Distribution of the Defatted Soy Milk and of the Two Fractions Separated by Ultracentrifugation.

Defatted soy milk was ultracentrifuged at $200,000 \times g$ for 50 min. The supernatant and precipitates were diluted with distilled water to a suitable concentration for the measurement of particle-size by light scattering.

*The percentage shows the ratio of protein in the supernatant and precipitates against the whole protein of defatted soy milk.

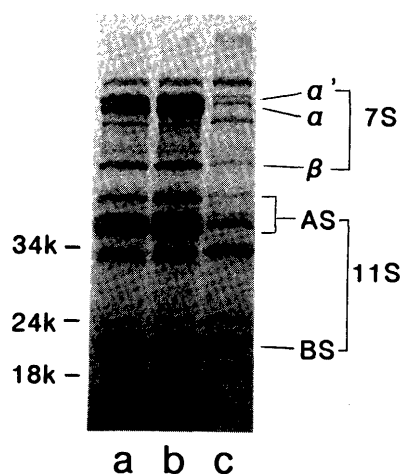


Fig. 2. SDS-PAGE Patterns of the Defatted Soy Milk and of the Two Fractions Separated by Ultracentrifugation.

Defatted soy milk with 1 M NaCl at pH 7.5 was ultracentrifuged at $200,000 \times g$ for 50 min. The protein ($30 \mu\text{g}$) from each fraction was applied to a 12% gel. a, whole defatted soy milk; b, supernatant of defatted soy milk; c, precipitates of defatted soy milk.

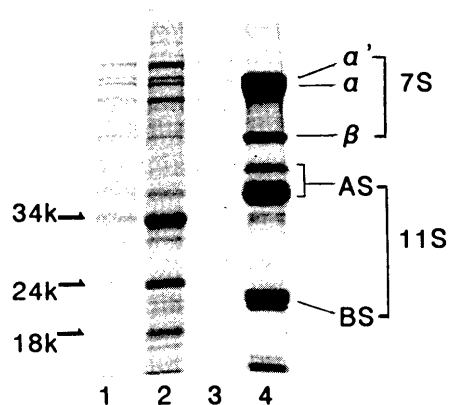


Fig. 3. SDS-PAGE Patterns of Proteins Precipitated with Different Concentrations of Ammonium Sulfate from Large Particles of Defatted Soy Milk.

The large particle proteins in 100 ml of defatted soy milk were precipitated by ultracentrifugation. The proteins were then dissolved in a buffer with 10 mM 2-mercaptoethanol, before the solution was fractionated with ammonium sulfate at pH 7.5. Following dialysis of the precipitates, the fractions were applied to an SDS-PAGE column. Lanes 1, 2, 3, and 4 show the fractions precipitated by 0–30%, 30–40%, 40–50%, and 50–100% of saturated ammonium sulfate, respectively.

Table Amino-terminal Sequence of the 34 kDa Protein

34 kDa protein	KKMKKEQYSCDHPPASWD
P34* (<i>Gly m I</i>)	KKMKKEQYSCDHPPASWDWR—

* P34, 34 kDa oil-body-associated protein and deduced amino-terminal sequence of amino acids.⁴⁾

nearly 100% (lane 4), with an overall yield of 78% and 70% of total soy protein, respectively.

Some people develop an allergy for soybean products, the causal protein for which was recently assigned as *Gly m I*,^{1,2)} and a monoclonal anti-body against it became available.^{2,9)} This made it possible for us to identify 34 kDa band as *Gly m I* and to develop a simple, efficient and practical procedure to remove the 34 kDa allergenic protein as presented in this report. This procedure has some advantages over an other theoretically possible procedure (not developed yet) using proteolytic enzymes to destroy the allergenic epitope sequence of soy protein.

Our method based on physicochemical separation without any side effects is easy to conduct and efficient (nearly 100% removal of the allergenic protein).

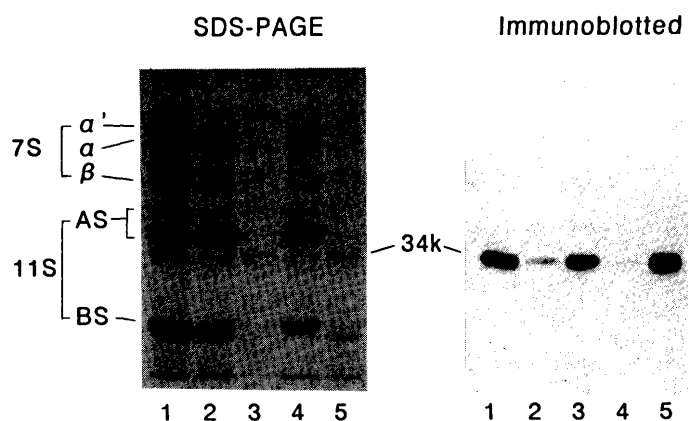


Fig. 4. SDS-PAGE and Immunoblotted Patterns of the Supernatant and Precipitate Separated by Centrifugation in 0.3 M or 1.0 M Na₂SO₄ at pH 4.5.

Soy milk (100 ml) was adjusted to pH 4.5 with 1 N HCl and then centrifuged at 10,000 × *g* for 10 min, after standing for 10 min at 25°C in Na₂SO₄ and 10 mM SBS (sodium bisulfite). The supernatant and precipitate were dissolved in distilled water, and their volume was adjusted to that before separation. A sample solution (50 μl) was mixed with 450 μl of an SDS-PAGE sample buffer, and 5 μl of the mixture were applied to an SDS-PAGE column. Lane 1, whole soy milk; lanes 2 and 3, supernatant and precipitates of soy milk containing 0.3 M Na₂SO₄; lanes 4 and 5, supernatant and precipitates of soy milk containing 1.0 M Na₂SO₄, respectively.

There is no significant loss of the functional soybean proteins, 7S and 11S, and thus the present method should find a wide application to food manufacturing.

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