

Specific Binding of Allergenic Soybean Protein *Gly m* Bd 30K with α' - and α -Subunits of Conglycinin in Soy Milk

Masahiko SAMOTO,[†] Chiaki MIYAZAKI, Takeshi AKASAKA, Hiroyuki MORI, and Yukio KAWAMURA^{*†}

Central Research Institute, Fuji Oil Co., Ltd., Kinunodai 4–3, Yawara, Ibaraki 300–24, Japan

^{*}Protein Science Laboratory, National Food Research Institute, Kannondai 2–1–2, Tsukuba, Ibaraki 305, Japan

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When defatted soy milk was ultracentrifuged, 34 kDa allergenic soybean protein *Gly m* Bd 30 K was more abundant in the precipitate than in the supernatant by an SDS–PAGE analysis. The addition of more than 10 mM of 2-mercaptoethanol (2-ME) to the soy milk resulted not only in further removal of the 34 kDa allergenic protein to the precipitate, but also in better recovery of conglycinin in the supernatant.

After a two-dimensional SDS–PAGE analysis (the first dimension, minus 2-ME; the second, plus 2-ME) of the precipitates, superimposition between the CBB-stained gel and the electroblotted membrane stained with a monoclonal antibody specific to *Gly m* Bd 30 K indicated that part of *Gly m* Bd 30 K was preferentially bound to the α' - and α -subunits of conglycinin, and that part of them had formed the dimer through a disulfide bond.

Key words: allergen; soybean protein; disulfide bond; soymilk

The incidence of allergy to soybean proteins, a major plant protein, is quite low in comparison with other major food proteins such as egg, milk, and wheat proteins, but is gradually increasing with the growing consumption of soybean products all over the world. The 34 kDa oil-body associated protein, *Gly m* Bd 30 K, has recently been assigned as the major allergenic protein of soybean by Ogawa *et al.*^{1,2)}

In the course of a light-scattering analysis of a defatted soymilk emulsion, we found a bimodal distribution of large and small protein particles. *Gly m* Bd 30 K was found to be enriched by the large particles, but not by the small ones. Based on this result, we developed a simple and efficient procedure for removing the allergenic protein from defatted soy milk by conventional centrifugal separation.³⁾ The presence of a reducing agent was critical for the effective separation of *Gly m* Bd 30 K from major soybean storage proteins such as glycinin and conglycinin.

Kalinski *et al.* have reported that the 34 kDa oil-body associated protein (the same protein as *Gly m* Bd 30 K) had 6 mol of cysteine per mole from the DNA sequence.⁴⁾ This suggests a possibility that binding of *Gly m* Bd 30 K with the other proteins may occur in soy milk through a disulfide bond.

In this paper, the mode of interaction of *Gly m* Bd 30 K with the other soybean proteins was investigated by two-dimensional SDS–PAGE, using the specific immuno-detection of *Gly m* Bd 30 K.

Materials and Methods

Materials. The soybean variety used was IOM (Indiana–Ohio–Michigan, 1994 crop) from U.S.A. The nitrocellulose membrane was purchased from Bio Rad Co., Ltd. (U.S.A.) and other reagents, including 2-mercaptoethanol (2-ME), were all of special reagent grade from Wako Chemical Co., Ltd. (Japan).

Preparation of defatted soy milk and precipitates. Defatted soybean meal was prepared by extracting ground soybean with *n*-hexane. Soy milk was prepared by dispersing the soy meal in distilled water (1:15, w/w), adjusting the pH value to 7.5 with 1 N NaOH, and mixing at room temperature for 3 h. The mixture was centrifuged at 10,000 $\times g$ for 10 min to remove the insoluble materials, the supernatant being used as defatted soy milk. This defatted soy milk was stood for 30 min at 25°C in the presence and absence of various concentrations of 2-ME and NaCl, and then ultracentrifuged at 200,000 $\times g$ for 50 min at 20°C to obtain the large particles as a precipitate.³⁾

SDS–polyacrylamide gel electrophoresis (SDS–PAGE). SDS–PAGE was carried out by using the buffer system of Laemmli,⁵⁾ a 12% slab gel and electrophoresis kit (Tefco Co., Japan) being used. The gel slabs after electrophoresis were fixed in a solution of methanol, acetic acid, and water (5:5:2), and then each stained with 0.1% Coomassie Brilliant Blue R-250 in the solution. Protein in the gels was quantified by densitometric scanning at 550 nm (reference, 490 nm) with a Shimadzu CS-9000 dual-wavelength scanner. The relative content of each subunit of the soy proteins was evaluated by the area ratio of each subunit in the precipitate to that of the subunit in the original soy milk. Two-dimensional SDS–PAGE (2D SDS–PAGE) was carried out with an 8% disc gel in the absence of 2-ME for the first dimension (1D) and with a 12% slab gel in the presence of 2-ME for the second dimension (2D).

The precipitate prepared by ultracentrifuging defatted soy milk without 2-ME was applied to 1D SDS–PAGE in the absence of 2-ME. After electrophoresis, the 1D gel was reacted with a 5% 2-ME solution containing 3% SDS at 40°C for 30 min. The treated gel was then applied to a 12% slab gel for the 2D electrophoresis in the presence of 2-ME.

Immunodetection of *Gly m* Bd 30 K by the second-dimensional SDS–PAGE analysis. After the 2D SDS–PAGE analysis, the gel was electroblotted to a nitrocellulose membrane. After the membrane had been blocked with 3% skim milk, it was reacted with a monoclonal antibody specific to *Gly m* Bd 30 K (kindly provided by Prof. T. Ogawa, University of Tokushima) at 37°C for 1 h, and with a peroxidase-conjugated anti-mouse antibody at 37°C for 1 h. This was followed by the determination of fluorescence with ECL immunodetection reagents (Amersham Institute plc, U.K.) on X-ray film.

[†] To whom correspondence should be addressed.

Abbreviations: 2-ME, 2-mercaptoethanol; ECL, enzyme chemiluminescence; 2D SDS PAGE, two-dimensional SDS polyacrylamide gel electrophoresis.

Results and Discussion

Effects of 2-ME on the precipitability of *Gly m Bd 30 K*

Defatted soy milk containing 1 M sodium chloride at pH 7.5 in the presence or absence of 2-ME was fractionated by ultracentrifuging at $200,000 \times g$ for 50 min, the resulting precipitates being applied to SDS-PAGE to evaluate the precipitability of the 34 kDa allergenic soybean protein (*Gly m Bd 30 K*) as shown in Fig. 1.

In the absence of 2-ME (Fig. 1a), about 52% of the 34 kDa protein in the original soy milk was recovered in the precipitated fraction, which contained about 20% and 15% of the conglycinin and glycinin in the original soy milk, respectively. In the presence of 10 mM 2-ME (Fig. 1b), the α' -, α -, and β -subunits of conglycinin in the precipitates had decreased to 24%, 24%, and 35% of the respective levels in the absence of 2-ME (Fig. 1a). The acidic (AS) and basic (BS) subunits of glycinin had decreased to 56% and 67% of those of Fig. 1a, respectively, while the contents of *Gly m Bd 30 K* had increased by about 10% of that of Fig. 1a. The addition of 100 mM 2-ME (Fig. 1c) resulted in a further but small decrease in both glycinin and conglycinin in the precipitate, and in a slight increase in *Gly m Bd 30 K*, compared with that at 10 mM 2-ME (Fig. 1b). This favorable recovery of the α - and α' -subunits of conglycinin in the supernatant by the 2-ME treatment is considered to have been attained by cleavage of the S-S bond between the conglycinin subunits and *Gly m Bd 30 K*. The cleaved conglycinin subunits are soluble in nature, whereas *Gly m Bd 30 K* become insoluble due to its inherent aggregative property,²¹ which brought about the increased recovery of *Gly m Bd 30 K* into the precipitates that was observed in the presence of 2-ME (Figs. 1a and b). These results indicate that the presence of a reducing agent facilitated both the removal of *Gly m Bd 30 K* into the precipitates and the recovery of glycinin and conglycinin into the supernatant (soy milk).

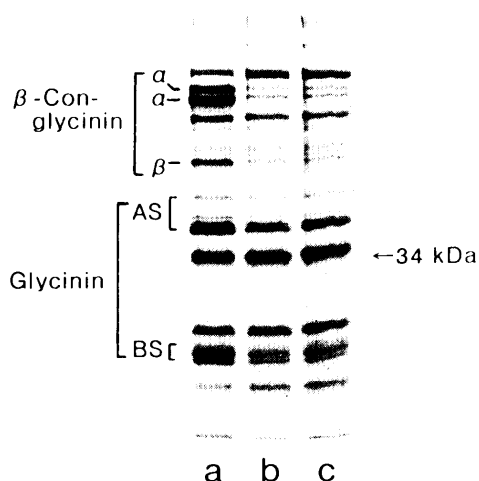


Fig. 1. SDS PAGE Patterns of the Precipitates Prepared by Ultracentrifuging Defatted Soy Milk in the Presence and Absence of 2-Mercaptoethanol (2-ME).

Defatted soy milk at pH 7.5 containing 1 M sodium chloride in the presence or absence of 2-ME was ultracentrifuged ($200,000 \times g$, 50 min). The resulting precipitates were dissolved in distilled water and made up to the volume of the original defatted soy milk before centrifugation. Fifty μ l of the solution was then mixed with 450 μ l of the SDS PAGE sample buffer, 30 μ l of the mixture being applied to a 12% gel. Lane a, 0 mM 2-ME; lane b, 10 mM 2-ME; lane c, 100 mM 2-ME.

Mode of interaction between *Gly m Bd 30 K* and the other soybean storage proteins

The improved separation of *Gly m Bd 30 K* in the presence of the reducing agent suggested its association with the other storage proteins through a disulfide bond. Thus, 2D SDS-PAGE was conducted in the absence (1D) and presence (2D) of 2-ME as shown in Fig. 2.

In the 1D SDS-PAGE analysis in the absence of 2-ME (Fig. 2a, the disc gel), several major protein bands were observed in a higher molecular weight region than that of the monomeric subunits of conglycinin and glycinin, their estimated molecular weights being as follows: a, 157 kDa; a', 150 kDa; b, 103 kDa; b', 95 kDa; c, 70 kDa; c', 64 kDa. The other dense protein bands near 53, 48, and 34 kDa were assigned as the acidic plus basic (AS+BS) subunits of glycinin, the β -subunit of conglycinin, and the monomer of *Gly m Bd 30 K*, respectively.

By 2D SDS-PAGE in the presence of 2-ME (Fig. 2a, the slab gel), the a and a' bands by 1D SDS-PAGE gave only one dense spot at the position of the α' - and α -subunits of conglycinin, their estimated molecular weights being nearly twice those of the α' - and α -subunits of conglycinin, respectively. Thus, the a and a' bands are considered to have been the homodimers of the α' - and α -subunits, respectively. Bands, b and b' produced a dense spot at the positions of the α' - and α -subunits of conglycinin, respectively, and an additional 34 kDa spot (Nos. 2 and 3) in common. This result and the molecular size of bands b and b' indicates that they were complexes of the α' - and α -subunits of conglycinin with the 34 kDa protein, respectively. The spots from bands c and c' were mostly on a diagonal line in the gel and their positions were the same as those of the α' - and α -subunits of conglycinin, suggesting that they were the monomeric α' - and α -subunits of conglycinin, respectively.

Since the band near 53 kDa by 1D SDS-PAGE was dense and broad, it was difficult to distinguish its constituting proteins by 2D SDS-PAGE. However, it seemed to give two main spots corresponding to the acidic and basic subunits of glycinin on the 2D gel, suggesting that this band was a complex of AS and BS of glycinin. The 48 kDa band just beneath 53 kDa seemed to give three spots, one at the position (on the diagonal) of the β -subunit of conglycinin, the second at the position (No. 4) of the 34 kDa protein, and the third at the position of a basic subunit of glycinin, suggesting the possibility that the β -subunit of conglycinin and the complex of the 34 kDa protein with the basic subunit (21 kDa) of glycinin had co-migrated into the 48 kDa band by 1D SDS-PAGE. The spot of the basic subunit of glycinin was too dense and broad to decide whether its complex formation with *Gly m Bd 30 K* or the dimerization of *Gly m Bd 30 K* itself had occurred. These possibilities are studied later. Band No. 5 by 2D SDS-PAGE is considered to have been the monomer of *Gly m Bd 30 K*, since it migrated on the diagonal by 2D SDS-PAGE.

Immunodetection of bound *Gly m Bd 30 K*

The electroblotted membrane of the 2D SDS-PAGE gel was stained with a monoclonal antibody specific to *Gly m Bd 30 K* as shown in Fig. 2b. Five spots were detected at the same position as that of *Gly m Bd 30 K*. Superimposition of the CBB-stained gel (Fig. 2a) and its immuno-stained membrane (Fig. 2b) show that the five CBB-stained spots

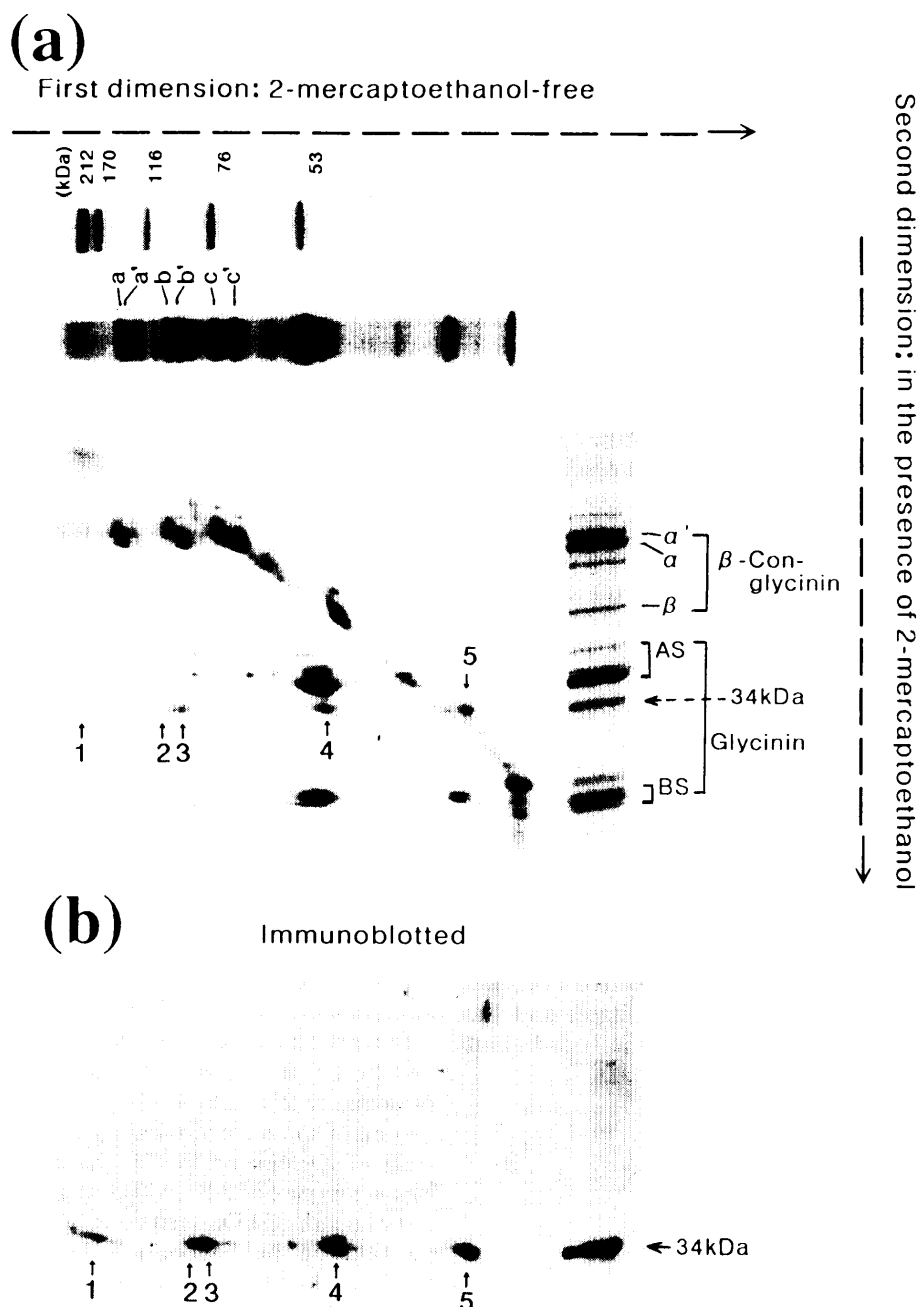


Fig. 2. Two-dimensional (2-D) SDS PAGE Patterns (first, minus 2-ME; second, including 2-ME) and the Immunoblotted Membrane of Soy Proteins in the Precipitate.

Thirty μ g of the precipitate prepared by ultracentrifuging defatted soy milk without 2-ME was applied to 8% gel for 1D SDS PAGE in the absence of 2-ME. The molecular weights of the bands by 1D SDS PAGE were as follows: a, 157 kDa; a', 150 kDa; b, 103 kDa; b', 95 kDa; c, 70 kDa; c', 64 kDa.

After 1D SDS PAGE, the gel was cut, and reacted with 5% 2-ME containing 3% SDS at 40 °C for 30 min. This gel was applied to 12% gel for 2D SDS PAGE in the presence of 2-ME. One of the two 2D SDS PAGE gels was stained with CBB (Fig. 2a) and the other was electroblotted to a nitrocellulose membrane which was then immunostained with a monoclonal antibody specific to *Gly m* Bd 30 K (Fig. 2b).

(Nos. 1–5) were completely coincident with the spots (Nos. 1–5) on the immuno-stained membrane.

These results strongly indicate that spots 1 to 5 were the soybean allergenic protein, *Gly m* Bd 30 K, which had bound to the other proteins. Spots 2 and 3 had bound to the α' - and α -subunits of conglycinin to form bands b and

b' by 1D SDS-PAGE through a disulfide bond. Spot 5 was the monomeric form of *Gly m* Bd 30 K, judging from the relative mobility on the 2D gel. Spot No. 1, although a very small amount, is considered to have been derived either from a high-molecular-weight complex of *Gly m* Bd 30 K with the α' - and α -subunits of conglycinin, or from non-

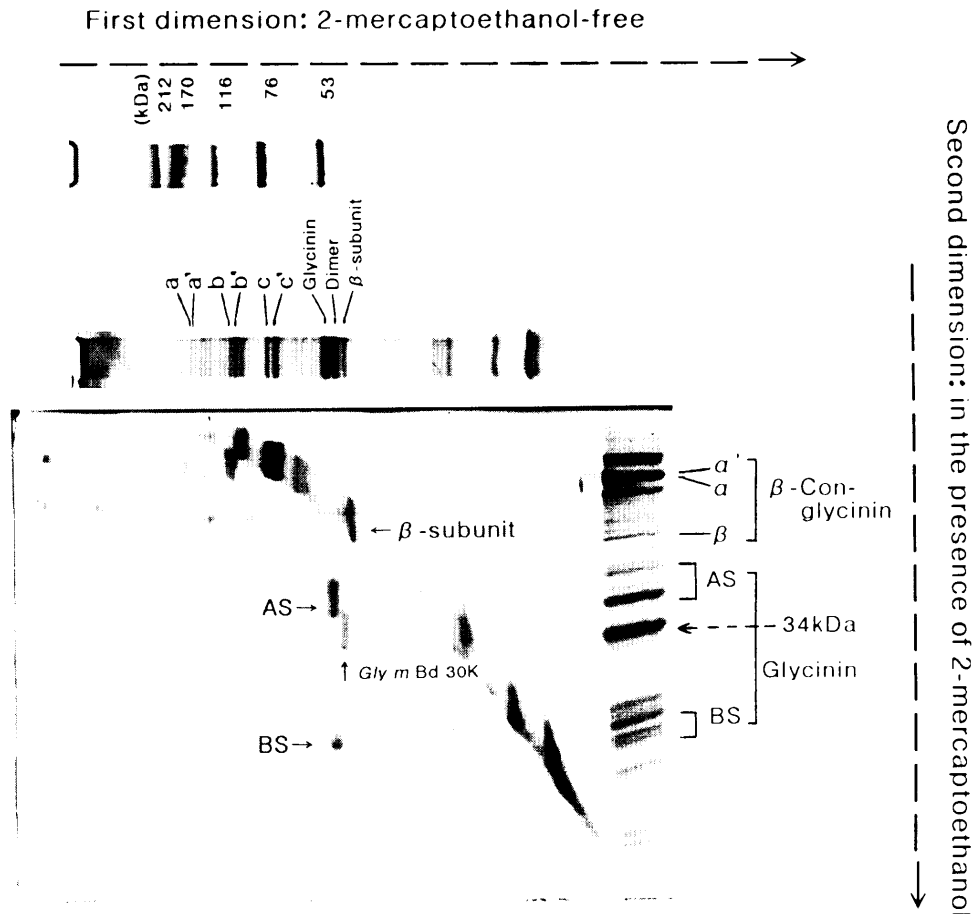


Fig. 3. Two-dimensional SDS-PAGE Patterns of the Precipitate Containing Less Glycinin.

Ten μ g of the precipitate prepared from soy milk at high ionic strength (in the presence of 3M NaCl) was applied to 2D SDS-PAGE as described in Fig. 2.

specific aggregation of *Gly m Bd 30 K* itself.

Spot No. 4 as a dimer of *Gly m Bd 30 K*

The 34 kDa spot No. 4 by 2D SDS-PAGE, which had been immunostained by a monoclonal antibody, migrated to near the position of the β -subunit (48 kDa) of conglycinin and the AS+BS subunit (53 kDa) of glycinin by 1D SDS-PAGE, which made it difficult to determine the precise relative position of No. 4 spot by 2D SDS-PAGE. To identify from which band No. 4 spot was derived, a precipitate with less conglycinin and glycinin was prepared from soy milk at high ionic strength (3M NaCl). The 2D SDS-PAGE results are shown in Fig. 3. The 1D gel shows three bands at around 53 kDa: the upper is AS+BS of glycinin, the middle is unassigned, and the bottom is the β -subunit of conglycinin. The 2D gel clearly shows that No. 4 spot was only one component derived from the middle band and was well resolved from those of AS and BS of glycinin and of the β -subunit of conglycinin. Since No. 4 spot was immunostained by an antibody against *Gly m Bd 30 K* (Fig. 2b) and the middle band migrated at around 53 kDa, this being nearly twice as large as that of *Gly m Bd 30 K*, the middle band was assigned to be a dimer of *Gly m Bd 30 K*.

The data presented here indicate that, in soy milk, part of soybean allergenic protein *Gly m Bd 30 K* specifically formed a complex through a disulfide bond with the α' - and α -subunits of conglycinin, but not with the glycinin sub-

units, having free SH groups, too.¹¹⁾ which led to a loss of conglycinin from soy milk to the precipitated fraction. There is one free SH group per molecule of the α' - or α -subunit of conglycinin from their amino acid sequence deduced by c-DNA.⁶⁻⁹⁾ Therefore, only one disulfide bond could be expected between 2 molecules of *Gly m Bd 30 K*, and between *Gly m Bd 30 K* and the α' - or α -subunit. In fact, the complex formed was not a multimer but a dimer of *Gly m Bd 30 K*, and there was one kind of complex (bands b and b') between *Gly m Bd 30 K* and the conglycinin subunits. This complex formation may explain our previous results¹⁾ that the efficient removal of *Gly m Bd 30 K* was attained by the presence of a reducing reagent with only a small loss of soybean storage proteins from soy milk.

Iwabuchi *et al.* have reported that 2-ME was needed to separate the minor soybean proteins from conglycinin and glycinin by fractionating with ammonium sulfate.¹⁰⁾ We have also observed that soy proteins forming large particles with *Gly m Bd 30 K* could be separated from β -conglycinin and glycinin by fractionating with ammonium sulfate in the presence of 2-ME, but not in its absence (M. Samoto, unpublished results).

Although soybean allergy is not common, the development of soybean cultivars lacking *Gly m Bd 30 K* or containing less the α' - or α -subunit of conglycinin is desirable. Until then, the technological and selective removal of *Gly m Bd 30 K* based on the present strategy will be effective to avoid soybean allergy.

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