

Purification and Characterization of Soybean Allergen Gly m Bd 28K

Hideaki TSUJI,[†] Noriko BANDO, Miki HIEMORI, Rintaro YAMANISHI, Masumi KIMOTO, Kiyoshi NISHIKAWA,* and Tadashi OGAWA

Department of Nutrition, School of Medicine, The University of Tokushima, Kuramoto-cho, Tokushima 770, Japan

*Department of Pediatrics, National Kagawa Children's Hospital, Zentuji 765, Japan

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At least 15 allergenic proteins have been found in soybean using the sera of soybean-sensitive patients with atopic dermatitis [T. Ogawa *et al.*, *J. Nutr. Sci. Vitaminol.*, 35, 555–565 (1991)]. In the present study, a monoclonal antibody (mAb) against Gly m Bd 28K, one of the major allergens of soybean, was prepared, and Gly m Bd 28K was purified from defatted soybean flakes by five purification steps, including immunoaffinity chromatography with the mAb as a ligand. The purified allergen was found to be a glycoprotein with a molecular mass of 26 kDa. During the purification process the allergen was converted to more acidic proteins with the same molecular mass, suggesting that the allergen is unstable. The sugar composition and amino acid sequence of Gly m Bd 28K suggest that the allergen is a new glycoprotein with an Asn-linked sugar moiety. The distribution of the allergen in soybean products was examined by an immunoblotting technique with the mAb.

Key words: soybean allergen; Gly m Bd 28K; Gly m Bd 30K; Gly m Bd 68K

Soybean is one of the principal foodstuffs as a protein source in Japan, from which soybean curd, soy sauce, miso, natto, and so on are produced. A soybean protein isolate (SPI) prepared from defatted soybean is also widely utilized as an ingredient in many processed foods such as hamburgers and meat balls. However soybean is known in Japan as one of the typical allergenic foods. Therefore, the characterization of soybean allergens has become an important problem to be solved for the healthy use of soybean and SPI.

We have recently examined the allergens in soybean using the sera of soybean-sensitive patients with atopic dermatitis and found at least 15 IgE-binding proteins.¹⁾ Among them, three allergens with molecular masses of 68, 30, and 28 kDa were found to be the major allergens in soybean, and these proteins were fractionated into the 7S globulin fraction of soybean proteins. The 68- and 30-kDa allergens have already been purified and characterized.^{2,3)}

These 68-kDa (Gly m Bd 68K) and 30-kDa (Gly m Bd 30K) allergens were respectively identified as α the subunit of β -conglycinin³⁾ and the 34-kDa oil body-associated protein (p34).²⁾ cDNA of the 34-kDa oil body-associated protein has been cloned and sequenced.^{4,5)} Interestingly, Gly m Bd 30K is a protein of the thiol protease family⁵⁾ and exhibits high homology with a house dust mite allergen, Der p 1.⁶⁾ On the other hand, Gly m Bd 28K has not been fully purified and characterized.

In the present study, we describe the preparation of a monoclonal antibody (mAb) against Gly m Bd 28K and the purification of the allergen from defatted soybean flakes. Furthermore, Gly m Bd 28K in various soybean products was examined by an immunoblotting technique with the mAb.

Materials and Methods

Materials. Ampholine and DEAE-Sepharose were obtained from Pharmacia (Uppsala, Sweden). A nitrocellulose membrane and polyvinylidene difluoride (PVDF) membrane were purchased from Bio Rad (Richmond, CA, U.S.A.). 2-Fluoro-1-methylpyridinium toluene 4-sulfonate (FMP)-activated cellulofine was from Seikagaku Kogyo Co. (Tokyo, Japan), and defatted soybean flakes (*Glycine max*, a mixture of IMO varieties) and SPI were from Fuji Oil Co. (Osaka, Japan). A CAP RAST RIA kit (¹²⁵I-labelled anti-human IgE antibodies, 1.63 × 10⁵ Bq/5.1 ml), which was a mixture of rabbit polyclonal anti-human IgE and mouse monoclonal anti-human IgE, was obtained from Pharmacia Diagnosis (Uppsala, Sweden). Peroxidase-conjugated sheep anti-mouse IgG was from Organon Teknika (West Chester, CA, U.S.A.). The 7S globulin fraction of soybean proteins was prepared as described previously,¹⁾ all other chemicals used being of the highest quality commercially available.

Patients and sera. Serum samples containing IgE antibodies to soybean proteins were obtained from patients with atopic dermatitis (mean age of 3.9 years). These patient had been diagnosed as sensitive to soybean by RAST and by one of the additional methods for diagnosing food allergy such as a skin prick test or history of dietary restriction at National Kagawa Children's Hospital.

Preparation of the mAb against Gly m Bd 28K. In order to obtain Gly m Bd 28K as an antigen, the allergen was partially purified from defatted soybean flakes that had been homogenized in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and 10 mM mercaptoethanol and centrifuged at 10,000 × g for 30 min. The 7S globulin fraction was obtained from the supernatant by the method of Thanh and Shibasaki.⁷⁾ This fraction was subjected to DEAE-Sepharose CL-6B, Sephacryl S-200, and CM-cellulofine chromatographies and isoelectric focusing on a 110-ml column. Gly m Bd 28K was detected by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and by an immunoblotting analysis with the patients' sera. Although the allergen preparation was shown to contain minor contaminants, it was used directly as an antigen to prepare the mAb.

Fifty μ g of this allergen was emulsified with complete Freund's adjuvant (1:1, v/v) and injected intraperitoneally into a 8-week-old female BALB/c mouse. At intervals of two weeks, the animal was intraperitoneally injected twice with 50 μ g of the antigen in a 1:1 mixture (v/v) of incomplete

* To whom all correspondence should be addressed (Tel: 0886-33-7088, Fax: 0886-33-7089).

Abbreviations: BSA, bovine serum albumin; FMP, 2-fluoro-1-methylpyridinium toluene 4-sulfonate; HPLC, high-performance liquid chromatography; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; SPI, soybean protein isolate.

Freund's adjuvant and phosphate-buffered saline (PBS). Thereafter, the animal was intraperitoneally injected with 50 μ g of the antigen in PBS as a final booster and killed 4 days later.

The immunized spleen cells were fused with P3U1 myeloma cells using 50% polyethylene glycol 4000 as a fusion agent as described previously.⁸⁾ Hybridoma cells were grown in hypoxanthine-aminopterin-thymidine selection medium containing 15% fetal bovine serum on wells of 96-well microplates (Becton Dickson, Oxnard, CA, U.S.A.). After 9 days, the supernatant of each culture was tested for the production of mAbs against the allergen by an enzyme-linked immunosorbent assay (ELISA). Positive colonies of hybridoma cells were subsequently cloned three times by the limiting dilution method, using a feeder layer of thymus cells obtained from 6-week old female BALB/c mice.

Female BALB/c mice, which had been primed with 0.5 ml of pristane per mouse one week before, were intraperitoneally injected with 5×10^6 hybridoma cells per mouse. After 14 days, ascitic fluid was obtained from the mice. The mAb was purified from the ascites by ammonium sulfate fractionation and protein A chromatography, and is designated mAb-C5 in this paper.

Preparation of mAb-C5-cellulofine affinity gel. An affinity gel was prepared by coupling mAb-C5 with FMP-activated cellulofine according to the manufacturer's instruction. Briefly, the FMP-activated gel (1.74 g) was washed with water, after which the gel was mixed with 21.6 ml of 50 mM NaHCO₃ containing the mAb (87 mg) and incubated at 4°C for 18 h with gentle shaking. The gel was filtered off and washed with 50 mM NaHCO₃. The remaining active groups in the gel were masked by a further 16-h incubation with 10 mM Tris-HCl buffer (pH 8.0) containing 0.1 M monoethanolamine at 4°C. After filtration, the gel was successively washed with 10 mM acetate buffer (pH 4.5), an acetate buffer containing 0.5 M NaCl, 10 mM Tris-HCl buffer (pH 8.0), and the Tris buffer containing 0.5 M NaCl. Cellulofine coupled with mAb-C5 as a ligand was used in the subsequent affinity chromatography and is designated as mAb-C5-cellulofine in this paper.

Purification of Gly m Bd 28K. All the steps were carried out at 4°C. Gly m Bd 28K was purified from the 7S globulin fraction that had been prepared from defatted soybean flakes (1 kg) by the method of Thanh and Shibasaki.⁷⁾ The proteins in the 7S globulin fraction were fractionated with solid (NH₄)₂SO₄. The precipitate formed between 50% and 70% saturation of (NH₄)₂SO₄ was collected by centrifugation and dissolved into 10 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 10 mM mercaptoethanol, and 6 M urea (buffer A). After the urea in the allergen solution, which would have inhibited the binding of the allergen to mAb-C5-cellulofine, had been removed by dialyzing against 10 mM Tris-HCl buffer (pH 8.0), the dialysate was applied on a mAb-C5-cellulofine column (1.4 $\times$ 13 cm) that had been equilibrated with the same buffer. The column was washed with the Tris buffer containing 0.15 M NaCl and then with the Tris buffer containing 1 M NaCl. The allergen adsorbed on the column was eluted with 0.1 M acetic acid, the eluted allergen being detected by immunoblotting with mAb-C5 in addition to the above-mentioned methods. Fractions containing the allergen were pooled and concentrated by ultrafiltration through a Diaflo YM1 membrane filter. The concentrate was then dialyzed against buffer A, the dialysate being loaded onto a DEAE-Sepharose CL-6B column (0.65 $\times$ 15 cm) that had been equilibrated with buffer A. The column was washed with buffer A containing 10 mM NaCl, and the allergen on the column was eluted with 500 ml of a linear gradient of NaCl (10 to 150 mM) in buffer A. The fractions containing the allergenic protein were pooled and concentrated by ultrafiltration through the above-mentioned membrane. The concentrate was subjected to gel filtration on a Sephacryl S-200 column (3.4 $\times$ 130 cm) that had been equilibrated with buffer A, the allergen fractions being pooled, collected and concentrated. The concentrate was used as the purified allergen in the subsequent experiments.

ELISA. ELISA was done in the same manner as that described previously.⁸⁾ Briefly, 50 μ l of the allergen (50 μ g/ml) was added to the wells of a microplate for incubation at 4°C overnight, the wells then being blocked with 200 μ l of 1% bovine serum albumin (BSA) dissolved in PBS. After washing three times, the wells were incubated with the culture supernatant or with the mAb and mixed with peroxidase-conjugated sheep anti-mouse IgG. Each immunocomplex on the wells was visualized by the peroxidase reaction with *O*-phenylenediamine and H₂O₂ as substrates.

Electrophoresis and immunoblotting. SDS-PAGE was done on 15%

polyacrylamide gels according to the method of Laemmli.⁹⁾ Two-dimensional gel electrophoresis was carried out in the presence of Ampholine (pH 3.5–10) containing a sample, a 5% polyacrylamide gel being prepared in a cylindrical tube (2.2 mm $\times$ 12 cm) as described previously.²⁾ The gel was focused at 4°C for 16 h at 400 V. After electrophoresis, the gel was taken out and equilibrated with the sample buffer for SDS-PAGE. The gel was loaded on the top of a 12% polyacrylamide slab gel and fixed with 1% agarose, SDS-PAGE being conducted in the same manner as that already described. The proteins on the gel were detected by staining with Coomassie Brilliant Blue R250 in methanol-acetic acid-water (5:1:4, by vol.). The sugar moiety of the protein on the gel was visualized with PAS staining according to the method of Zaccharius *et al.*¹⁰⁾ For the immunoblotting analyses, the proteins separated by the above-mentioned SDS-PAGE were electroblotted onto nitrocellulose membranes in the same manner as that described previously.¹⁾ After the membranes had been blocked with 1% BSA in PBS at 37°C for 1 h, the allergen on the membranes was reacted at 37°C for 1 h with mAb-C5 or at 4°C overnight with IgE antibodies in the patients' sera. After washing three times, the immunocomplex of the allergen was incubated with peroxidase-labelled anti-mouse IgG at 37°C for 1 h, or with ¹²⁵I-labelled anti-human IgE at room temperature overnight. Radio-autography was done as described previously.¹⁾

Amino acid analysis. Amino acids were analyzed by a Hitachi L8500A automatic amino acid analyzer. The samples were hydrolyzed with 6 M HCl at 115°C for 24 h according to the method of Moore and Stein.¹¹⁾ The total half-cystine content was determined as cysteic acid after performic acid oxidation by the method of Moore.¹²⁾ The tryptophan content was spectrophotometrically determined according to the method of Edelhoch.¹³⁾

Sugar analysis. The sugar components of the purified allergen were analyzed by hydrolyzing the allergen sample in a mixture of 4 M HCl and 4 M trifluoroacetic acid (1:1, v/v) at 100°C, acetylating with acetic anhydride and then pyridylaminating with 2-aminopyridine. The pyridylamino derivatives of the sugar components were analyzed by a high-performance chromatograph equipped with a PALPAK type A column (Takara, Kyoto, Japan).

Amino acid sequence analysis. The purified allergen was subjected to two-dimensional electrophoresis as already described. After two-dimensional electrophoresis, the allergen was electrotransferred on a PVDF membrane and detected by staining with Coomassie Brilliant Blue R250. The band corresponding to the allergen was cut off and subjected to N-terminal amino acid sequencing, the amino acid sequence of the allergen being analyzed with a Hewlett Packard G1005A automatic sequencer.

Results

Preparation of the mAb against Gly m Bd 28K and the affinity gel

Fusion of the immunized spleen cells with P3U1 myeloma cells was done as described under Materials and Methods. Finally a positive hybridoma was established. The light chains and heavy chains of the mAb (mAb-C5) produced by the hybridoma were κ and γ_{2a} , respectively, and the apparent dissociation constant was 4.8×10^{-10} M. The mAb-C5-cellulofine was found to specifically bind to Gly m Bd 28K, and the complex between mAb-C5-cellulofine and the allergen to be easily dissociated by 0.1 M acetic acid. This result suggests that mAb-C5-cellulofine would be a powerful tool for use as an immunoaffinity gel in the purification of the allergen.

Purification of Gly m Bd 28K

When soybean proteins were fractionated by the conventional method,⁷⁾ the three major allergens (Gly m Bd 68K, Gly m Bd 30K, and Gly m Bd 28K) were recovered in the 7S globulin fraction.¹⁾ As shown in Fig. 1A, this fraction contained large amounts of Gly m Bd 68K and the β subunit of β -conglycinin, as well as a considerable amount

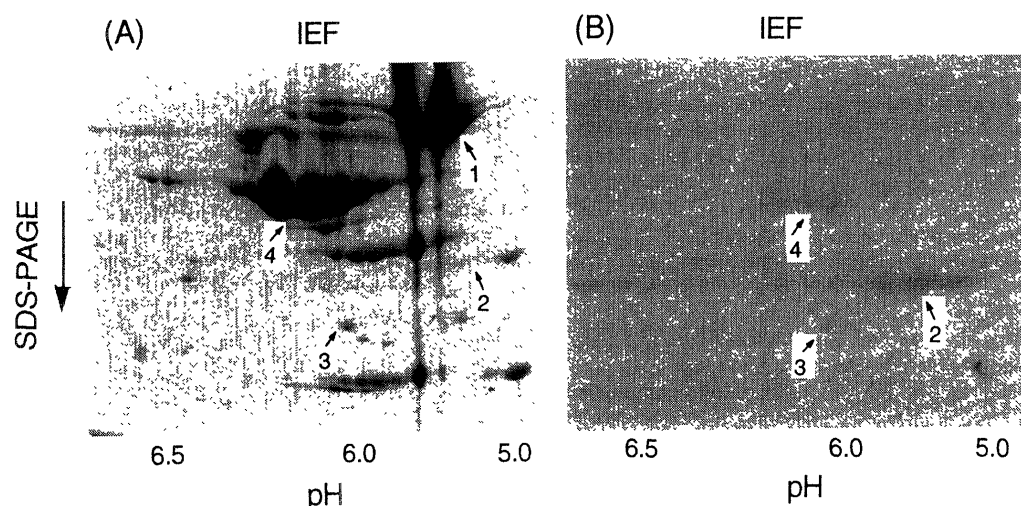


Fig. 1. Two-dimensional Electrophoresis of the 7S Globulin Fraction.

The 7S globulin fraction was prepared as described in the text. The 7S globulin proteins (300 μ g) were isoelectrically focused on two 5% cylindrical polyacrylamide gels (2.2 mm $\times$ 12 cm) and then the proteins on the gels were electrophoresed onto two slab polyacrylamide gels. After the two-dimensional electrophoresis, the proteins on one gel were stained with Coomassie Brilliant Blue R250 (A). The proteins on the other gel were electroblotted onto a nitrocellulose membrane and immunoblotted with patients' sera (B). Arrows 1, 2, 3, and 4 represent the spots corresponding to Gly m Bd 68K, Gly m Bd 30K, Gly m Bd 28K, and the β subunit of β -conglycinin, respectively.

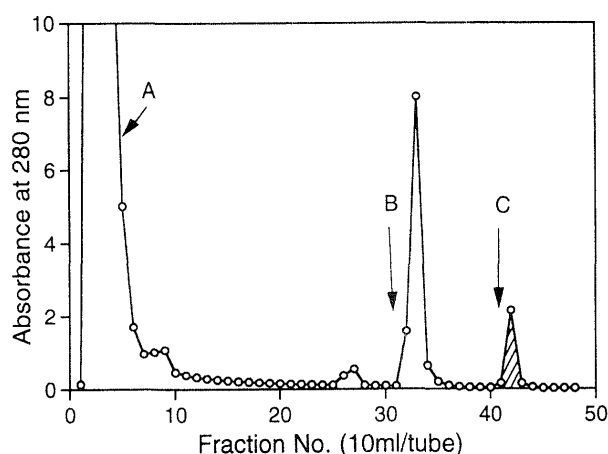


Fig. 2. Immunoaffinity Chromatography of Gly m Bd 28K on MAb-C5-Cellulofine Column.

The fraction containing Gly m Bd 28K was obtained from soybean flakes by isoelectric precipitation and ammonium sulfate fractionation as described in the text. A, washed with 10 mM Tris-HCl (pH 8.0) containing 0.15 M NaCl; B, washed with Tris buffer containing 1 M NaCl; C, eluted with 0.1 M acetic acid. The hatched peak represents the allergen fraction.

of Gly m Bd 30K. Gly m Bd 30K was immunoblotted with the sera of the soybean-sensitive patients used in this work, but Gly m Bd 68K could not be immunoblotted with the patients' sera (Fig. 1B). These results suggest that the patients were insensitive to Gly m Bd 68K. The β subunit of β -conglycinin was immunostained with the sera, but the immunoreaction was non-specific. On the other hand, Gly m Bd 28K was found to exist in a very low concentration in the 7S globulin fraction by staining with Coomassie Brilliant Blue R250, although the protein was clearly immunostained with the sera of the patients and ^{125} I-labelled anti-human IgE. Gly m Bd 28K could not be detected, or existed in a negligible concentration, in the other fractions (data not shown). These results show Gly m Bd 28K to be the minor protein component in soybean, as well as one of the potent major allergens.

We purified the allergen from defatted soybean flakes by affinity chromatography. Soybean proteins were extracted

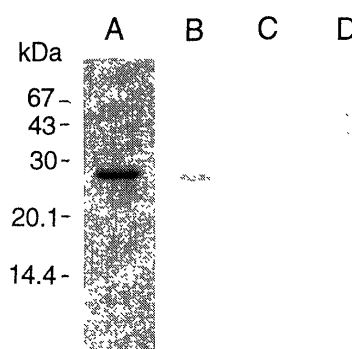


Fig. 3. SDS-PAGE of the Purified Allergen.

The purified allergen (2 μ g/lane except for 10 μ g/lane in lane D) was electrophoresed on a 12% slab polyacrylamide gel. The protein on the gel was electroblotted onto a nitrocellulose membrane, and the membrane was cut into several pieces. The protein on one piece of the membrane was stained with Amido Black 10B (A), and that on the other pieces was immunostained with the patients' sera (B) or mAb-C5 (C), or stained with the PAS reagent (D). The standard markers are shown on the left (in kDa).

from defatted soybean flakes. Most of the glycinin (11S globulin) and the bulk of the 7S globulin were respectively removed from the soybean proteins by isoelectric precipitation at pH 6.4 and by 50% saturation with $(\text{NH}_4)_2\text{SO}_4$. The precipitate formed between 50% and 70% saturation was then collected and dissolved in buffer A, in which urea was supplemented to make the precipitate easy to dissolve. After dialyzing against 10 mM Tris-HCl buffer (pH 8.0), the proteins in the dialysate were separated on a mAb-C5-cellulofine column as described under Materials and Methods (Fig. 2). The allergenic protein was further purified by DEAE-Sepharose CL-6B chromatography. Finally, the contaminated proteins present in the allergen preparation were removed by gel filtration on a Sephacryl S-200 column. Fifteen mg of the purified allergen was obtained from 1 kg of defatted soybean flakes. The purified allergen was shown to be homogeneous by SDS-PAGE (Fig. 3A), and immunoblotting with the patients' sera and the mAb demonstrated that purified protein to be Gly m Bd 28K (Figs. 3B and 3C).

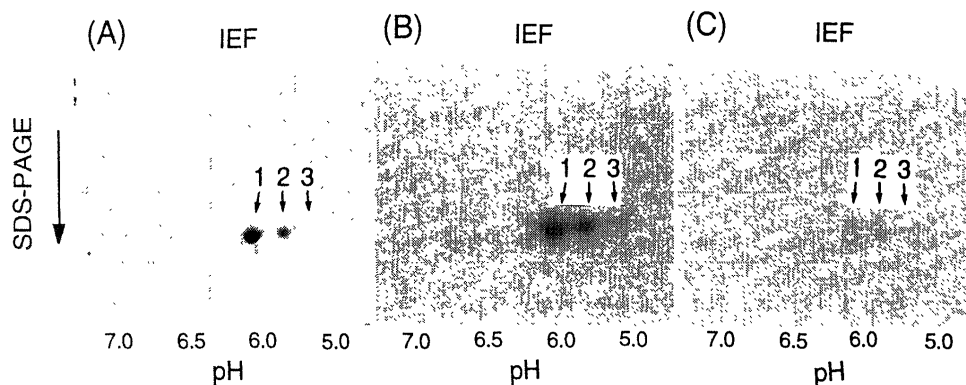


Fig. 4. Two-dimensional Electrophoresis of the Purified Allergen.

The purified allergen (5 μ g) was subjected to two-dimensional electrophoresis on three polyacrylamide gels as described in the text. After the two-dimensional electrophoresis, the allergen on each gel was electroblotted onto a nitrocellulose membrane. The allergen on one membrane was stained with Amido Black 10B (A), and the protein on the other membranes was immunostained with the patients' sera (B) or with mAb-C5 (C). Spot 1 corresponds to the intact allergen.

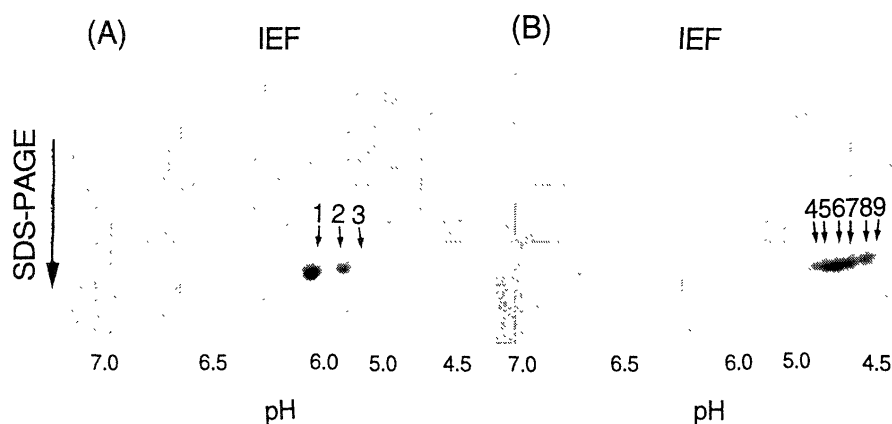


Fig. 5. Two-dimensional Electrophoretic Patterns of the Freshly Purified Allergen (A) and That Stood at 37°C for 2 Days (B).

Two-dimensional electrophoresis was conducted under the same conditions as these described in the legend to Fig. 4. Spot 1 corresponds to the intact allergen.

Stability of Gly m Bd 28K

The purified Gly m Bd 28K sample showed only one band on polyacrylamide gel by one-dimensional electrophoresis, suggesting that it was homogeneous in molecular mass (Fig. 3A). However, when this preparation was subjected to two-dimensional electrophoresis, two protein spots with different isoelectric points of 5.8 and 5.5 were observed on the gel, in addition to a spot corresponding to the original allergen with an isoelectric point of 6.1 (Fig. 4A). The original allergen and the other proteins derived from the original allergen showed the same molecular masses, but their isoelectric points were quite different from one another. Furthermore, the protein derivatives were also immunoblotted with the mAb and the patients' sera (Figs. 4B and 4C). Spot 1 corresponds to the position of the original allergen, its N-terminal amino acid sequence being determined to be FHDDEGGDKK. The N-terminal amino acid sequences for spots 2 and 3 were FHDDEGGDKK and FHD, respectively. When the purified allergen was stood in buffer A at 37°C for 2 days, the three above-mentioned spots disappeared and six new spots with more acidic isoelectric points appeared (Fig. 5). The N-terminal amino acid sequences of the proteins corresponding to the four spots (FHDDEGGD for spot 5, FHDDEGGD for spot 6, FHDDEGGD for spot 7, and FHDDEG for spot 8) were found to be identical with that of the original allergen, although the products corresponding to spots 4 and 9 could not be sequenced due to being beyond the

detection limit.

Sugar composition of Gly m Bd 28K

The allergen was stained with the PAS reagent (Fig. 3D), indicating it to be a glycoprotein. The allergen was hydrolyzed with a mixture of 4 M HCl and 4 M trifluoroacetic acid (1:1, v/v), its sugar components being aminopyridylated and then analyzed by HPLC. The sugar analysis results show that the sugar chain of the allergen consisted of mannose, *N*-acetylglucosamine, xylose, and fucose in a molar ratio of 3:2:1:1. No *N*-acetylgalactosamine or glucose could be detected in the purified allergen.

Amino acid composition of Gly m Bd 28K

The amino acid composition of the allergen is summarized in the Table. The allergen was rich in aspartic acid, glutamic acid, glycine, isoleucine, leucine, and phenylalanine. On the assumption that one mol of cysteine is included in one mol of the allergen, the molecular weight of the allergen was estimated to be 25,304, which is compatible with that obtained by SDS-PAGE (ca. 26,000), even if the sugar moiety of the allergen is taken into consideration.

N-Terminal amino acid sequence of Gly m Bd 28K

The N-terminal amino acid sequence of the allergen was determined as already described by direct N-terminal amino acid sequencing of Gly m Bd 28K electroblotted on a PVDF membrane, after SDS-PAGE. The sequence of the allergen

was determined to be FHDDEGGDKKSPKSLFLMS-STR.

Distribution of Gly m Bd 28K in soybean products

Soybean and SPI are supplemented into various processed foods as an ingredient. Therefore, Gly m Bd 28K in the soybean products was analyzed by an immunoblotting technique with mAb-C5. As shown in Fig. 6, the allergen was shown to be contained in a high concentration in tofu, yuba, abura-age, and soy milk. SPI contained a significant amount of the allergen. However, the allergen could not be detected in fermented soybean products of natto, soy sauce, or in SPI-containing processed foods such as meat balls and fish sausage.

Table Amino Acid Composition of Purified Gly m Bd 28K

Amino acid	mol%	Residues/mol ^a
Aspartic acid ^b	9.09	20
Threonine	3.63	8
Serine	10.00	22
Glutamic acid ^b	10.00	22
Proline	4.09	9
Glycine	10.00	22
Alanine	4.55	10
Valine	4.55	10
Methionine	1.82	4
Isoleucine	7.27	16
Leucine	9.09	20
Tyrosine	2.73	6
Phenylalanine	7.73	17
Lysine	5.00	11
Histidine	3.64	8
Arginine	5.45	12
Half-cystine ^c	0.45	1
Tryptophan ^d	0.9	2

^a The nearest integral number was calculated on the basis of a molecular mass of 26 kDa obtained by SDS-PAGE.

^b The aspartic and glutamic acid values include asparagine and glutamine, respectively.

^c Determined as cysteic acid.

^d Determined spectrophotometrically as described in the text.

Discussion

In the present study, we prepared a mAb against Gly m Bd 28K using the partially purified allergen and applied mAb-C5 to immunoaffinity chromatography for purifying the allergen. Five purification steps, including the immunoaffinity chromatography, enabled the allergen to be purified to homogeneity by one-dimensional electrophoresis (Fig. 1). The fact that only 15 mg of the purified allergen was obtained from 1 kg of defatted soybean flakes suggests that Gly m Bd 28K may be a minor protein component and occur two or three orders lower in concentration than Gly m Bd 30K.²⁾ The molecular mass and isoelectric point of the allergen were found to be 26 kDa and 6.1, respectively. Analysis of the sugar components of the allergen showed it to be an Asn-linked glycoprotein, suggesting that its sugar composition was the same as that of Gly m Bd 30K.¹⁴⁾ No homologous protein could be found by a homology search with respect to the N-terminal amino acid sequence of the allergen, indicating it to be a new glycoprotein with an Asn-linked sugar moiety. The sugar composition of Gly m Bd 28K was found to be similar to that of such Asn-linked glycoproteins as pineapple stem bromelain,¹⁶⁾ horseradish peroxidase,¹⁷⁾ and ascorbate oxidase from *Cucurbita pepo*,¹⁸⁾ in addition to that of Gly m Bd 30K.¹⁴⁾ Batanero *et al.*¹⁹⁾ have recently reported that the sera of patients allergic to olive pollen was bound not only to Ole e I, the major olive pollen allergen, but also to the three above-mentioned plant glycoproteins, which do not exhibit any homology with Ole e I, but which have the same sugar moieties as Ole e I. This observation suggests that the sugar moieties of these glycoproteins might constitute an epitope that is recognized by IgE antibodies. It would be of great interest to examine whether the sugar moieties of the two soybean allergens might be involved in their allergenicity.

When the 7S globulin fraction were subjected to two-dimensional electrophoresis, only one spot corresponding to the allergen could be detected by immunoblotting with the mAb (Fig. 1). Unexpectedly, the purified allergen gave two more spots by two-dimensional electrophoresis, in addition to the spot corresponding to the original allergen (Fig. 4). The molecular masses of the two products were the same as that of the original allergen, but the isoelectric points of the allergen and derivative were clearly different

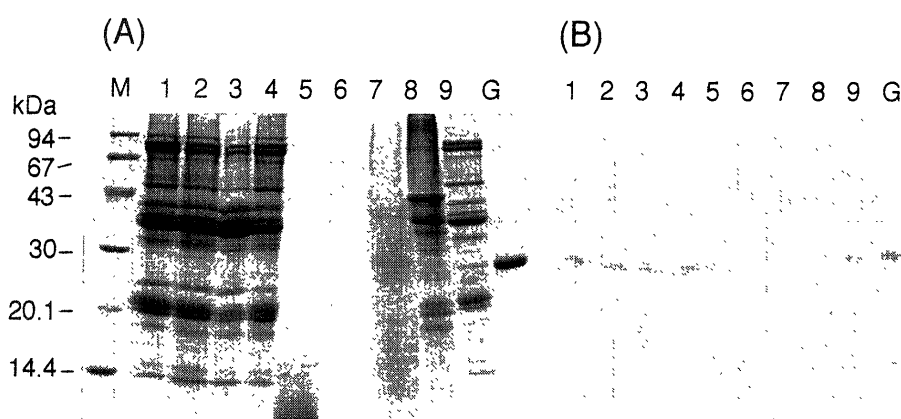


Fig. 6. SDS-PAGE and Immunoblotting Pattern of Gly m Bd 28K in Soybean Products.

Gly m Bd 28K in soybean products was extracted in the same manner as that described in the previous paper.¹⁵⁾ The proteins (20 µg/lane) in each extract were separated on two 15% polyacrylamide gels. The proteins from the extracts on one gel were stained with Coomassie Brilliant Blue R250 (A). The proteins on the other gel were electroblotted onto a nitrocellulose membrane, and the allergen on the membrane was immunostained with the mAb (B) as described in the text. M, marker proteins; 1, tofu; 2, yuba; 3, abura-age; 4, soy milk; 5, natto; 6, soy sauce; 7, meat balls; 8, fish sausage; 9, SPI; G, the purified allergen.

from one another. Since these protein species were found to be immunostained with the patients' sera and with mAb-C5, they are likely to have been derived from the original allergen in the course of the purification. By standing for 2 days at 37°C, the purified allergen preparation gave six other spots on another two-dimensional electrophoretic pattern which were quite different from those of the three proteins originally present in the purified allergen preparation. Interestingly, the N-terminal amino acid sequences and the molecular masses of all the sequenced products were identical with those of the original allergen, although their isoelectric points were different from one another. When the allergen was heated for 10 min in a boiling water bath, the same phenomenon was observed (data not shown). This finding indicates that the conversion of the original allergen to the other products during the incubation at 37°C was not likely to have been due to a proteolytic enzyme contaminating in the purified allergen and that the products may have been artifacts during the process. During the exposure of the allergen to urea, the cyanate and/or isocyanate formed by the decomposition of urea may result in carbamylation of the allergen,²⁰⁾ so that the positive charge of the allergen may be reduced by carbamylating amino acid residues such as lysine to convert the allergen to more acidic artifacts. Alternatively, the asparagine and glutamine residues of the allergen might undergo spontaneous deamidation.^{20,21)} The mechanism by which the allergen was converted to acidic protein products without cleavage of its peptide bonds still remains unresolved.

It is very important to elucidate the distribution of Gly m Bd 28K in processed foods which contain soybean or SPI as an ingredient, because soybean-sensitive patients are heavily exposed to soybean allergens every day. Such soybean products as tofu, yuba, abura-age, and soy milk were each found to contain a high concentration of Gly m Bd 28K (Fig. 6). This indicates that the major part of the allergen in soybean seeds would be retained in the products during processing, in the same manner as Gly m Bd 30K was.¹⁵⁾ However, Gly m Bd 28K could not be detected in fermented soybean products such as natto and soy sauce, suggesting that the allergen may have been rapidly digested to small peptides during fermentation. Interestingly, SPI contained a significant amount of the allergen, but such processed foods as meat balls and fish sausage, which are supplemented with SPI, were shown to contain no Gly m Bd 28K. In contrast with Gly m Bd 28K,

Gly m Bd 30K has been found to occur significantly in meat balls,¹⁵⁾ which may be explained by the following reasons: first, the content of Gly m Bd 28K in SPI was much less than that of Gly m Bd 30K and, it is possible that the amount of Gly m Bd 28K was below the detection threshold; second, Gly m Bd 28K would be degraded more easily than Gly m Bd 30K during the processing of meat balls. These findings provide useful information to select hypoallergenic soybean products for patients allergic to soybean.

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