Summary  About 15 soybean proteins were shown to be recognized by sera of soybean-sensitive patients with atopic dermatitis. Three of them were identified as major allergens and designated as Gly m Bd 60K, Gly m Bd 30K, and Gly m Bd 28K, respectively. Gly m Bd 60K is an α subunit of β-conglycinin well known as a major soybean storage protein. Gly m Bd 30K is also known as a soybean oil-body-associated glycoprotein with a molecular weight of 34,000, which is homologous to Der p (or f) 1, a major allergen of house dust mite, classified under the papain super family. Gly m Bd 28K is a vicilin-like glycoprotein with a molecular weight of 26,000, a minor component fractionated into 7S globulin fraction. The reduction of allergenicity of soybean and soybean products has been developed with respect to the above-mentioned major three allergens as the targets by the use of the combined techniques of a chemical breeding, a physico-chemical treatment, and an enzymatic digestion. Among the three major allergens, the α subunit of β-conglycinin and Gly m Bd 28K were eliminated from soybean seeds by the development of a mutant line, Tohoku 124, introduced by a chemical breeding technique. The strongest allergen, Gly m Bd 30K, was almost completely removed from defatted soymilk prepared from Tohoku 124 by a salting-out technique and a centrifugation under the limited pH and ionic strength and alternatively by an enzymatic digestion. By the application of these procedures, several hypoallergenic soybean products, such as cooked soybean grains, soybean curd (Tofu), and fermented soybean paste (Miso), soymilk, and a jelly-like soybean cake have been made to evaluate their usefulness by a challenge test for soybean-sensitive patients. It has been demonstrated by a preliminary trial that about 80% of the soybean-sensitive patients could ingest these hypoallergenic products without any adverse reactions.

Key Words  soybean, allergen, allergenicity, hypoallergenic, chemical breeding

Abbreviations: RAST; radioallergosorbent assay, SDS-PAGE; sodium dodecyl-sulfate polyacrylamide gel electrophoresis, ECL; enzyme chemiluminescence, ELISA; enzyme-linked immunosorbent assay, SPI; soybean protein isolate, KSTI; Kunitz soybean trypsin inhibitor.
antibodies in sera of soybean allergic patients showed a cross reactivity among the 2S-, 7S-, and 11S-globulin fractions by the RAST-inhibition analyses. But this method could not characterize the individual protein component responsible for the cross reactivity. He demonstrated that most allergenic fraction was the 2S-globulin fraction, and 7S- and 11S- in this order. Several investigators wrote about the features of soybean allergens, but no detailed information has been presented. The allergenicity of soybeans is known to reside in the protein fractions, not in soybean oil itself (5), whereas oxidized soybean oil has been shown to enhance IgE-binding ability of soybean or other food proteins (6). Burks et al. (7) showed that allergenic proteins in soybean predominated in the 7S- or 11S-globulin fractions rather than in the 2S-globulin fraction as a result of immunochemical analysis using sera of soybean-sensitive patients with atopic dermatitis. Recently, Herian et al. (8) reported that the sera of patients sensitive to both peanuts and soybeans bound to several protein components with molecular weights ranging from 50,000 to 60,000 (probably subunits of β-conglycinin) and also to a component with a molecular weight of about 20,000, not identical with KSTI, which was strongly recognized by the IgE from the patients allergic only to soybeans. Rodrigo et al. (9) reported that the inhalation of soybean dust accidentally caused asthma in Barcelona. The patients with asthma raised against soybean dust had specific IgE antibodies for the glycoproteins with molecular weights lower than 14,000, which were assumed to be degradation products of β-conglycinin or unique protein species occurring in soybean hulls, designated as Gly m 1 and 2. Herian et al. (8) described different soybean-allergic subjects being sensitive to quite different proteins and able to be classified into three categories according to the immunoblotting patterns. Our results also showed that the IgE-binding proteins varied among the patients but the patients could not be classified into distinct groups according to their immunoblotting patterns (1). Recently, we demonstrated the occurrence of about 15 protein components binding with IgE antibodies in sera of soybean-sensitive patients, three of them named as Gly m Bd 30K, Gly m Bd 28K, and Gly m Bd 60K were shown to be major allergenic proteins (1). Based on this information about allergenic proteins in soybeans as the target to be removed, many approaches to reduce the allergenicities of soybeans and soybean products have been proposed: (a) physico-chemical procedures such as heat denaturation and precipitation, (b) destruction and modification of allergenic structures such as an introduction of polysaccharide moieties and enzymatic digestion, (c) breeding (selection of an allergen-deficient variety or induction of mutants), (d) genetic engineering, and (e) fabrication of non-allergic constituents. Furthermore, there have concurrently been developed more selective and sensitive methods for an evaluation of allergenicity of soybean products, which can be applicable during the course of processing. The convenient methods to detect and determine the major allergens by immunoblotting and enzyme-linked immunosorbent assay (ELISA or sandwich ELISA) have been established using allergen specific monoclonal antibodies. The present paper reviews recent information on the molecular, biochemical and immunological properties of the major allergens from soybeans and the development of hypoallergenic soybean products.

**Major Soybean Allergens**

1) Gly m Bd 30K

The soybean allergenic protein, Gly m Bd 30K (1) which is most strongly and frequently recognized by the IgE antibodies in sera of soybean-sensitive patients with atopic dermatitis, has been characterized as a soybean seed 34 kDa oil-body-associated protein (10). This protein had been identified by Kalinski et al. (11) from the fractionated soybean oil body membrane, whereas the cDNA was isolated and cloned as a vacuolar storage protein P34 with close homology to thiol proteases classified under a group of papain super family. The primary structure of Gly m Bd 30K was shown to have about 30% homology or 54% similarity with Der p 1, a house dust mite allergen that is thiol protease found in feces of *Dermatophagoides pteronyssius* (12). As shown in Fig. 1 the mature P34 vacuolar protein consists of 257 amino acid residues which is derived by a removal of a part of N-terminal 122 amino acid residues from a precursor protein with a molecular weight of about 47,000 during the maturation in a vacuole (11). The glycosylation site of Gly m Bd 30K was established to be located on Asn₁⁷₀ residue of a mature protein (13), which consists of mannose, N-acetylgalcosamine, xylose, and fucose in a molar ratio of 3:2:1:1, respectively, indicating one of typical plant asparagine-N linked high mannose type glycans with xylose and fucose branch. The localization of Gly m Bd 30K (P34) in vacuoles of soybean cotyledons was confirmed by an electron microscopic immunostaining technique (14). In recent years, IgE binding sites (B-cell epitopes) located on Gly m Bd 30K were investigated by using synthetic peptides and identified to be located on the 3-12, 100-110, 229-238, 299-308, and 331-340 amino acid residues, respectively (15). Interestingly, all the epitope sites recognized by human IgE antibodies were shown to be quite different from those on house dust mite allergen, Der p 1. The epitope of the monoclonal antibody of F5 (IgG), which was raised against Gly m Bd 30K using BALB/c mouse, is identified on the 115-132 amino acid residue (16). Gly m Bd 30K was specifically associated with the proteins in 7S-globulin fraction through the disulfide linkage. This property added an important piece of information to the strategy of development of hypoallergenic SPI. Furthermore, there is no soybean variety lacking Gly m Bd 30K in the stock culture of soybean. The cDNA was cloned and the recombinant allergen without glycans moiety was prepared from *E. coli*, which was recognized by sera of soybean-sensitive patients, suggesting that rGly m Bd 30K can be applicable for a diagnostic use as an allergen standard of RAST (17). In addition, the distribution of Gly m Bd
30K as the index of a soybean allergenicity in soybean varieties and soybean products can be selectively determined by the use of monoclonal antibodies, F5 and H6 (18).

2) GlymBd28K

A minor protein component in soybean recognized by soybean-sensitive patients with about 25% incidence, one of major allergens named Gly m Bd 28K, was isolated and purified from 7S-globulin fraction prepared from defatted soybean flakes (products from Indiana-Ohio-Michigan, U.S.A.) (19). The purified allergen was shown to be a glycoprotein with the molecular mass and isoelectric point of 26 kDa and 6.1, respectively and an Asn-N linked glycan moiety with the same sugar composition as that of Gly m Bd 30K was identified to be located on Asn20 residue of Gly m Bd 28K. The N-terminal amino acid sequence analysis gave a result of FHDDEGGDKKSPKSLFMSDSTRVFK and no homologous proteins (peptides) could be found in a data base of proteins (20). However, the translated complementary DNA sequence completely coincides with a part of the sequence of unknown cDNA clones reported from Glycine max (GenBank accession no. A1416520), which is assumed to encode a vicilin-like protein similar to that reported from peanuts (21). When the soybean varieties lacking this 28 kDa allergen were screened in the Japanese stock cultures and imported soybean seeds, about 80% of varieties examined were shown to lack...
the allergen, Gly m Bd 28K (Takahashi M. personal communication). The SPI prepared from defatted soybean flakes (IOM) was shown to contain this allergenic protein and the processed foods with plant proteins as ingredients (SPI) were also demonstrated to contain Gly m Bd 28K as well as Gly m Bd 30K (22).

3) Gly m Bd 60K (α subunit of β-conglycinin)

The other allergenic protein in the 7S-globulin fraction, which was recognized by about 25% of sera from soybean-sensitive patients with atopic dermatitis, was identified as an α subunit of β-conglycinin (23). The IgE antibodies recognizing the α subunit showed no cross-reactivity against either α' or β subunit of β-conglycinin known to be highly homologous to α subunit. α Subunit of β-conglycinin is a glycoprotein with the molecular weight of 57,000, and with pI of 4.90 (24). The amino acid sequence of the precursor deduced from the cDNA consisted of 543 amino acid residues (25). The epitope(s) of the IgE antibodies were shown to be located on the peptide of 232-383 residue from N-terminal, which is highly homologous to α' subunit and phaseolin, a storage protein of Phaseolus vulgaris (23) (Fig. 3).

4) Other allergenic proteins in soybean

Soybean low molecular weight proteins identified as allergens eliciting Barcelona asthma by Rodrigo et al. (9) were identified as Gly m 1.0101 (Gly m 1A) and Gly m 1.0102 (Gly m 1B) which are isofoms with different molecular weights of 7,500 and 7,000, respectively (26). Their amino acid sequences are well matched to a part of the hydrophobic protein first reported by Odani et al. (27), which is synthesized in the endocarp on the inner ovary wall and is deposited on the seed surface during development of soybeans (28). Patients with Barcelona asthma have specific IgE antibodies for this unique glycoprotein located in a part of the hulls of the grain surface. Several reports on glycinin as allergens could be found, and acidic subunits A1a, A1b, A2, A3 and A4 were identified to be allergenic (31). The IgE epitope on the acidic chain of glycinin Gl is located on 192-306 amino acid residue (32). KSTI was first identified as a soybean allergen using sera of patients with asthma working in laboratory and being sensitized through the air way by dealing with a fine powder of KSTI as a reagent (3), and also causing sensitization of occupationally exposed bakers (33). We examined sera of the patients and found a few patients have IgE against KSTI (frequency of sensitization; about 1.5% in soybean-sensitive patients with atopic dermatitis (1)).

Development of hypoallergenic soybean products

1) Chemical breeding

A new soybean line (Glycine max Tohoku 124) lacking the α subunit of β-conglycinin was induced by irradiation with 20 kR (1.0 kR/h) gamma-ray to Kariki 434 with a marked decrease in the level of the α-, α'- and β-subunits of β-conglycinin (34). The SDS-PAGE pattern of protein fraction of Tohoku 124 indicates that the seeds lack one of the major allergens, the α subunit of β-conglycinin (Fig. 4). Recently, it was confirmed that this mutant Tohoku 124 also lacks another major allergen, Gly m Bd 28K, together with the α subunit of β-conglycinin from a result of the immunoblotting analysis using monoclonal antibody C5 (19) specific to Gly m Bd 28K as shown in Fig. 3 (35). This fact indicates that an application of Tohoku 124 for processing of soybean products is beneficial for developing hypoallergenic soybean products because of the absence of the two major
allergens, Gly m Bd 28K and 60K, in advance. However, a mutant lacking Gly m Bd 30K could hardly be found even by screening the soybean varieties and mutants available in the stock culture of the soybean breeding laboratory of Tohoku National Agricultural Experiment Station (Takahashi M, personal communication).

2) Physicochemical approach

Heat treatment is a general method in food processing and induces denaturation of protein structures. Epitope structures of most allergens are, however, assumed to be sequential, so that the reduction of allergenicity due to heat denaturation would not be expected. It has been reported that the IgE-binding activity of Gly m Bd 30K is remarkably enhanced by an autoclave treatment (36). As a unique technique of the hypoallergenic process, the selective removal of Gly m Bd 30K from soy milk or defatted soy milk by centrifugation under a specified condition had been achieved. The selective removal of Gly m Bd 30K was dependent on the unique characteristic of solubility different from those of the major storage proteins, glycinin and β-conglycinin. In the case of non-defatted soy milk, about 90% of Gly m Bd 30K could be removed into the oil pad layer formed by centrifugation in the presence of reducing agents (37). In the case of defatted soy milk, about 97% of Gly m Bd 30K could be removed as the precipitate in the presence of a reducing agent (10 mM sodium bisulfite) under the specified condition (1 M Na2SO4 in acidic pH of 4.5). The major storage soybean proteins, both glycinin and β-conglycinin, remained in the supernatant after centrifugation (38). A small amount of Gly m Bd 30K, however, could not be removed from supernatant in the absence of reducing reagents. It indicates a possible formation of disulfide linkage between Gly m Bd 30K and α (α') subunit of β-conglycinin (39). This hypothesis was proved by the following fact. By using a mutant soybean Tohoku 124 lacking the α and α' subunit, the removal ratio of Gly m Bd 30K from defatted soy milk was improved to 99.8% from 97% (normal soybean) without addition of reducing agents for reductive cleavage of disulfide linkage between Gly m Bd 30K and the α or α' subunit of β-conglycinin (39). Accordingly, as a result of combination of an application of Tohoku 124 and a physicochemical procedure, the substantially complete removal of the three major allergenic proteins (Gly m Bd 30K, α subunit of β-conglycinin, and Gly m Bd 28K) from defatted soy milk was attained (38) (Fig. 5). The average removal rate of the three allergens was attained to almost 99.9% based on the results of the densitometric measurement of ECL immunofluorescent intensity on X-ray film (Fig. 5). Since these procedures for reduction of allergenicity do not include the methods of modifying protein structures, especially digestive cleavages, the processing functionality of soybean storage proteins could not be changed to be applicable for making the traditional soybean products, for example, Tofu (soybean cake) and Ganmodoki (cooked soybean cake).

3) Enzymatic digestion

An enzymatic treatment of whole soybean seeds effectively reduces the allergenicity. Autoclaved soybean was treated by certain proteases from Bacillus sp. at 37°C for 20 h, which is the same condition as that of the fermentation procedure for Natto with Bacillus natto, the Japanese traditional fermented food (40). The product has a Natto-like texture while it has no Natto-like flavor or taste (plain). When the residual allergens were examined by the immunoblot and ELISA (ELISA inhibition), the product showed no binding activity against monoclonal antibody F5 and patients’ sera and all the proteins in the enzyme-treated soybean grains were hydrolyzed into the peptides with molecular weights of less than 10,000 (Fig. 6). Miso (fermented soybean paste) also showed no residual immuno-react-
Fig. 5. SDS-PAGE and immunoblotting patterns of defatted soy milk. Defatted soy milk with 1 M Na$_2$SO$_4$ without reducing agents was centrifuged to precipitate Gly m Bd 30K. The supernatant was treated with SDS-PAGE sample buffer and then run on a 12% gel for CBB staining (A) and for immunostaining with a monoclonal antibody C5 specific to Gly m Bd 28K (B) and for immunostaining with a monoclonal antibody F5 specific to Gly m Bd 30K (C). Lane 1, IOM soybean; lane 2, Tohoku 124. (adapted from ref. 35).

Fig. 6. Hydrolysis of soybean proteins by Protease N. Soybean grains soaked in water overnight were autoclaved at 121°C for 20 min. Ten milliliter of protease solution per gram of soybean (dry weight basis) was added to soybean grains, which was incubated for 20 h at 37°C with gentle shaking. Lanes 1, control (0 unit); 2, 1×10$^3$ units; 3, 5×10$^3$ units; 4, 25×10$^3$ units; 5, 12.5×10$^4$ units; M, molecular marker proteins. (adapted from ref. 40).

4) Chemical modification

An attempt to mask the allergenic site of soybean proteins using the Maillard-type polysaccharide conjugation was examined. Acid-precipitated soybean proteins (APP) and galactomannan mixed in weight ratio of 1 : 5 were dissolved in water at 10% (W/V) and freeze-dried. Maillard reaction was then induced at 60°C under 79% relative humidity (RH) in a desiccator for several days. The allergic potential of soybean like cheese but not Tofu, and no bitter taste which generally appears in hydrolyzates. They also managed to produce the hypoallergenic Tofu-like-textured food by use of a coagulant (e.g. polysaccharide) and an enzyme-treated hypoallergenic soymilk (37). Recently, a novel hydrolytic processing of soybean proteins was reported (42). Under the limited hydrolytic condition, the selective digestion of β-conglycinin (but not glycinin) was attained. The key point of selective digestion is based on the different denaturation temperatures between β-conglycinin and glycinin at neutral pH. The digestion of denatured soybean proteins could proceed more rapidly than that of native proteins with proteases at 70°C. Among Bacillus proteases used for the treatment, Prolateather FG-F (Amano Pharmaceutical Co.) was found to be effective for the selective hydrolysis of Gly m Bd 30K as well as β-conglycinin. The product obtained was proved to lose its reactivity against the monoclonal antibody specific to these two allergens and sera of soybean-sensitive patients. As a result of the treatment with proteases under optimum condition, the three major allergens could be digested. The product containing glycinin showed the processing functionality such as gelation to produce Tofu and emulsification activity remained intact (42).
proteins can be reduced by the conjugation of galactomannan residues to APP (43).

5) Extrusion cooking

Ohishi et al. (44) reported that antigenicity of soybean meal against calves’ sera was reduced to 0.1% of the original activity by an extrusion cooking with screws containing kneading-disc elements and die-end temperatures exceeding 66°C. SDS-PAGE analysis of the cooked meal indicated that the reduction of antigenicity was due to destruction or modification of protein molecules.

Evaluation of hypoallergenic soybean products and perspectives

Hypoallergenic soybean products have been developed and subjected to the evaluation of usefulness under the observation of physicians and dieticians. In vitro examination of IgE binding activity was done by an extrusion cooking with screws containing kneading-disc elements and die-end temperatures exceeding 66°C. SDS-PAGE analysis of the cooked meal indicated that the reduction of antigenicity was due to destruction or modification of protein molecules.

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Soybean Allergens

Gly m Bd 30K with alpha and alpha'-subunit of beta-


