

## Reduction of the Allergenicity of Soybean by Treatment with Proteases

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**Summary** Soybeans were treated with proteases to reduce allergenicity. By immunoblotting with a monoclonal antibody against a major soybean allergen (*Gly m Bd 30K*), two of eight proteases so far tested were selected to achieve a reduction in allergenicity. Both antigenicity to the monoclonal antibody and allergenicity to the sera from soybean-sensitive patients proved to be markedly reduced by processing with either protease. Thus, soybeans treated with an appropriate protease may possibly be supplied as a hypoallergenic foodstuff for patients.

**Key Words** soybean, allergenicity, *Gly m Bd 30K*, protease, reduction, monoclonal antibody

Soybean is an allergenic food which can sensitize individuals through either an airway or oral pathway. Several components of soybean have been reported to date for inhalant (1-3) or dietary allergens (4-6). In respect to soybean-sensitive Japanese patients, who are thought to have been sensitized by the oral route, a protein with a molecular weight of 30,000 was a major allergenic protein for which 65% of the sera contained specific IgE (6). This allergen, named *Gly m Bd 30K*, was demonstrated to be identical to the 34 kDa oil-body-associated protein stored in the vacuoles of soybean cotyledons (7).

Recently, monoclonal antibodies against *Gly m Bd 30K* were prepared and applied for detecting or quantifying the allergen in soybean-related products (8,9). The sandwich enzyme-linked immunosorbent assay and immunoblotting using these antibodies revealed that the fermented products of soybean contained too little *Gly m Bd 30K* to detect (9). Especially in the experiments with both monoclonal antibody and patient's serum, the Japanese traditional fermented

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soybean food "natto" was demonstrated to be a hypoallergenic soy product; its allergenicity having been considerably reduced by the action of secretory proteases from *Bacillus subtilis* (10).

In this report, we treated soybean proteins with various proteases and monitored alterations in their antigenicity to the monoclonal antibody or allergenicity to patients' sera for the purpose of developing hypoallergenic soybean foods.

#### MATERIALS AND METHODS

**Materials.** The monoclonal antibody (F5) against Gly m Bd 30K was prepared as described previously (8). The proteases used in this experiment were as follows: Newlase F from *Rhizopus niveus*; Pancreatin from porcine pancreas; Protease N, Protease S and Proleather from *Bacillus subtilis*; Protease A and Protease M from *Aspergillus oryzae*; and Protease P from *Aspergillus melleus*. The proteases were provided by Amano Pharmaceutical Co. (Nagoya, Japan). The activities of these proteases were assayed as described in the manufacturer's instructions, and each unit was defined as the amount of enzyme capable of generating a 100  $\mu$ g tyrosine equivalent of trichloroacetic acid-soluble fraction for 60 min. Peroxidase-conjugated sheep anti-mouse IgG was purchased from Cappel (Durham, NC, USA). Anti-Gly m Bd 30K human serum was donated from patients with atopic dermatitis, who were diagnosed to be soybean-sensitive by the radioallergosorbent test. The CAP RAST RIA kit was purchased from Pharmacia Diagnostics AB (Uppsala, Sweden).

**Proteolysis of soybeans.** Soybeans soaked in water overnight were autoclaved at 121°C for 20 min. Ten milliliters of protease solution per gram of soybean (on a dry weight basis) was then added and incubated at 37°C for 20 h with gentle shaking.

**Extraction of soybean proteins.** The soybeans were homogenized with 50 mM sodium phosphate buffer (pH 6.8) containing 4% sodium dodecylsulfate (SDS) and 20 mM 2-mercaptoethanol (5 ml per gram of soybean). The homogenate was boiled for 5 min and centrifuged at 30,000  $\times g$  for 20 min. The precipitate was similarly reextracted with the same phosphate buffer. The supernatants were then combined and subjected to the following experiments.

**Calculation of protein equivalent contents.** The nitrogen content in the extract was determined according to the AOAC method (11). The protein equivalent content was calculated by multiplying the nitrogen content by 5.71 (i.e., the nitrogen-protein conversion factor for soybean) (12).

**Immunoblotting.** Fifty micrograms of protein equivalent extract from proteolyzed soybeans was separated by means of SDS electrophoresis on 14% polyacrylamide gels (13). The resulting protein bands were transferred electrophoretically onto a nitrocellulose membrane. The membrane was immunoblotted against the monoclonal antibody using peroxidase-conjugated sheep anti-mouse IgG (8) or against the allergic patients' serum using <sup>125</sup>I-labeled anti-human

IgE (6).

## RESULTS

Soybeans merely soaked overnight were fairly resistant to the proteolysis with proteases (data not shown). For this reason, autoclaved soybeans were homogenized and extracted in the above-mentioned manner. The extracts of soybeans which were incubated with a respective protease were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Even after autoclaving, the soyproteins were still resistant to proteolysis with these proteases, but were digested to some extent by Proleather and to a lesser extent by Protease N (Fig. 1A). Similarly *Gly m Bd* 30K was decomposed markedly by Proleather and slightly by Protease N (Fig. 1B). On this account, we selected Proleather and Protease N to develop hypoallergenic soybeans.

Next, we examined the most suitable ratio between these two proteases and soybeans. Soybeans treated with Proleather or Protease N at various ratios were analyzed by SDS-PAGE and Western blotting. The increased ratio of Proleather to soyprotein affected the digestion profile of the soyproteins (Fig. 2A). *Gly m Bd* 30K was decomposed in accordance with the hydrolysis of other soyproteins and could not be detected by immunoblotting analysis using the monoclonal antibody when more than 250 units of Proleather per gram of soybean was used (Fig. 2B). Excess Proleather ( $5.0 \times 10^3$  units/g of soybean) produced no additional changes. Similar to the case of Proleather, an increased ratio of Protease N elevated the hydrolysis of soyproteins (Fig. 3A). *Gly m Bd* 30K could no longer be detected by the use of monoclonal antibody when more than  $5.0 \times 10^3$  units per gram of soybean was used (Fig. 3B). Excess Protease N ( $125 \times 10^3$  units/g of soybean) did not

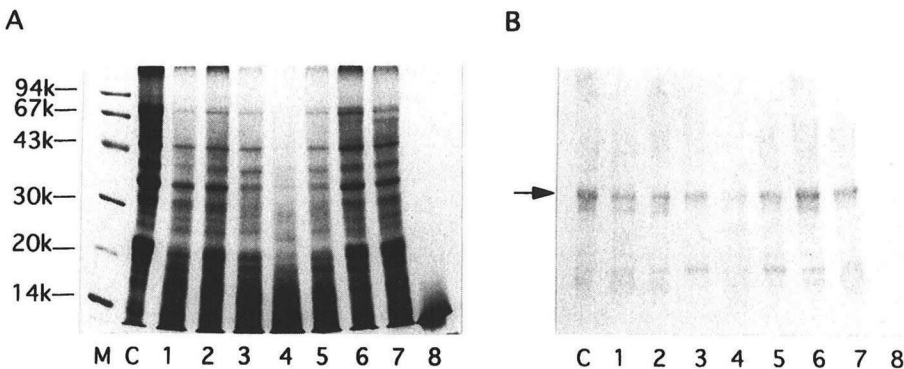


Fig. 1. Hydrolysis of soybean proteins by various proteases. M, molecular weight markers; C, no protease (control); 1, Newlase F; 2, Protease M; 3, Protease A; 4, Protease N; 5, Protease P; 6, Protease S; 7, Pancreatin; 8, Proleather. A. Stained with Coomassie brilliant blue; B. Detected with anti *Gly m Bd* 30K monoclonal antibody (F5) and HRP-conjugated anti mouse IgG.

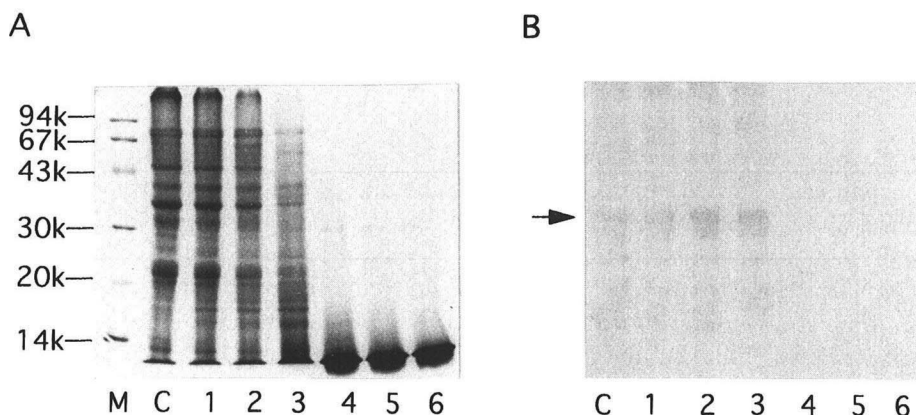


Fig. 2. Hydrolysis of soybean proteins by various ratios of Proleather. M, molecular weight markers. The following amount of Proleather was used per g of soybean: C, 0 (control); 1, 2 units; 2, 10 units; 3, 50 units; 4,  $25 \times 10^2$  units; 5,  $10 \times 10^2$  units; 6,  $50 \times 10^2$  units. A. Stained with Coomassie brilliant blue; B. Detected with anti *Gly m Bd 30K* monoclonal antibody (F5) and HRP-conjugated anti mouse IgG.

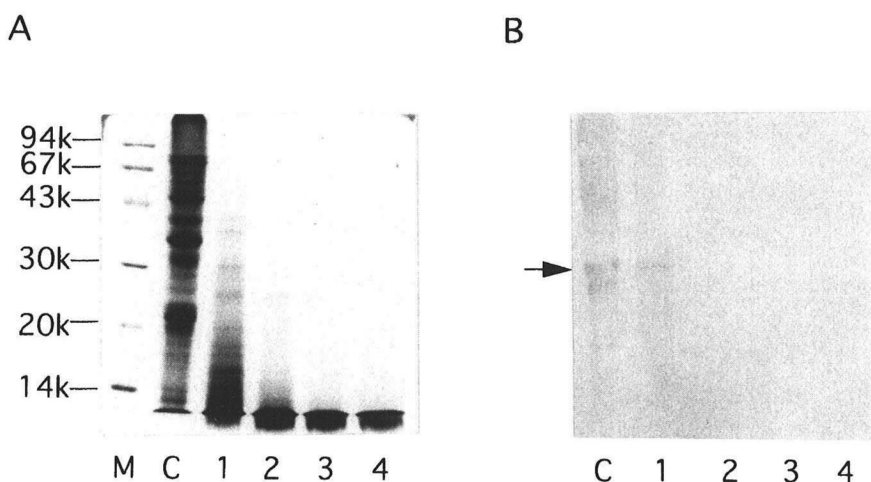


Fig. 3. Hydrolysis of soybean proteins by various ratios of Protease N. M, molecular weight markers. The following amount of Protease N was used per g of soybean: C, 0 (control); 1,  $1.0 \times 10^3$  units; 2,  $5.0 \times 10^3$  units; 3,  $25 \times 10^3$  units; 4,  $125 \times 10^3$  units. A. Stained with Coomassie brilliant blue; B. Detected with anti *Gly m Bd 30K* monoclonal antibody (F5) and HRP-conjugated anti mouse IgG.

induce further changes in the digestion profile.

Subsequently, the soybeans treated with either 250–1,000 units of Proleather or  $5.0$ – $25 \times 10^3$  units of Protease N were examined for their allergenicity; that is, the

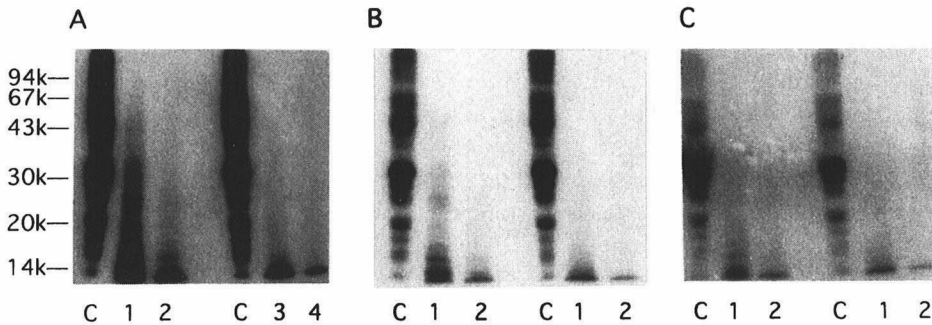


Fig. 4. Changes in allergenicity of hydrolyzed soybean proteins by Proleather or Protease N. The protease and amount (unit/g of soybean) used to hydrolyze soybean proteins are as follows: C, no protease (control); 1, Proleather ( $0.25 \times 10^3$  units); 2, Proleather ( $1.0 \times 10^3$  units); 3, Protease N ( $5.0 \times 10^3$  units); 4, Protease N ( $25 \times 10^3$  units). Detected with patients' sera and  $^{125}\text{I}$ -labeled anti-human IgE. Patients' profile (sex, old and RAST or MAST class for soybean) are as follows: Patient A, male, 23, RAST class 2; Patient B, female, 21, RAST class 3; Patient C, female, 8, RAST class 3.

antigenicity for IgE contained in patients' sera (Fig. 4). In either case, the autoradiographic patterns revealed that the higher the concentration of each protease, the weaker the IgE binding bands became.

#### DISCUSSION

From the eight proteases we tested, we selected Proleather and Protease N, both of which are produced by *Bacillus subtilis*, as the most suitable proteases for alleviating the allergenicity of soybean. As these two proteases were not specific for *Gly m Bd* 30K, hydrolyzing all the proteins in soybean was required for the destruction of this major allergen. In processing soybeans, we monitored only the antigenicity of the major allergen (*Gly m Bd* 30K) using its specific monoclonal antibody. Consequently, the protease-treated soybean, where the antigenicity of *Gly m Bd* 30K was not detectable in practice, was lacking in the allergenicity to the patients' sera, which could detect some other proteins as well as *Gly m Bd* 30K. Thus, the protease-treated soybean we prepared this time may be appropriate as a hypoallergenic and safe food for the many patients who are sensitive to soybean. In addition, the protease-treated soybean has no peculiar flavor or taste as opposed to most fermented products. Thus, we intend to utilize the protease-treated soybean as a foodstuff for "boiled beans."

Several hypoallergenic foods are commercially available today. For example, in Japan, we can purchase hypoallergenic infant formula and hypoallergenic rice at the market. Such foods are beneficial to patients who are allergic to these foods. Soybean is not always a common allergenic food in the western world. In Japan,

however, soybean is considered to be a member of three major allergenic foods with a high frequency of occurrence. Thus, the development of hypoallergenic soybean foods is meaningful in this country. In the western world, it has been reported that an allergy to soybean occurs when soya milk has been used as a substitute for cow's milk (14). If people in these areas began to ingest soyproteins more often, the number of allergic patients sensitized by soybean would increase.

The two proteases we selected were effective in hydrolyzing autoclaved soybeans, although not so effective for merely soaked soybeans. When these enzymes were added to extract protein from soybean, it was possible to hydrolyze *Gly m Bd 30K* as well as other proteins in soybean (data not shown). This suggests that some structural barrier of the intact soybean inhibited the penetration of protease into the soybean cotyledons. If it were possible to remove such barrier, the intact soybean could be treated as a modifying target to reduce allergenicity.

Of course, to thoroughly evaluate the reduction of allergenicity, skin and oral challenge tests are necessary. The oral challenge test of the protease-treated soybean is now in progress.

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