

Genetically Modified and Wild Soybeans: An Immunologic Comparison

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ABSTRACT

Most traits introduced into genetically engineered crops result from the expression of new proteins. As the first step toward assessing the allergenic potential of genetically modified organism (GMO) food, immunologic and physicochemical characterizations are needed. We prepared crude extract from GMO soybeans, wild soybeans, curd, and soy milk and then performed sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After acidification with HCl, the samples were separated to globulin and whey. To evaluate changes in protein composition, either the samples were heated or pepsin was added. Polymerase chain reaction with primer encoding the 35S-promotor and the 3-enol-pyruvyl-shikimat-5-phosphat-synthase gene were performed, respectively, to detect the GMO component. SDS-PAGE results showed definite protein bands at 80 kDa in GMO soybean, 50 kDa in wild soybean, and a similar distribution of protein bands was noticed below 40 kDa. It was difficult to observe protein distribution because of modifications that occurred during processing in soybean-processed products. After heating, proteins of GMO and wild soybeans showed similar distributions and no distinct bands

were detected at 50 and 80 kDa. Although SDS-PAGE analyses of raw GMO and wild soybeans differed, the same protein bands of 68, 37, and 20 kDa were observed in the globulin fraction after acidification. After adding pepsin, 20- and 68-kDa bands were found preserved in GMO and wild soybeans. The polymerase chain reaction procedures with primers specific to GMO soybeans showed that GMO soybeans and some curd samples included a GMO component. The skin test results of 49 patients showed 13 positive results to wild soybeans and 8 positive results to GMO soybeans. One patient had a positive skin test result to GMO soybeans only. Sera from nine patients with positive skin tests to the crude extract and a positive capsulated allergen product test to the soybean antigen were used for the immunoblotting of GMO and wild soybeans. GMO soybeans revealed a unique strong immunoglobulin E binding band at 25 kDa in some patients and wild soybeans showed a strong immunoglobulin E binding band at 30–36 kDa. To assess the allergenicity of GMO food, more research, including a selection of controlled sample materials and immunoassays of qualified sera, is needed. (Allergy and Asthma Proc 26:210–216, 2005)

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After successfully achieving the genetic control of plant inheritance, more genetically engineered plant products are being encountered in the marketplace. Traits being introduced into these crops include insect protection, delayed ripening, virus resistance, modified starch makeups, herbicide tolerance, modified oil contents, disease resistance, male sterility, and many others.¹ Before market introduction, these genetically modified organisms (GMOs) must undergo a safety assessment. In 1990 the first joint consultation between the Food and Agriculture Organization and the World Health Organization concluded that the safety assessment of new foods from genetically modified plants should be based on the principles of “Substantial Equivalence,” which entails considerations of the molecular

characterization of the GMO, its phenotypic characteristics, and its key compositional components, such as nutrients and the presence of any toxicants.²

Food allergy is a group of disorders characterized by abnormal or exaggerated immunologic responses to specific food proteins, which result in a variety of symptoms. Of the general population 1.5% experience food allergic disorders³; thus, an agreed method of assessing the allergenic potential of a new protein in GMOs is needed. Racial and cultural backgrounds also may influence the prevalence of food allergy and offending food listings. Soybeans are an economically important crop in Asian countries, and the inflow of GMO soybeans continues to increase. As a first step toward assessing the allergenicity of GMOs, we compared GMO soybeans and wild soybeans in terms of their immunologic and physicochemical characteristics and then quantified specific immunoglobulin E (IgE) and performed immunoblotting in sensitive patients.

MATERIALS AND METHODS

Production of Crude Extracts

Wild soybeans were obtained from the Department of Seed Distribution at the National Seed Management Office (Anyang, Korea) and GMO soybeans were chosen from among GMO soybeans introduced into Korea. Soybean-processed foods, including tofu and soy milk, were purchased locally. All of these materials were finely ground in liquid nitrogen and stirred for 18 minutes in phosphate-buffered saline (PBS; 137 mM of NaCl, 1.8 mM of KH_2PO_4 , 10 mM of Na_2HPO_4 , and 27 mM of KCl, pH 7.4). The supernatant was obtained by centrifugation at 15,000 rpm for 30 minutes and dialyzed for 3 days using a 3500-mol wt dialysis membrane, freeze dried, and kept at -20°C until required.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

According to Laemmli's method,³ each sample was dissolved in loading buffer (60 mM of Tris-HCl, 25% glycerol, 2% sodium dodecyl sulfate [SDS], 14.4 mM of 2-mercaptoethanol, and 0.1% bromophenol blue) and reacted in boiling water for 5 minutes. Samples then were separated on 13.5% gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein bands were stained with coomassie blue. The distributions of protein antigens in the different samples were compared.

Heat Treatment

After boiling, 20 g of GMO soybeans or wild soybeans for 30 minutes at 100°C , the samples were mixed with PBS buffer at a 1:10 ratio, homogenized, and then centrifuged for 20 minutes at 15,000 rpm. The supernatant obtained was purified by dialysis, freeze dried, and kept at -20°C until required. Then, the prepared crude antigen was diluted in distilled water, to a concentration of 1 mg/mL;

loading buffer was added; and the mix was heated for 5 minutes. Samples then were loaded into 5 and 13.5% SDS-PAGE. The isolated gel containing the protein antigen was stained with Coomassie blue and analyzed.

Enzyme Treatment

After grinding, 50 g of GMO soybeans and wild soybeans, using liquid nitrogen, PBS buffer was added to the ground soybean samples. The mixture was homogenized and then centrifuged for 20 minutes at 15,000 rpm, to collect the supernatant, which was dialyzed further, freeze dried, and kept at -20°C until required. The crude antigen prepared according to the foregoing method was diluted in distilled water to a concentration of 3 mg/mL; 10,000 U of pepsin solution (pH 4.0) (Sigma, St. Louis, MO) were then added at a ratio of 1:18; and digestion was continued for 0, 5, 15, 60, and 120 minutes. The digestion was stopped by adding Na_2CO_3 and SDS-PAGE was performed.

Polymerase Chain Reaction

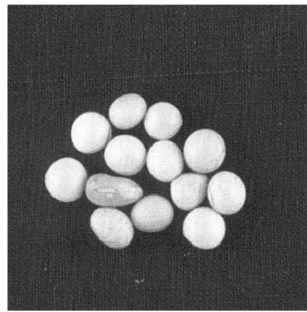
DNA was extracted from 0.5 g of the ground samples using extraction buffer (700 mM of NaCl, 50 mM of Tris-HCl, pH 8.0, 10 mM of ethylenediaminetetraacetic acid, 1% 2-mercaptoethanol, and 1% hexadecyltrimethylammonium bromide; Sigma). The absorbances of the extracted DNA samples were measured using a spectrophotometer (UV-1601PC; Shimadzu, Tokyo, Japan) at 260/280 nm to determine purity. The purest DNA sample was selected for study. Polymerase chain reaction (PCR) was performed using pro-3s1, pro-3s2 to amplify the promoter 35S regions and a primer specific for the recombinant soybean gene, 3-enolpyruvyl-shikimate-5-phosphate-synthase (EPSPS), and the extracted DNA templates. PCR was performed by predenaturation for 3 minutes at 94°C , followed by 40 cycles of denaturation for 30 seconds at 94°C , annealing for 30 seconds at 57°C , and synthesis for 30 seconds at 72°C . Extension was performed for 5 minutes at 72°C . PCR was performed after distributing the template DNA into a PCR kit (Takara, Osaka, Japan). Results of PCR were observed under UV after electrophoresis in 1.5% agarose gel.

Skin Testing

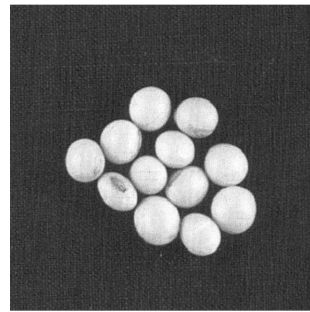
Skin-prick testing was performed on 49 atopic children who visited the pediatric allergy clinic at Severance Hospital, Yonsei University, and Ajou University, using 60 different antigens, including soybeans (Tori, Tokyo, Japan). The sum of two perpendicular diameters was calculated for each wheal after 20 minutes. Wheal sizes of ≥ 3 mm after subtraction of the control were considered positive.

Capsulated Allergen Product (CAP) Test

CAP testing was performed on the antigens of dust mites, egg whites, buckwheat, peanuts, soybeans, and shrimp in the sera of skin-tested children by using a UniCAP kit (Pharmacia & Upjohn, Uppsala, Sweden).



WILD Korean soybeans



GMO soybeans

Figure 1. Wild and GMO soybeans.

IgE Immunoblotting

After SDS-PAGE, bands were transferred to a nitrocellulose membrane for 80 minutes at 350 mA and reacted in 5% bovine serum albumin for 1 hour to block nonspecific protein binding. Based on the results of skin and CAP testing, pooled sera were prepared by mixing the sera from sensitive children. This pooled serum was diluted 1:4, reacted for 18 hours at 40°C, and diluted 1:2000 in goat anti-human IgE conjugated to alkaline phosphatase at room temperature for 1 hour. Color was developed by adding nitroblue tetrazolium/bromochloroindolyl phosphate.

RESULTS

Preparation of Crude Antigen and SDS-PAGE of Soybeans and Soybean Product Proteins

Glycin max is the most common species in Korea and is a major source of variable soy-processed products. Grossly, it was difficult to differentiate between wild soybeans and GMOs (Fig. 1). A Bradford assay using PBS buffer (pH 7.4) was performed to confirm the protein concentrations of each sample. The results showed that the protein concentrations of GMO soybeans, wild soybeans, tofu (P Co. and H Co.), and soy milk (S Co. and D Co.) were 530, 620, 260, 230, 120, and 100 ug/mL, respectively. SDS-PAGE performed on GMO soybeans and wild soybeans commonly showed 10 protein bands, between 10 and 80 kDa. GMO soybeans, e.g., revealed a distinct protein band at 80 kDa, and wild soybeans revealed a distinct protein band at 50 kDa. Below 40 kDa, these two samples showed similar protein band distributions (Fig. 2). The determination of accurate protein distributions was difficult in the soybean products because the protein compositions were altered by processing (Fig. 3).

Comparison of the Physically Treated GMO Soybeans and Wild Soybeans

When proteins were extracted from the soybean samples after boiling for 30 minutes, to duplicate the commercial heat treatment process, the protein distributions

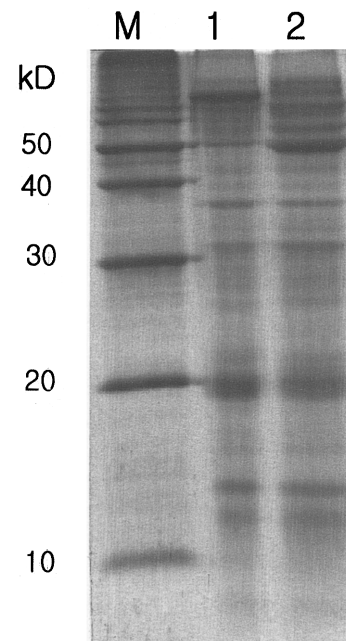


Figure 2. SDS-PAGE of crude soybean extracts. Lane 1 shows crude extracts from GMO soybeans and lane 2 shows crude extracts from wild soybeans (M, molecular weight marker).

of the heat-treated wild soybeans and the GMO soybeans were similar, and neither showed protein bands at 50 and 80 kDa, as shown by raw samples (Fig. 4).

Comparison of Chemically Treated GMO Soybeans and Wild Soybeans

After acidification of the protein solution using 0.5 N HCl to a pH of 4.6, the whey fraction and the globulin fraction were separated and compared. We found a distinct protein band at ~20 kDa in both the wild soybeans and the GMO soybeans in the whey and globulin fractions. Furthermore, both samples showed a protein band at 68, 37, and 20 kDa in the globulin fraction, whereas the whey fraction had bands in the range of ~20–37 kDa.

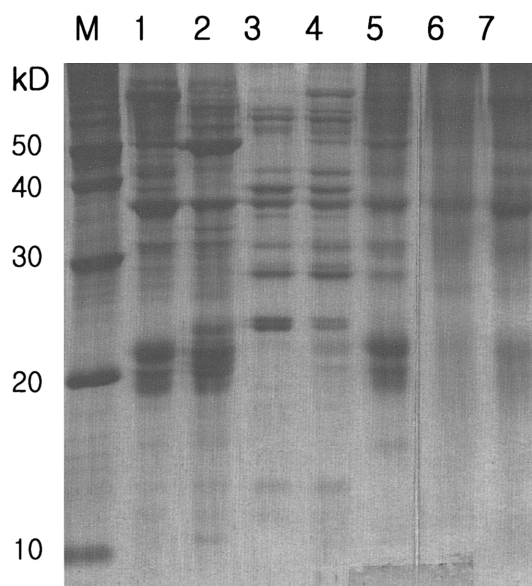


Figure 3. SDS-PAGE of soybean extracts and extracts of its products. Lane 1 shows GMO soybeans; lane 2 shows wild soybeans; lane 3 shows Tofu A; lane 4 shows Tofu B; lane 5 shows skin test extract from T company; lane 6 shows soy milk A; lane 7 shows soy milk B (M, molecular weight marker).

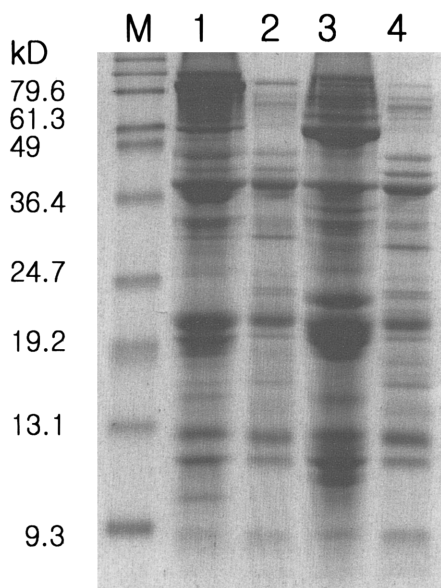


Figure 4. SDS-PAGE of soybeans after heating. lane 1, shows GMO soybeans; lane 2 shows GMO soybeans after heating; lane 3 shows wild soybeans; lane 4 shows wild soybeans after heating (M, molecular weight marker).

When wild soybeans and GMO soybean protein solutions were treated with pepsin, the overall protein concentrations were markedly reduced because the proteins were partially digested, suggesting the possibility that the allergen structure had changed somewhat or was dissociated, thus affecting the allergic reaction (Fig. 5).

Confirmation of GMO Gene Contamination Within the Soybean Products

After preparing PCR primers to amplify the 35S promoter region, which is commonly shared by gene-modified food products, amplification was confirmed. We found that in the GMO soybean sample a 195-bp fragment was amplified, which corresponds to the 35S promoter region, especially in one of the tofu samples that was sold unwrapped (Fig. 6).

On the other hand, we analyzed for GMO in each product by amplifying with a primer specific for the recombinant soybean gene, EPSPS. This analysis showed that the GMO soybean, *e.g.*, had a 570-bp fragment corresponding to the EPSPS gene. The EPSPS gene was found also to be present in one tofu sold unwrapped (Fig. 7). According to the results of two tests, we found that GMO is present in GMO soybeans and in one commercially available tofu sample.

Skin Testing

Skin testing was done using 60 antigens, including those of soybeans, in 49 children with allergies who presented to the Pediatric Allergy Clinic at Severance Hospital and Ajou University Hospital. The results showed that 13 children reacted to wild soybeans and 8 children reacted to GMO soybeans. Of those who showed a positive reaction, one child showed a positive reaction only to GMO.

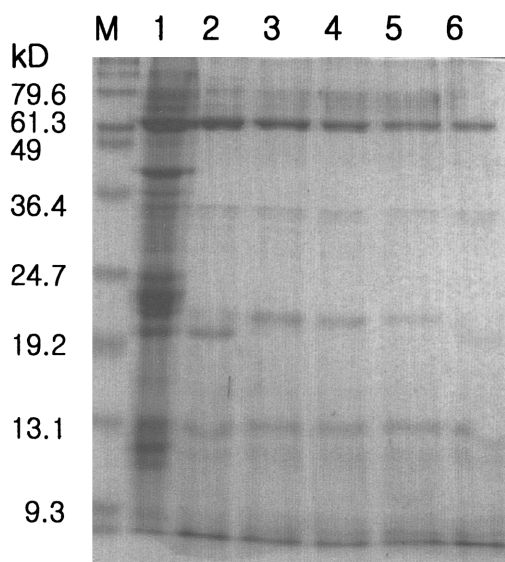
CAP Testing

CAP, using a UniCAP kit, was used to determine the concentration and degree of specific IgE to specific antigen. These were rated as absent, low, moderate, high, and very high.

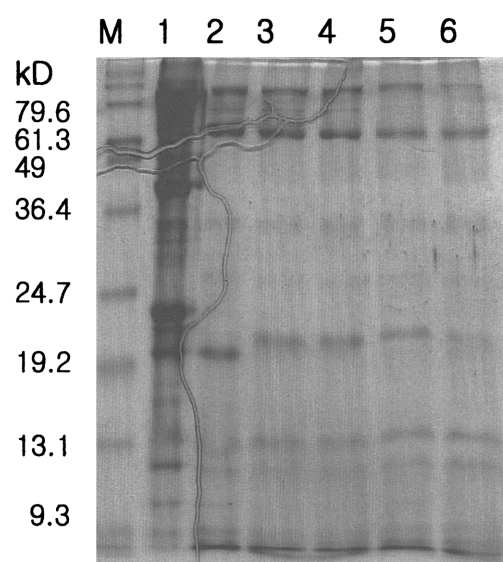
Among the various allergens that caused an allergic skin reaction, specific IgE was measured in children by selecting only six typical allergens (dust mites, egg whites, buckwheat, peanuts, soybeans, and shrimp). In 30 of the 41 children, sera elicited a positive reaction, and among these 30 children, 19 showed a reaction to one or more allergens. Specific IgE to soybean allergen was identified in nine children with a distribution of specific IgE to soybeans between low and moderate.

IgE Immunoblotting

Based on the results of skin testing and CAP testing performed in sera obtained from children who visited the allergy clinic, we found that the sera of nine children showed a positive result to soybeans. Two pooled sera were prepared by mixing these sera. Pooled serum 1 was comprised of the sera of five patients <2 years old with atopic dermatitis or food allergy, and pooled serum two of four patients >2 years old with respiratory allergy (Table I). Two sera were reacted with the proteins present in wild soybeans and GMO soybeans to observe the IgE binding protein bands. The protein bands that reacted with sera were mainly between 20 and 65 kDa, and protein bands of wild



A



B

Figure 5. SDS-PAGE of soybeans after pepsin treatment. Lanes 1–6 show soybeans digested with pepsin for 0, 5, 30, 60, 120, and 180 minutes (M, molecular weight marker; panel A, wild soybeans, and panel B, GMO soybeans).

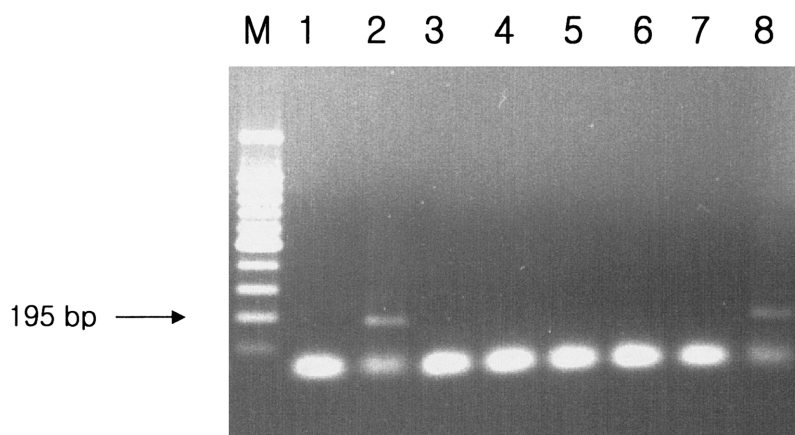


Figure 6. Detection of promotor 35s amplified by PCR in soybean extracts and in extracts of its products. Lane 1 shows soybeans without CP4; lane 2 shows soybeans inserted CP4; lane 3 shows wild soybeans; lanes 4–8 show different types of tofu (M, 100-bp DNA ladder).

soybeans and GMO soybeans were commonly observed at ~200–25, ~30–36, and ~50–65 kDa. GMO soybeans uniquely showed a strong protein band, which reacted with IgE, at ~25 kDa in lane 1 but not in lane 2 (Fig. 8). Wild soybeans exhibited a moderately strong protein band of ~30–36 kDa, which reacted with IgE, in lane 1 (Fig. 9).

DISCUSSION

The genetic manipulation of crops presents risks in terms of the unintentional introduction of allergens into food. An example of the introduction of new allergens into food caused by a change in a recipe was provided by the Brazil nut allergen in transgenic soybeans.⁴ Although, the nutritional quality of soybeans was improved by the intro-

duction of the methionine-rich 2S albumin of the Brazil nut, the introduced allergen caused IgE responses in sensitive individuals.

Soybeans are consumed widely in Asian countries, including Korea, and over 20 GMO soybeans have been developed. Soybeans contain multiple allergens, *e.g.*, Gly m 1 (a 30-kDa protein), a minor constituent of the 7S globulin fraction that also is a major allergen, a 68-kDa allergen (the α -subunit of β -conglycinin), and a KST1 20-kDa protein.⁵ However, the prevalence of soybean allergy is not known with precision. In general, identified food allergens are water-soluble glycoproteins with molecular weights ranging from 5 to 60 kDa and are stable to heat, acid, and protease treatments.⁶ Epitopes are either conformational or linear.

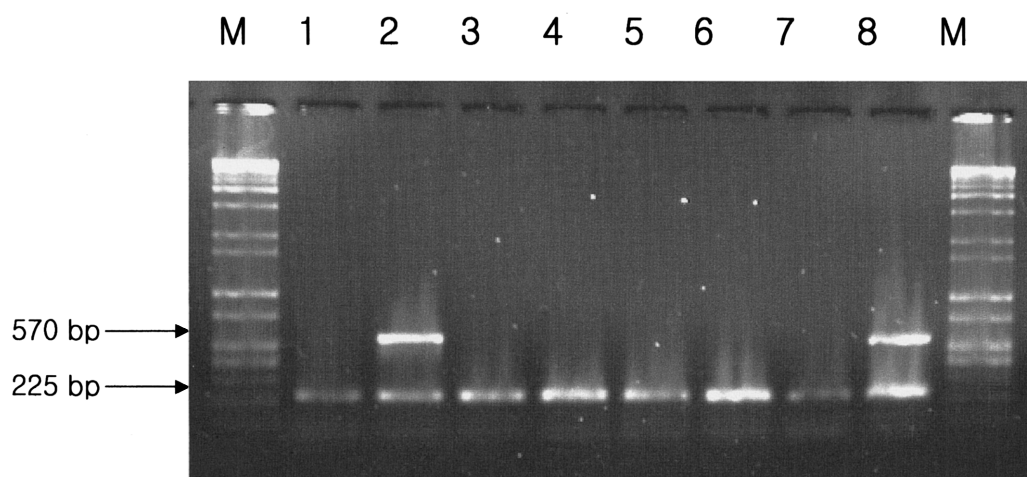


Figure 7. Detection of the CP4 gene amplified by PCR in soybean extracts and in extracts of its products. Lane 1 shows soybeans without CP4; lane 2 shows soybeans inserted CP4; lane 3 shows wild soybeans; lanes 4–8 show different types of tofu (M, 100-bp DNA ladder).

TABLE I

Clinical and Laboratory Profiles of Patients

Patient	Sex	Age	Diagnosis	Total IgE (kU/L)	CAP-soy (kU/L)	CAP-Peanut (kU/L)
Pooled Serum 1						
1	M	9 mon	AD	68	1.14	0.65
2	M	10 mon	AD,FA	1868	2.54	16.4
3	F	1 yr 7 mon	AD	127	0.77	0.65
4	M	11 mon	AD,FA	166	2.02	1.65
5	F	1 yr	AD,FA	1046	19.97	81.3
Pooled Serum 2						
1	F	3 yr 4 mon	AD,AR	55	0.41	0
2	F	2 yr 10 mon	AS	Not done	3.38	1.38
3	F	4 yr 10 mon	AS	670	0.36	0
4	M	9 yr 11 mon	AD,AR	2326	0.48	0

AD = atopic dermatitis; FA = food allergy; AR = allergic rhinitis; AS = asthma.

The generally held notion is that T-cell epitopes are linear, whereas the B-cell epitopes are conformational.⁷

SDS-PAGE of GMO and wild soybeans showed some differences. They both were found to have unique strong protein bands at 80 and 50 kDa, respectively, but after acidification of the crude extracts and fractionation into whey and globulin, no differences in their protein compositions were evident. Bands that were 20, 37, and 68 kDa were found in the globulin fraction after acidification. Moreover, the proteins of most soy products such as tofu and soy milk change during processing, and it is impossible to define the origin of soybeans by SDS-PAGE. Heating caused the unique protein bands at 80 kDa in GMO soybeans and 50 kDa in wild soybeans to disappear from SDS-PAGE (Fig. 4). Thus, if these proteins were allergenic, then heat treatment would have removed their associated allergenicities.

The addition of pepsin weakened most protein bands in both extracts (Fig. 5). Generally, important food allergens

are stable to digestion in gastric fluid. Thus, stability to digestion is a significant and valid parameter for differentiating food allergens and nonallergens.⁸

Although, limited by the various pretreatments and contaminations and by its sensitivity and specificity, PCR remains the most valuable method of identifying GMO crops today.⁹ We used a primer to amplify the 35S promoter and to confirm that a known GMO soybean extract and one of the curd samples were made from GMO products. Trials with the EPSPS insert confirmed these results.

Only eight patients had a positive reaction to GMO soybeans, among the 13 who showed a positive reaction to wild soybeans by the skin test. Only one patient had a positive skin reaction to GMO soybeans but a negative reaction to wild soybeans. When specific IgE immunoblotting was performed using the pooled sera of sensitized patients, protein bands of soybeans that bonded with the sera were found at ~20–65 kDa, whereas wild soybeans and GMO soybeans commonly contain protein bands at

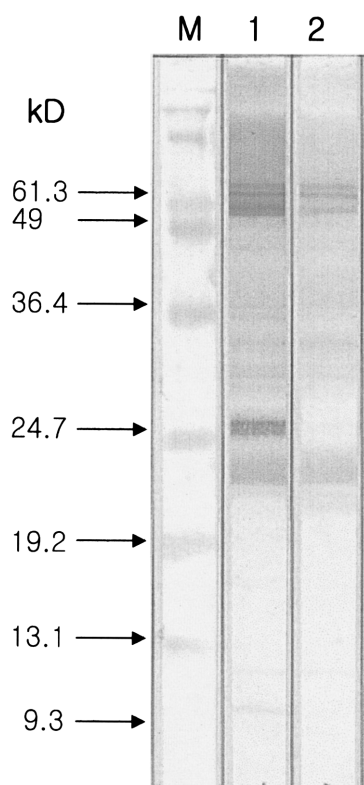


Figure 8. IgE immunoblot of GMO soybeans. Lane 1 shows pooled serum 1; lane 2 shows pooled serum 2 (M, molecular weight marker).

~20–25, ~30–36, and ~50–65 kDa. Wild soybeans showed a strong IgE protein band at ~30–36 kDa in lane 1, which was presumably the band at 34 kDa, a known main soybean allergen. GMO soybeans showed a strong IgE protein band at ~25 kDa in lane 1, but this was not present in lane 2.

We could not conclude that the 25-kDa protein band observed in GMOs on immunoblot is allergenic. Based on the results obtained, we propose two hypotheses. First, the 25-kDa band may be a protein unique to the GMO soybean, an intermediate product in response to the gene recombination process, and that IgE binding may be the result of nonspecific binding. Second, our soybean materials, Glycine max, may evolve differently under different harvesting environments. Therefore, additional studies of materials harvested in controlled environments are necessary.

Our study has some limitations. Its major shortcoming concerns the collection of patients' sera. According to the guidelines recommended by the Food and Agriculture Organization/World Health Organization, >25 serum samples showing high IgE are needed, which is in contrast to the pooled sera used in the present study.¹⁰ In addition, the collection of sera from children who did not have any severe clinical symptoms to soybeans also was a limitation.

In conclusion, to assess the allergenicity of GMOs, further research is needed using a more extensive collection of

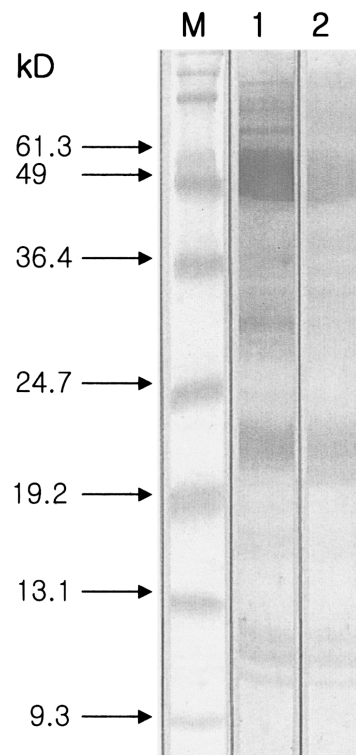


Figure 9. IgE immunoblot of wild soybeans. Lane 1 shows pooled serum 1; lane 2 shows pooled serum 2 (M, molecular weight marker).

patients with a convincing clinical history and using materials prepared in a controlled environment.

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