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Assessment of the Potential Allergenicity of Genetically Modified Soybeans and Soy-based Products

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Abstract A comprehensive safety evaluation was conducted to assess the potential allergenicity of newly introduced proteins in genetically modified (GM) crops. We assessed the allergenicity of CP4 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in GM soybeans. This assessment was performed by IgE immunoblotting with soy-allergic children's sera, amino acid sequence homology with known allergens, and the digestibility of CP4 EPSPS. No differences in IgE-antigen binding by immunoblotting were found between GM soy samples and the corresponding non-GM samples. Based on the comparison of EPSPS amino acid sequence homology with current allergen databases, no known allergen was found. In addition, CP4 EPSPS protein was rapidly digested by simulated gastric fluid (SGF). Taken together, these results indicate that GM soybeans have no allergenicity in children and are as safe as conventional soybeans.

Keywords: allergenicity, 5-enolpyruvylshikimate-3-phosphate synthase, GM soybeans, immunoblotting, simulated gastric fluid

Introduction

Commercially available genetically modified (GM) crops include soybean, maize, cotton, canola, potato, and tomato. GM crops for food use have been evaluated for safety according to each country's safety laws. In most countries, the safety assessment of new foods made from GM crops relies on comparison with a food generally recognized as safe made from the corresponding non-GM crop (1, 2). The detailed safety assessment involves the following 4 steps: characterization of the parent crop; characterization of the transformation process and of inserted recombinant DNA; safety assessment of the introduced gene products; and identification of any other targeted and unexpected alterations in the GM crops (3-5). Among these assessments, food allergy due to introduced gene products is the main safety concern of GM crops. Based on the recommendation of FAO/WHO and the Codex Alimentarius Commission, the assessment of the allergenic potential of foods derived from biotechnology has been suggested (6, 7). This assessment comprises the analysis of contiguous amino acid sequence homology to known allergens, specific or targeted serum screens for immunoglobulin E (IgE) cross-reactions to known allergens, digestibility studies of the proteins in simulated gastric and/or intestinal fluids, and animal studies (8, 9).

Most GM soybeans used in commercial production are tolerant to glyphosate herbicide due to a gene expression cassette encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) isolated from *Agrobacterium* sp. strain CP4 (10). EPSPS is a novel protein that needs to be assessed for its potential allergenicity before it is introduced into the food market. However, there is some debate about its allergenicity in herbicide-tolerant GM soybeans (11-14). Also, there are some concerns of possible allergic reactions to GM crops in infants, babies, and children.

In this study, CP4 EPSPS protein in GM soybeans was assessed for its potential allergenicity by IgE immunoblotting with soy-allergic children's sera, by contiguous amino acid sequence similarity to known allergens, and by its stability when exposed to pepsin in an *in vitro* digestion assay.

Materials and Methods

Samples Non-GM and GM soybeans (Roundup Ready Soybean; Monsanto Co., St. Louis, MO, USA) were provided by the American Soybean Association. Non-GM and GM soybean processed foods such as tofu, soy milk, bean-curd, and soybean paste were manufactured in our laboratory.

Sera of allergic patients Sera from 10 children with soy allergy were obtained from Samsung Seoul Hospital (Seoul, Korea). Sera were taken from allergy patients and stored at -20°C until used. Diagnostic measurements of allergen-specific IgE were performed with the CAP system FEIA (Pharmacia & Upjohn, Uppsala, Sweden) (15). Equal volumes of the 10 sera were mixed and used for the detection of soybean allergens.

Protein extraction One hundred mg of each non-GM and GM soybean processed food was treated with 1.0 mL

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of lysis buffer containing 2 M thiourea, 7 M urea, 4%(w/v) 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 1% dithiothreitol (DTT) for 12 hr at room temperature. Samples were then centrifuged for 12 min at 22,000×g. Each supernatant minus the fat phase was transferred to a new tube and centrifuged for 10 min at 15,000×g. The supernatant was filtered and stored at -20°C until used. The protein content of each supernatant was determined using the Bradford method.

Cloning, expression, and purification of recombinant CP4 EPSPS CP4 EPSPS was amplified from GM soybeans using PCR with a forward flanking primer (5'-CGCGCGCGCATATGTCGCACGGTGCAAGCAGCCG GC-3') and a reverse primer (5'-GCGCCTCGAG TCATCAGGCAGCCTTCGTATCGGAGAGT-3'). The *Nde*I and *Bam*HI sites in each primer are underlined. PCR was carried out using Ex *taq* DNA polymerase (Takara, Tokyo, Japan). The PCR product was purified with a Qiagen PCR purification kit (Qiagen GmbH, Hilden, Germany) and digested with *Nde*I and *Bam*HI. This PCR product was ligated into *Nde*I and *Bam*HI cleaved pET15b expression vector (Novagen, Madison, WI, USA) and sequenced to verify the integrity of the construct.

The *Escherichia coli* BL21 (DE3) strain (Novagen) was trans-formed with the recombinant CP4 EPSPS expression plasmid to, and a single positive colony was cultured overnight at 37°C. Ten mL of overnight culture was reinoculated into 1 L of media, and grown until the optical density at 600 nm was 0.4. The expression of recombinant CP4 EPSPS was induced with 0.4 mM Isopropyl β-D-thiogalactoside, IPTG) for 4 hr. After 4 hr incubation at 37 °C, cells were collected and lysed by sonication. After centrifugation to collect the supernatant at 10,000×g for 15 min, the supernatant was loaded onto nickel resin (Novagen). The bound protein was eluted with 0.5 M imidazole buffer. The recombinant CP4 EPSPS containing 10 histidine residues at N-terminal was purified and analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown).

SDS-PAGE and Western blot The proteins were separated by SDS-PAGE using a 12% acrylamide gel, and western blotting was performed to detect CP4 EPSPS protein from GM soybeans. SDS-PAGE was performed using a Mini-PROTEAN3 apparatus (BIO-RAD, Hercules, CA, USA) at 100 V for 2 hr. Separated proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 1% skim milk with 0.05% Tween 20 in phosphate buffered saline (PBS) (10 mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl) for 1 hr at room temperature and washed five times with PBST (PBS with 0.05% Tween 20). Polyclonal antibody (16) specific for the EPSPS protein was diluted 2,000-fold and incubated with the membrane for 1 hr at room temperature. The membrane was treated with a 1:10,000 dilution of secondary antibody (Anti-rabbit IgG; Sigma, St. Louis, MO, USA) for 1 hr at room temperature. The secondary antibody was then visualized using an ECL kit (Amersham Pharmacia, Björkgatan, Sweden).

Crude protein extracts from non-GM and GM soybeans, and purified CP4 EPSPS protein expressed in *E. coli* and each processed food (tofu, soy milk, bean-curd, and soybean paste made from non-GM and GM soybean) were compared by SDS-PAGE and Western blot analysis using rabbit anti-CP4 EPSPS polyclonal antiserum.

Immunoblotting After transferring the separated proteins to a nitrocellulose membrane by tank blotting, the membrane was blocked in PBS containing 1% skim milk and 0.05% Tween 20 for 1 hr to avoid nonspecific binding. The membranes were then probed with the pooled sera from children with soy allergy at a final dilution of 1:10 for each for 2 hr at room temperature. The membrane was treated with a 1:2,000 dilution of secondary antibody (horse radish peroxidase-conjugated goat anti-human IgE (ε) (KPL, Gaithersburg, MD, USA) and visualized using an ECL kit.

Digestion by simulated gastric fluid The digestibility of recombinant EPSPS protein produced in *E. coli* was tested in tubes containing simulated gastric fluid (SGF) incubated at 37°C. The final SGF formulation contained 0.32%(w/v) pepsin A (Sigma), 0.084 N HCl and 35 mM NaCl, pH 1.2. Each protein sample (164 ng of the recombinant EPSPS protein) was dissolved in 200 μL of prewarmed SGF. Digestion proceeded at 37°C with continuous shaking and an aliquot of each digest was periodically withdrawn. The time points analyzed in this study were 0, 1/3, 2/3, 1, 3, 5, and 10 min. Aliquots were quickly mixed with 25 μL of a sample buffer containing 2.5% SDS, 5% 2-mercaptoethanol, and 10% glycerol to quench the reactions at desired time points. Each mixture was boiled for 5 min and stored at -20°C until further analysis. The zero time point of digestion was quenched by the addition of 0.2 M Na_2CO_3 prior to the addition of CP4 EPSPS protein. The digestibility of recombinant CP4 EPSPS protein in SGF was assessed using SDS-PAGE and Western blot analysis.

Screening of amino acid sequence homology in the allergen database All EPSPS protein amino acid sequences were analyzed for similarity to the amino acid sequences of all known allergens in the Allergenonline database (http://allergenonline.com) developed by Monsanto and Nebraska University (Lincoln, NE, USA) using an 80

Table 1. Characteristics of ten patients' sera

Patient No.	Age (year)	Sex	CAP IgE FEIAsoybean IgE (kU_A/L)
1	1	M	4.75
2	9	F	1.51
3	1	M	0.88
4	5	F	0.94
5	6	M	1.38
6	1	M	2.04
7	7	M	3.29
8	2	M	19.7
9	1	F	0.49
10	2	F	0.42

amino acid window size. Proteins with more than 35% identity in an 80 amino acid window, or more than 8 contiguous identical amino acids with known allergens were considered to have a high possibility of cross reaction between the compared proteins.

Results and Discussion

SDS-PAGE and Western blot analysis of non-GM and GM soybeans and processed foods Extract of non-GM and GM (CP4 EPSPS-introduced) soybeans was loaded on an SDS-PAGE gel and separated by electrophoresis. The electrophoretic patterns of non-GM and GM soybean proteins on SDS-PAGE are shown in Fig. 1. The electrophoresis pattern of non-GM soybeans was similar to that of 100% GM soybeans. Western blot analysis was performed to confirm that the CP4 EPSPS protein expressed in GM soybeans was of the expected size and identity. We also performed SDS-PAGE and Western blot analysis of non-GM and GM processed foods (Fig. 2). Western blot analysis showed that CP4 EPSPS proteins with a molecular weight of 47.5 kDa were detected in GM soybeans, GM soy milk, and GM bean curd derived from GM soybeans, however, none were detected in GM tofu and GM soybean paste. This result indicates that CP4 EPSPS protein in tofu and soybean paste derived from GM soybeans was degraded during processing. During most food processing, proteins are degraded due to processing methosds such as grinding, milling, drying, boiling, and heating (17).

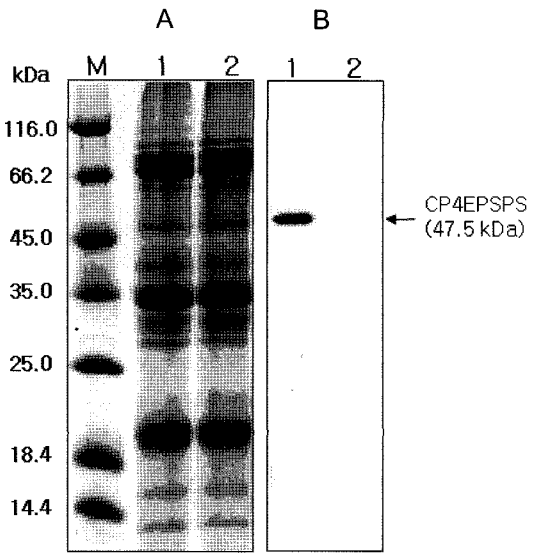

Fig. 1. SDS-PAGE and Western blot of non-GM and GM soybeans. Lane M, protein molecular weight marker; lane 1, GM soybeans; lane 2, non-GM soybeans.

Immunoblotting of non-GM and GM soybeans and processed foods The allergenicity of major soybean component proteins was determined by SDS-PAGE and immunoblotting using sera from children with soy allergy (Fig. 3). Sera from 10 individuals with soy allergy as determined by specific CAP testing did not recognize recombinant CP4 EPSPS proteins (Lane 1 in Fig. 3) produced in *E. coli*, however, they recognized extracts of non-GM and GM soybeans and processed foods as shown in Fig. 3. These results show that GM soybeans do not present any measurable differences in allergenicity compared with non-GM soybeans.

Digestibility of CP4 EPSPS protein in SGF Proteins digested by pepsin in the human stomach are considered less likely to be allergenic. The digestibility of recombinant CP4 EPSPS protein from *E. coli* was assessed by western blot (Fig. 4). Digestion samples of the time zero were quenched by the addition of 0.2 M Na_2CO_3 to SGF prior to the addition of CP4 EPSPS protein. For each digestion, 2 ng of recombinant CP4 EPSPS protein from *E. coli* was used. The digestion of CP4 EPSPS protein was completed after 20 sec in SGF. A control digestion prepared without

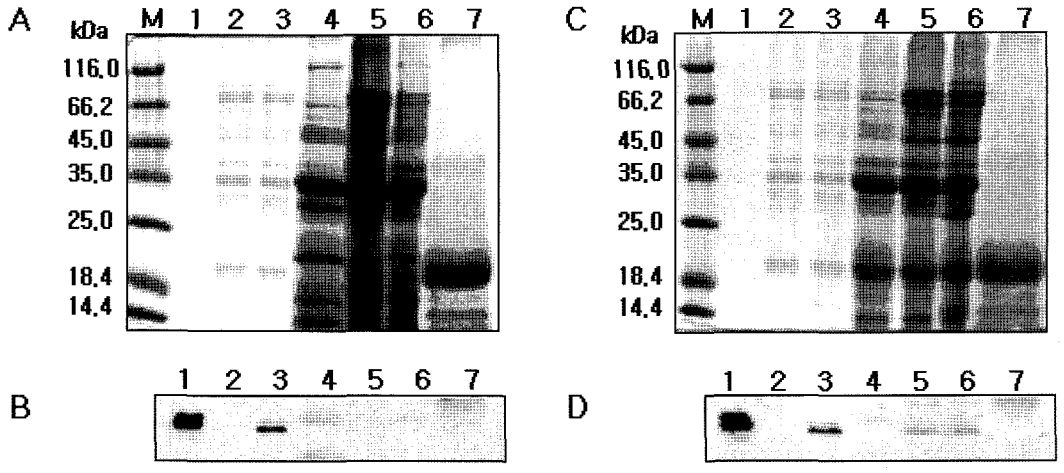

Fig. 2. SDS-PAGE and Western blot of non-GM and GM foods. Lane M, protein molecular weight marker; lane 1, CP4 EPSPS protein (1 ng); lane 2, non-GM soybeans; lane 3, GM soybeans; lane 4, tofu; lane 5, soy milk; lane 6, bean-curd; lane 7, soybean paste. A and B: SDS-PAGE and western blot of non-GM foods; C and D, SDS-PAGE and Western blot of GM foods. CP4 EPSPS proteins with a molecular weight of 47.5 kDa were detected in GM soybeans, GM soy milk, and GM bean curd derived from GM soybeans.

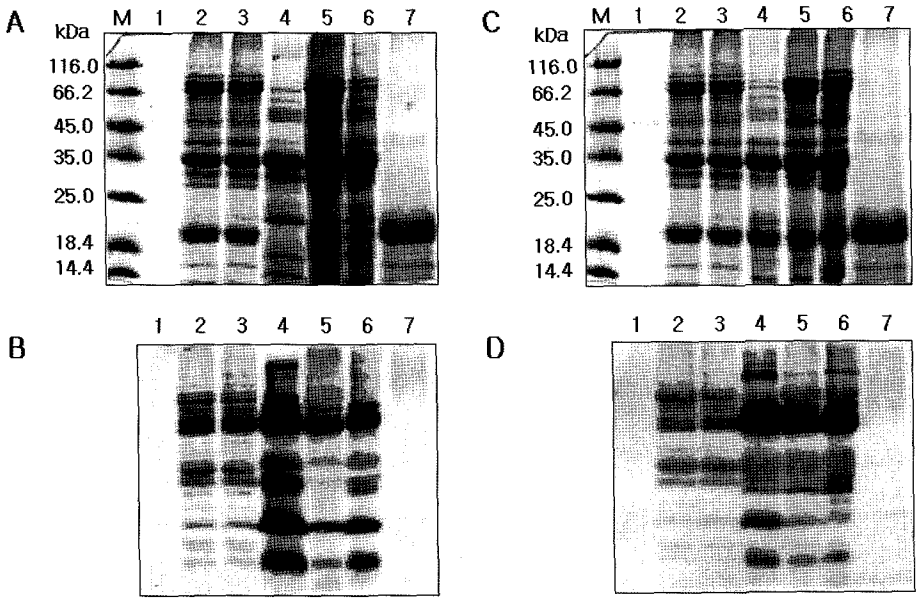

Fig. 3. SDS-PAGE and Immunoblot of non-GM and GM foods. Lane M, protein molecular weight marker; lane 1, CP4 EPSPS protein (1 ng); lane 2, non-GM soybeans; lane 3, GM soybeans; lane 4, tofu; lane 5, soy milk; lane 6, bean-curd; lane 7, soybean paste. A and B, SDS-PAGE and Immunoblot of non-GM foods; C and D, SDS-PAGE and Immunoblot of GM foods.

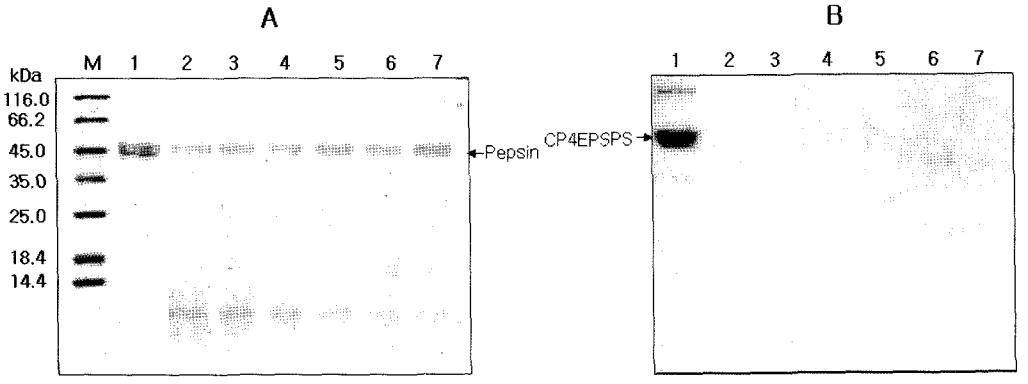

Fig. 4. Digestibility of CP4 EPSPS protein by SGF. Lane M: protein molecular weight marker; lanes 1-7: CP4 EPSPS protein in SGF (Incubation time: 0, 1/3, 2/3, 1, 3, 5, and 10 min). Reactions were stopped at set times and a portion of each reaction mixture was assayed by SDS-PAGE (A) and western blot (B). CP4 EPSPS protein was loaded at 2 ng per lane based on the pre-digestion concentration.

pepsin demonstrated that the degradation of recombinant CP4 EPSPS protein from *E. coli* was due to digestion by SGF and not instability of the test substance at pH 1.2 during incubation at 37°C (data not shown).

Sequence similarity between CP4 EPSPS and known allergens A comparison of the amino acid sequence of an introduced protein with that of known allergens is a useful indicator of its allergenic potential. Sequence comparisons are used to indicate potential unexpected cross reactivity to existing allergens and to assess the potential for developing new sensitivities. The amino acid sequences of most major allergens, including food allergens, have been reported, and the important IgE binding epitopes of many allergenic proteins have been mapped (18, 19). The optimal peptide length for binding is at least 8 amino acids (20). After comparing the CP4 EPSPS amino acid sequence to proteins in the Allergenonline database, no homology of more than 35% in any 80 amino acid window, or more than 8 consecutive identical amino acids were found. These results indicate that there is little possibility of cross reactivity between CP4 EPSPS protein and other known allergens based on amino acid sequence homology.

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