

The 5-Enolpyruvylshikimate-3-Phosphate Synthase of Glyphosate-tolerant Soybean Expressed in *Escherichia coli* Shows No Severe Allergenicity

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The recombinant gene was amplified from the chromosomal DNA of genetically-modified (GM) soybeans and identified as *epsps* encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) which renders glyphosate resistance. The *epsps* structural gene was introduced in the pET28(a) plasmid for its expression in *Escherichia coli* BL21(DE3). It was confirmed that the maximal productivity of the EPSPS protein was achieved when cultivating the recombinant strain in a LB broth for 2 h after supplementing 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) in a 2 h-culture broth. Since the expressed EPSPS protein was found as an insoluble form in the inclusion body, it was extracted by 6 M urea after sonication, and then purified through immobilized nickel-affinity column chromatography to isolate EPSPS having a molecular mass of 57 kDa. When incubated in simulated gastric fluid containing pepsin at pH 1.5, the purified EPSPS protein was completely digested within 1 min. In addition, the passive cutaneous anaphylaxis reaction of the purified EPSPS protein was not observed in the Sprague Dawley rat system that was administered either orally or subcutaneously. Furthermore, treatment of the EPSPS protein to the culture of the sensitized peritoneal mast cells, or unsensitized but antisera-labeled mast cells, showed neither a remarkable change in the histamine release nor a cytokine production, including interleukin-4 (IL-4) and tumor necrosis factor- α (TNF- α). Thus, it can be concluded that the EPSPS protein in the GM soybean showed no significant al-

lergenicity in the Sprague Dawley rats.

Keywords: 5-Enolpyruvylshikimate-3-Phosphate Synthase; Allergenicity; Cytokine Production; Gene Expression; Genetically-Modified Soybean; Glyphosate-tolerant Soybean; Histamine Release; Passive Cutaneous Anaphylaxis; Pepsin Digestion.

Introduction

The continual demand for agricultural products for human foodstuffs, as well as livestock feedstuffs, brought the creation of GMO for quality improvement (Kim *et al.*, 1990), pest-tolerance (Stewart *et al.*, 1996), herbicide-tolerance (Padgett *et al.*, 1995; Song *et al.*, 1996), drought-tolerance (Yeo *et al.*, 2000), salt-tolerance (Jeong *et al.*, 2001), etc. (Hemmer, 1997). However, the emergency of GMO in the human food chain created some concern about food safety (Gaskell *et al.*, 1999; Hino, 2002; Moseley, 1999).

In the case of soybeans, the Monsanto Company developed 2 transgenic soybean lines, denoted 40-3-2 and 61-67-1,

Abbreviations: α -MEM, α -minimum essential medium; BSA, bovine serum albumin; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; GM; genetically modified; GMO, genetically-modified organism; GTS, glyphosate-tolerant soybean; HEPES, N-(2-hydroxyethyl) piperazine-N'-(4-ethanesulfonic acid); IgE, immunoglobulin E; IL-4, interleukin-4; IPTG, isopropyl- β -D-thiogalactopyranoside; LB, Luria-Bertani; SDS-PAGE, sodium dodecyl sulfate-denatured polyacrylamide gel electrophoresis; SGF, simulated gastric fluid; TNF- α , tumor necrosis factor- α .

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which are resistant to herbicide glyphosate (Roundup) for the effective control of weeds during cultivation (Padgett *et al.*, 1995). These GTS lines called, Roundup Ready, possess the *epsps* gene of *Agrobacterium* sp. CP4 encoding glyphosate-tolerant EPSPS. The substantial equivalence of GTS with conventional soybean was verified through a proximate analysis and compositional comparison of the amino acid, fatty acid, lectin, isoflavin, etc. (Padgett *et al.*, 1996; Taylor *et al.*, 1999). The animal feeding values of the processed GTS meal were also reportedly comparable to that of the conventional one in rats, chickens, catfish (Hammond *et al.*, 1996), and pigs (Cromwell *et al.*, 2002). Further, the gastric digestibility and acute toxicity of the EPSPS protein that is expressed in GTS were also conducted (Harrison *et al.*, 1996). Throughout these works, it was concluded that GTS is safe, based on the following facts: no remarkable acute toxicity in rats fed with GTS, the rapid digestion of the EPSPS protein in gastric and intestinal fluid, its presence as an unglycosylated form in GTS, and its concentration lower than 0.03% in GTS seed.

However, any direct evaluation of the allergenic potential of the GM soybean has not been tried until now. In our group, the allergenic potential of the GM soybean homogenate in Sprague Dawley rats was examined (Chang *et al.*, 2001). The result implied that the GM soybean homogenate induced no severe allergenicity in the rat system. However, it is still unclear whether the GM soybean homogenate presented higher IgE-specific immune responses, because of the co-existence of a certain immune-suppressing substance in the soybean homogenate.

In order to confirm the allergenic potential of the recombinant protein itself in the GM soybean, the recombinant gene in the GM soybean was cloned and expressed in *Escherichia coli*. Using the recombinant protein that was purified from *E. coli*, the pepsin digestibility and further *in vivo*, *ex vivo* as well as *in vitro* evaluation of allergenicity in Sprague Dawley rats, were attempted.

Materials and Methods

Materials A GMO detection kit was purchased from I. J. Biotech (Korea), and PCR primers for gene amplification were supplied from Bioneer Co. (Korea). A pGEM-T-easy vector from Promega Corp. (USA) and *Escherichia coli* XL1-Blue [*recA1 supE44 endA1 hsdR17 gyrA46 relA1 thi lac⁻ F'[proAB⁺ lacI^f lacZ M15 Tn10(tet^r)*] were employed in the cloning of the recombinant gene from the GM soybean. A pET-28(a) plasmid from Novagen Inc. (USA) was used in its gene expression in *E. coli* BL21(DE3) [*F⁻ ompT hsd SB(rB⁻ mB⁻) gal dcm (DE3)*]. For the determination of the nucleotide sequences, T7 and SP6 primers from Promega Corp. (USA) and a DNA sequencing kit from Perkin-Elmer Biosystems (USA) were employed. The gel

extraction kit and plasmid miniprep kit from Qiagen Inc. (USA) were used for DNA purification. The immobilized nickel affinity resin (His-Bind resin) was purchased from Novagen Inc. (USA) for protein purification. The restriction enzymes were provided from TaKaRa Korea (Korea), and all of the other reagents used were in pure grade from the Sigma Co. (USA).

Experimental animals Specific pathogen-free male Sprague Dawley rats (4–5 weeks old) were purchased from Daehan Laboratory Animal Research Center Co. Ltd. (Korea). Four animals were housed in a cage and fed with tap water and rodent pellet feedstuff (Samyang Co., Korea). The animal room was kept at 23 ± 3°C with humidity of 50 ± 10% and illuminated repeatedly at 150–300 Lux with 12 h intervals. All of the animals were adapted for at least 1 week before the experiment.

Confirmation of the recombinant gene from the GM soybean

The chromosomal DNAs from the GM soybean and non-GM soybean were isolated using a GMO detection kit (I. J. Biotech). Following the PCR amplification procedures that were reported previously (Chang *et al.*, 2001), the chromosomal DNA was subjected to 40 cycles of DNA amplification using primers of 35S-1 (5'-gct cct aca aat gcc atc a-3'; sense strand) for 35S promoter and NOS-3 (5'-tta tcc tag ttt geg cgc ta-3'; antisense strand) for NOS terminator, in a GeneAmp 2400 Thermocycler (Perkin-Elmer, USA). The amplified gene product was then eluted by a Qiagen gel extraction kit (Qiagen Inc., USA), and inserted into a pGEM-T vector by ligating with T4 DNA ligase at 4°C. The transformed *E. coli* XL1-Blue cells were screened on a LB agar plate (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar, pH 7.2) containing 50 µg/ml of ampicillin (Sambrook *et al.*, 1989). The amplified gene product in the pGEM-T vector was isolated by digesting with *EcoRI* and purified by a Qiagen miniprep kit (Qiagen Inc., USA). The base sequence was determined using T7 and SP6 primers and DNA sequencing kit by ABI PRISM 377 Automatic DNA Sequencer (Perkin-Elmer Biosystems, USA). The determined sequences were searched on the Entrez Nucleotides database of the National Center for Biotechnology Information (USA).

Cloning and expression of the recombinant gene

In order to amplify the recombinant gene itself, two primers were prepared as follows: G-f primer (5'-cac ata tgg cac aaa tta aca ac-3') that had a *NdeI* site at the 5'-end and a G-r primer (5'-cga agt cga cct cat cag gc-3') that had a *SalI* site at the 3'-end. The recombinant gene that was amplified from the previously mentioned plasmid by the same PCR procedure was then introduced into a pGEM-T vector and transformed into *E. coli* XL1-Blue. From the positive clone, the inserted gene was isolated by digesting the plasmid with *NdeI* and *SalI*, and re-cloned into the pET-28(a) expression plasmid. The transformed *E. coli* BL21(DE3) cell was screened on a LB agar plate containing 30 µg/ml of kanamycin. The selected recombinant strain of *E. coli* BL21(DE3) was subjected to the fermentation by seeding 1% (v/v) inoculum in 3 L of a LB broth with 30 µg/ml kanamycin in a 5 L-jar fermentor (Kobio-

tech Co., Korea) at 37°C with the agitation of 300 rpm and aeration at 1.0 vvm. After a 2 h-cultivation, 1 mM of IPTG was supplemented for the gene expression and the fermentation continued for longer than 2 h.

Purification of the recombinant protein The recombinant *E. coli* BL21(DE3) cells were recovered by centrifugation at 10,000 rpm for 10 min, suspended in 1/50 volume of a binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), and subjected to ultrasonication for 15 min at 4°C. The recombinant protein in the form of a inclusion body was then separated by centrifugation at 14,000 rpm for 20 min, and dissolved in 3 volume (v/w) of a binding buffer containing 6 M urea for 1 h on ice. The urea extract that was recovered by centrifugation at 12,000 rpm for 15 min was then loaded on an immobilized nickel affinity column (2.5 × 10 cm) that was pre-equilibrated with the same binding buffer by the procedure reported previously (Hochuli, 1990; Hochuli *et al.*, 1988), washed with 10 volume of the same buffer, followed with 6 volume of a washing buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) containing 6 M urea, and finally eluted with an elute buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.9) containing 6 M urea. The fractions containing the recombinant protein were collected and dialyzed against distilled water, and finally recovered after lyophilization.

Digestibility of the recombinant protein by simulated gastric fluid One hundred µl of the suspension of the purified recombinant EPSPS protein was treated with 200 µl of the simulated gastric fluid [SGF; 0.32% (w/v) pepsin, 0.03 M NaCl, pH 1.2] at 37°C. The reaction was completed by adding 75 µl of 160 mM sodium carbonate, according to the method of Astwood *et al.* (1996).

Passive cutaneous anaphylaxis test Sprague Dawley rats were divided into 6 groups. Three groups were exposed orally 3 times a week for 3 weeks with saline (control), 0.5 or 2.0 mg/kg of the recombinant EPSPS protein in saline. The other 3 groups were sensitized subcutaneously with the same dose. One week after 9 times of sensitization, the animals' abdomens were cut open after anesthetization with ether. The blood was taken from the inferior vena cava and left at room temperature for 30 min for agglutination. The sera were obtained by centrifugation of the agglutinated blood at 2,500 × g for 10 min at 4°C, and stored at -80°C until use. The sensitized sera was diluted from 1/10 to 1/320, and injected intradermally onto the clipped back of the unsensitized rats. Then 24 h later, the recombinant EPSPS solution containing 1% Evans blue was injected intravenously with 20 mg/kg of recombinant EPSPS protein. The release of Evans blue by immunoglobulin E (IgE)-specific reaction was observed for 30 min.

Histamine release and cytokine production in mast cells The mast cells were isolated from the peritoneal cavity of sensitized or unsensitized rats. For the isolation of sensitized mast cells,

the rats were orally exposed 3 times a week for 3 weeks with saline (control), 5 or 20 mg/kg of the recombinant EPSPS protein in saline. After anesthetizing the rats with ether, 20 ml of Tyrode buffer B (137 mM NaCl, 5.6 mM glucose, 12 mM NaHCO₃, 2.7 mM KCl, 0.3 mM NaH₂PO₄) was injected into the peritoneal cavity, and the abdomen was smoothly massaged for 90 s. After cutting the abdomen open, the peritoneal fluid was taken into a sterilized centrifuge bottle, and the cells were separated by centrifugation at 150 × g for 10 min at room temperature. The mast cells were then isolated using metrizamide by the procedure of Jippo-Kanemoto *et al.* (1993). The mast cells that were obtained were resuspended in 1 ml of α-MEM containing 15 mM of HEPES and 10% fetal bovine serum. The purity of the mast cells was checked by Giemsa staining, and the cell viability was tested by the trypan blue-staining method. The sensitized mast cells were treated with 10 µg/ml of the EPSPS protein and cultivated in a 5% CO₂ incubator at 37°C for 30 min. In the case of the unsensitized mast cells, a 16-h pretreatment with 5% antisera that was obtained in the passive cutaneous anaphylaxis test was done before exposing to 10 µg/ml of the EPSPS protein for 30 min. From the supernatant that was obtained by centrifugation of the culture broth of both of the mast cells, the amount of histamine was assayed by the method of Shore *et al.* (1959). The amount of cytokines was measured using ELISA kits for TNF-α and IL-4 (BD Biosciences Pharmingen, USA).

Statistical analysis All of the experimental results were expressed as mean value ± standard error. A Dunnett's *t*-test was employed to analyze the significance of the obtained data.

Results

Cloning and confirmation of the recombinant gene in the GM soybean In the genomic DNA from the GM soybean, it was previously confirmed that there is a 35S promoter and NOS terminator that are introduced during the gene manipulation (Chang *et al.*, 2001). Thus, the recombinant gene was amplified using the combined sets of the 35S-1 primer (forward) and NOS-3 primer (reverse) to give 2.1 kb of the recombinant gene from the genomic DNA of the GM soybean. The amplified gene product was inserted into the pGEM-T vector to construct pGMO-T. The base sequence was determined using the T7 primer and SP6 primer. By the BLAST search, it was revealed that the recombinant gene is an *epsps*-coding EPSPS protein, which renders herbicide glyphosate resistance. However, the N-terminal region was *Petunia x hybrida* EPSPS (Swissprot accession No. P11043) and C-terminal was *Agrobacterium* sp. CP4 EPSPS (Swissprot accession No. Q9R4E4), which implies that the glyphosate-resistant EPSPS from *Agrobacterium* sp. was fused with petunia EPSPS for stable gene expression in GTS, as reported previously (Padgett *et al.*, 1995).

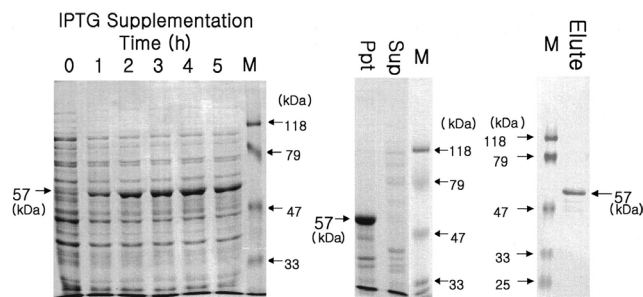


Fig. 1. Expression and purification of the recombinant EPSPS protein in *E. coli* BL21(DE3). **A.** The expression of the EPSPS protein in *E. coli* BL21(DE3) was achieved by supplementing 1 mM IPTG to a 2-h culture broth of *E. coli* BL21(DE3). The whole cell extracts, sampled at each culture times (from 0 to 6 h), were run on 10% SDS-PAGE with a molecular marker (M). **B.** After sonication of the cultured *E. coli* BL21(DE3) cells, the localization of the recombinant EPSPS protein was confirmed. The supernatant (Sup) and precipitate (Ppt) fractions after centrifugation were loaded on 10% SDS-PAGE with a molecular marker (M). **C.** The recombinant proteins in the inclusion body of *E. coli* BL21(DE3) were purified through an immobilized nickel affinity column. The recombinant EPSPS protein that was obtained by eluting with 1 M imidazole was confirmed by 10% SDS-PAGE with a molecular marker (M).

In order to clone only the *epsps* structural gene, a G-f primer that has a *Nde*I site and a G-r primer that has a *Sal*I site were newly-designed and employed for DNA amplification in order to give 1.7 kb of the PCR product. The amplified-DNA fragment was first introduced into the pGEM-T vector. The *epsps* structural gene was separated by a cut-out with *Nde*I and *Sal*I, followed by re-cloning in the pET28(a) expression vector to make the pET28(a)-GM plasmid.

Production and purification of the recombinant EPSPS protein During the fermentation study of the *E. coli* BL21(DE3) strain that harbored the pET28(a)-GM plasmid, it was observed that the recombinant *epsps* gene was induced well when cultivated for longer than 2 h after supplementing 1 mM IPTG to a 2-h culture (Fig. 1A). It was also found that the EPSPS protein that was produced was localized in an insoluble inclusion body (Fig. 1B).

Thus, the recombinant EPSPS protein was purified by a nickel-affinity column chromatography after dissolving in 6 M urea. The pure EPSPS protein was eluted by 1 M imidazole, and the fraction showed a single band of 57 kDa on 10% SDS-PAGE (Fig. 1C). The molecular mass of the purified EPSPS protein was the same as *Mycobacterium* EPSPS (Oliveira *et al.*, 2001), but somewhat larger than the EPSPS protein in GTS that was reported previously by Harrison *et al.* (1996). This is due to the His-tag moiety (2.1 kDa) that fused with the EPSPS protein at the

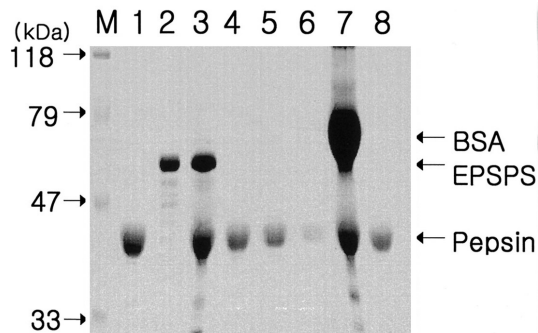


Fig. 2. Stability of the recombinant EPSPS protein in simulated gastric fluid (SGF). Ten % SDS PAGE was employed to assess digestion of the recombinant EPSPS protein. Lane M, molecular marker; lane 1, SGF; lane 2, EPSPS protein only; lane 3, EPSPS treated with SGF for 0 min (control); lane 4, EPSPS treated with SGF for 1 min; lane 5, EPSPS treated with SGF for 5 min; lane 6, EPSPS treated with SGF for 15 min; lane 7, BSA treated with SGF for 0 min (control); lane 8, BSA treated with SGF for 1 min.

N-terminus.

Digestibility of the recombinant EPSPS protein by simulated gastric fluid In order to assess the allergenic potential of the recombinant EPSPS protein in the GM soybean, the purified EPSPS was subjected to the SGF containing 0.32% (w/v) pepsin and 0.03 M NaCl (pH 1.2). As shown in Fig. 2, it was observed that the recombinant protein was completely digested by SGF within 1 min. This implies that the recombinant EPSPS protein was unable to produce severe allergenicity when administered orally.

In vivo allergenicity test of the recombinant EPSPS protein For the confirmation of the possibility of *in vivo* allergenicity by the passive cutaneous anaphylaxis test, the recombinant EPSPS protein was administered orally or subcutaneously to Sprague Dawley rats. The dosage was determined as 0.5 and 2.0 mg/kg, based on the report of Harrison *et al.* (1996) that considered the amount of soybean that are consumed annually. During 9 times of sensitization, no significant effect on rat growth, nor any apparent symptoms, was observed.

The rat antisera that were obtained by oral or subcutaneous sensitization with the recombinant EPSPS protein was then subjected to the passive cutaneous anaphylaxis test by injecting intradermally on the clipped back of the unsensitized rats. As seen in Table 1, no positive release of Evans blue by the IgE-specific reaction was observed when challenged with the antisera of rats that were exposed either orally or subcutaneously. This means that the recombinant EPSPS protein elicited no severe allergenicity in the range of soybean consumption.

Table 1. The passive cutaneous anaphylaxis test in male Sprague Dawley rats.

Administered recombinant EPSPS protein	Challenged antigen	Body weights of recipient rats (g)	Positive ratio
Oral			
Saline	20 mg/kg, iv	298 ± 7	0/5
0.5 mg/kg	20 mg/kg, iv	286 ± 13	0/5
2.0 mg/kg	20 mg/kg, iv	293 ± 6	0/5
Subcutaneous			
Saline	20 mg/kg, iv	285 ± 9	0/5
0.5 mg/kg	20 mg/kg, iv	283 ± 12	0/5
2.0 mg/kg	20 mg/kg, iv	293 ± 15	0/5

The antisera from individual groups of 5 animals that were sensitized either orally or subcutaneously were diluted to 10-, 20-, 40-, 80-, 160-, and 320-folds. Each diluted serum (100 µl) was injected intradermally on the clipped back of the unsensitized recipient animal with saline control. After 24 h, each animal was challenged intravenously with 20 mg/kg of an antigen solution containing 1% Evans blue. The decision was made by the release of Evans blue by IgE-specific reaction for 30 min.

Ex vivo allergenicity test of the recombinant EPSPS protein The mast cells from the Sprague Dawley rats that were sensitized orally were subjected to an *ex vivo* allergenicity test. On exposing the mast cells to 10 µg/ml of antigen (recombinant EPSPS protein) at 37°C for 30 min, no significant changes in histamine release as well as cytokine production, including IL-4 and TNF-α in sensitized mast cells, were observed (Fig. 3). This result shows that the mast cells from sensitized rats bear no IgE for the EPSPS protein on the surface.

In vitro allergenicity test of the recombinant EPSPS protein In order to check the *in vitro* allergenicity of the recombinant EPSPS protein, the mast cells from the unsensitized rats were first labeled with 5% sensitized antisera for 16 h, then treated with 10 µg/ml of antigen (recombinant EPSPS protein) at 37°C for 30 min. No significant changes in histamine release or cytokine production, including IL-4 and TNF-α in the mast cells, were observed (Fig. 4). This means that there is not enough IgE for the EPSPS protein in sensitized antisera for the activation of the unsensitized mast cells.

Discussion

The newly-introduced GMO in the human food chain brought consumer concern about food safety. The majority of people have negative perceptions about GMO, especially in developed countries, based on ethical considerations or safety concerns (Gaskell *et al.*, 1999; Hino, 2002; Moseley, 1999). Their concerns include the poten-

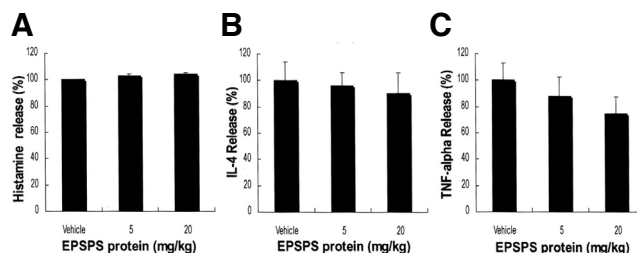


Fig. 3. Effects of the recombinant EPSPS protein on the histamine and cytokine release from sensitized mast cells. Male Sprague Dawley rats were sensitized orally with the recombinant EPSPS protein 3 times a week for 3 weeks. Peritoneal mast cells were isolated and cultured for 30 min in the presence of 10 µg/ml of the recombinant protein. The amount of histamine (A), TNF-α (B), and IL-4 (C) that was released to the culture supernatant from the mast cells was determined. Each bar represents the mean percent of vehicle control ± SE of six animals.

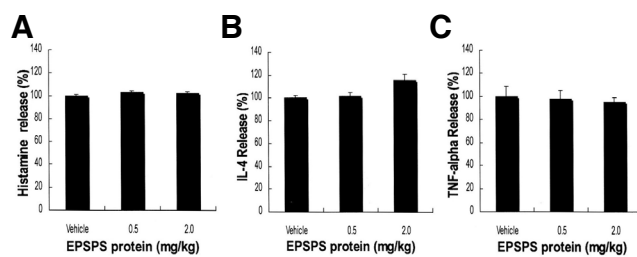


Fig. 4. Effects of the recombinant EPSPS protein on the histamine and cytokine release from unsensitized mast cells. Male Sprague Dawley rats were sensitized orally with recombinant EPSPS protein 3 times a week for 3 weeks. Peritoneal mast cells were isolated from unsensitized rats and precultured for 16 h with 5% antisera that was obtained during the passive cutaneous anaphylaxis test. Then the cells were incubated with 10 µg/ml of the recombinant EPSPS protein for 30 min. The amount of histamine (A), TNF-α (B), and IL-4 (C) that was released to the culture supernatant from the mast cells was determined. Each bar represents the mean percent of vehicle control ± SE of five animals.

tial harmfulness of insert or selectable marker genes and/or their expressed proteins in food, and the potential nutritional change of food by genetic disturbance of plants. Possibly, the intake of GMO may result in potential acute or chronic toxicity, potential carcinogenicity or mutagenicity, potential allergenicity, and potential pathogenicity by nutritional alteration.

Especially, the potential of antigenicity or allergenicity of the GM food has been a major concern, because the target products in GMO are the newly expressed proteins from the GM genes in plants (Lehrer and Reese, 1997; Metcalfe *et al.*, 1996; Moneret-Vautrin, 1998; Shirai *et al.*, 1998). New approaches to routinely evaluate the antigenicity or allergenicity of GM food have been designed,

because very few foods that are consumed today have been subjected to conventional toxicological studies (Gendel, 1998a; 1998b). The decision tree has recently been adopted by a Joint WHO/FAO Expert Consultation, which includes a sequence homology search from allergen database, specific serum screening, targeted serum screening, pepsin resistance, and animal model study (WHO/FAO, 2000; 2001).

In our previous report (Chang *et al.*, 2001), the GM soybean homogenate was tested for its potential of allergenicity. Throughout the passive cutaneous anaphylaxis reaction test and the analysis of histamine that is released from mast cells, it was concluded that the GM soybean extract might not act as a strong allergen in Sprague Dawley rats. For a precise examination, the allergenicity test of the recombinant protein itself in the GM soybean was attempted in this study.

To do this, the recombinant gene in the GM soybean was isolated by PCR amplification, and cloned in an *E. coli* vector. The determination of the nucleotide sequences revealed that the recombinant gene was introduced in the order of P35 promoter, *Petunia x hybrida epsps* gene at the N-terminal (not resistant to glyphosate), *Agrobacterium* sp. *epsps* gene at the C-terminal (resistant to glyphosate), and NOS terminator, as originally reported by Padgett *et al.* (1995). This implies that the GM soybean that was used in this study was a GTS line, which was originally constructed by fusing the bacterial *epsps* gene resistant to glyphosate with the plant *epsps* gene for the stable expression in plant cells.

The identified-*epsps* gene itself was then re-cloned into the *E. coli* expression vector for large-scale production. After gene induction with 1 mM IPTG, the produced EPSPS protein was recovered from the inclusion body, dissolved in 6 M urea, and purified through an immobilized nickel-affinity column. The purified-EPSPS protein showed a single protein of 57 kDa on 10% SDS-PAGE. The molecular mass was somewhat larger than the EPSPS protein that was expressed in GTS (Harrison *et al.*, 1996), because it contained the His-tag moiety (2.1 kDa) that was fused with the EPSPS protein at the N-terminus. Because the proteins in the GTS seed are stored in insoluble forms, the EPSPS protein from the inclusion body of *E. coli* was directly subjected to an evaluation for allergenic potential.

When the purified-EPSPS protein was incubated with SGF, it was completely degraded within 1 min. This indicates that this recombinant protein is easily decomposed in gastric juice to elicit its allergenicity *in vivo* after taking it orally (judged from the report of Astwood *et al.*, 1996, that most of allergenic protein persisted for longer than 2 min, up to 1 h in SGF). In fact, the EPSPS protein showed no significant allergenicity in the passive cutaneous anaphylaxis reaction test in Sprague Dawley rats that were sensitized through either the subcutaneous or oral

route.

The most common type of food allergy is mediated by allergen-specific IgE antibodies. These antibodies attach on the surfaces of mast cells or basophils, thus provoking the immune system to react upon subsequent exposure to the specific antigen. When the mast cells in the peritoneal cavity of sensitized rats were again isolated and *ex vivo* exposed to antigen (recombinant EPSPS protein), no significant increase in histamine release or cytokine production was observed. These results suggest that the mast cells might not be sensitized with the IgE antibody for the EPSPS protein on the surface during oral administration.

Alternatively, the mast cells from unsensitized rats were *in vitro*-labeled with antisera from sensitized rats, then treated with antigen (recombinant EPSPS protein). Even in this case, no severe change of histamine release and cytokine production was observed. This implies that the IgE antibody that was sufficient to activate mast cells was not present in antisera by the oral or subcutaneous challenge of the EPSPS protein.

All of the data that were obtained from the *in vivo*, *ex vivo*, and *in vitro* allergenic tests of the EPSPS protein in the rat system were negative. It was generally accepted that most of the food allergens are present, though not always, as major components and in a glycosylated form that has a molecular mass of 10 to 70 kDa (Taylor *et al.*, 1987). Following the report of Padgett *et al.* (1995), the level of EPSPS in GTS seeds was approximately 0.03% of the fresh weight and 0.08% of the total protein. It was also reported by Harrison *et al.* (1996) that the EPSPS protein that is expressed in the GTS seed was not glycosylated. In addition, the rapid degradation of the EPSPS protein in SGF supports its low allergenicity. Considering these results as well as our previous report (Chang *et al.*, 2001), it can be concluded that the allergenic potential of the EPSPS protein in GTS seed is very low.

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