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Allergenicity Test of Genetically Modified Soybean in Sprague Dawley Rats

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Allergenicity of genetically-modified (GM) soybean was evaluated in male Sprague Dawley rats. To confirm the GM soybean used in this study, the polymerase chain reaction (PCR) was performed using the chromosomal DNA of soybeans. The PCR result provided the clear discrimination of genetically-modified (GM) soybeans. To evaluate the allergenicity of GM soybean and non-GM control one, the soybean homogenate was sensitized subcutaneously 3 times a week for 3 weeks. The doses of soybean were 0, 2 and 20 mg/kg in the protein basis. A week after the last sensitization, antisera were recovered from individual animals. When the sera were injected intradermally on the clipped back of unsensitized rats with various dilutions, followed by a challenge with 20 mg/kg of soybean homogenate containing 1% Evans blue, no sign of passive cutaneous anaphylaxis reaction was detected. In addition, when the sera were treated in the cultures of peritoneal mast cells, the increase of histamine release by anti-(GM soybean) sera was not observed when compared to that by anti-(non-GM soybean) sera. The present results indicate that the GM soybean might not act as a strong allergen in male Sprague Dawley rats.

Key words: Allergenicity, Genetically-modified soybean, Polymerase chain reaction, Passive cutaneous anaphylaxis, Histamine release, Sprague Dawley rats

INTRODUCTION

Continuous increase of human population as well as gradually increased demands of livestock feedstuffs leads the shortage of human foodstuffs. However, agricultural cultivation of plants by traditional breeding or fertilizer improvement cannot provide any more increased supply of foodstuffs. In order to overcome this problem, genetically-modified organisms (GMO) have been introduced for quality improvement, pest-tolerance, herbicide-tolerance, and so on (Kim *et al.*, 1990; Hemmer, 1997; Moseley, 1999).

New emergency of GMO in human food chain brought some fear in food safety. The presumed potential hazards of GMO on human health will include the potential 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 (Gaskell *et al.*, 1999). Even though any case has not been reported till now, the possible apparent results from the potential hazards of GMO will be the potential acute or chronic toxicity, the potential carcinogenecity or mutagenicity, the potential allergenicity, and the potential pathogenicity by nutritional alteration (Metcalfe *et al.*, 1996; Moneret-Vautrin, 1998). Especially, genetically-modified (GM) food will have high potentials of antigenicity or allergenicity, because the target products in GMO are the newly expressed proteins from GM genes in plants (Lehrer and Reese, 1997; Gendel, 1998a; Gendel, 1998b).

As the allergenicity test of biotechnology products in Korea and Japan, the passive cutaneous anaphylaxis test in mouse-rat or guinea pig-guinea pig system, and the anaphylactic shock test in guinea pig have been recommended (Korea Food and Drug Administration, 1999). This method of toxicity test gives only end-point results, regardless of any immunological processes in molecular level.

In this study, allergenicity of GM soybean was evaluated

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for the purpose to establish more scientific and convenient method of GMO safety test. Especially, using GM soybean homogenate, the passive cutaneous anaphylaxis by the sensitized sera and the histamine release in unsensitized mast cells of Sprague Dawley rats were tested.

MATERIALS AND METHODS

Materials

GMO detection kit was purchased from I. J. Biotech (Ansan, Korea), and PCR primers for gene amplification were supplied from Bioneer Co. (Cheongwon, Korea). Metrizamide, A23187, gelatin and glucose from Sigma Chemical Co. (MO, USA), α-MEM (minimal essential media), fetal bovine serum and HEPES from Gibco-BRL (NY, USA), and Histamine and o-phthalaldehyde from Aldrich (WI, USA) were used in this study.

Experimental animals

Specific pathogen-free male Sprague Dawley rats (4~5 weeks old) were purchased from Daehan Laboratory Animal Research Center Co. Ltd. (Eumsung, Korea). Four animals were housed in a cage and fed with tap water and rodent pellet feedstuff (Samyang Co., Ulsan, 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 interval. All animals were adapted for at least 1 week before experiment.

Identification of GM soybean

The chromosomal DNAs from GM soybean and non-GM soybean were isolated using GMO detection kit (I. J. Biotech). Following the PCR amplification procedures recommended by IUPAC (Lipp *et al.*, 1999), the chromosomal DNA was subjected to 40 cycles of DNA amplification with denaturation at 94°C for 3 min, annealing at 54°C for 40 sec and extension at 72°C for 1 min, and following the final extension at 72°C for 3 min. The primers used were 35S-1 (5'-gct cct aca aat gcc atc a-3'; sense strand) and 35S-2 (5'-gat agt ggg att gtg cgt ca-3'; antisense strand) for 35S promoter, and NOS-1 (5'-gaa tcc tgt tgc cgg tct tg-3'; sense strand) and NOS-3 (5'-tta tcc tag ttt gcg cgc ta-3'; antisense strand) for NOS terminator. The PCR amplification was performed using PCR master mix (520 μl of sterile deionized water, 105 μl of 10 × PCR buffer, 105 μl of 25 mM $MgCl_2$ solution, 52 μl of 4 mM dNTPs, 26 μl of 20 μM oligonucleotide solution, 25 units of DNA polymerase, and 10 μl of template DNA solu-tion) in GeneAmp 2400 Thermocycler (Perkin-Elmer, CT, USA)

Preparation of soybean homogenate

GM soybean and non-GM soybean were treated in saline solution at 4°C for 2 days, and homogenized with a blender. After removing debries by centrifugation at 2,500 × g, the supernatant was taken as soybean homogenate. The protein content was determined by Bradford method (Bradford, 1976) using bovine serum albumin as a standard. The soybean homogenate was diluted with saline before sensitization.

Sensitization of Sprague Dawley rat with soybean homogenate

Sprague Dawley rats were divided into 5 groups and each group was sensitized subcutaneously 3 times a week for 3 weeks with saline solution as control, 2 or 20 mg/kg of GM soybean homogenate, and 2 or 20 mg/kg of non-GM soybean homogenate. One week after 9 times of sensitization, animals were cut the abdomen open after anesthetization with ether, and 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 agglutinated blood at 4°C and 2,500 × g for 10 min, and stored at -80°C until use.

Passive cutaneous anaphylaxis test

The sensitized sera was diluted from 1/2 to 1/256, and challenged intradermally onto the de-haired clipped back of the unsensitized rats. After 48 h, the soybean homogenates containing 1% Evans blue were injected intravenously, and the release of Evans blue by immunoglobulin E (IgE)-specific reaction was observed.

Histamine release in mast cells

In the peritoneal cavity of the unsensitized rat after anesthetizing with ether, 20 ml of Tyrode buffer B (137 mM NaCl, 5.6 mM glucose, 12 mM $NaHCO_3$, 2.7 mM KCl, and 0.3 mM NaH_2PO_4) was injected, and the abdomen was smoothly massaged for 90 seconds. After cutting the abdomen open, the peritoneal fluid was taken into a sterilized centrifuge tube, and the cells were separated by centrifigation at 150 × g for 10 min. The mast cells was separated using metrizamide by the procedure of Jippo-Kanemoto *et al.* (1993). The finally obtained mast cells were resuspended in 1 ml of α-MEM containing 15 mM of HEPES and 10% fetal bovine serum. The purity of mast cells was detected by Giemsa staining, and the cell viability was tested by tryphan blue staining method. The degree of histamine release in mast cells was measured after treating with 5% sensitized sera with 1 μg/ml of antigen or 0.5 μg/ml of A23187 as control, and cultivating in CO_2 incubator at 37°C for 30 min. From the supernatant obtained by centrifugation of cultured broth, the amount of histamine was assayed by the method of Shore *et al.* (1959).

Statistical analysis

All the experimental results were expressed as mean

value ± standard error. Dunnett's t-test was employed to analyze the significance of data obtained.

RESULTS

Identification of GM soybean

When the chromosomal DNA of GM soybean was amplified by PCR, 195-bp of DNA fragment at S35 promoter region (lane 1) and 180-bp of DNA fragment at NOS terminator region (lane 2) were observed as shown in Fig. 1. Comparatively, non-GM soybean did not give any distinctive PCR bands (data not shown). From this result, GM soybean was discriminated from non-GM soy-bean, and subjected to *in vitro* and *in vivo* allergenecity test.

Sensitization of GM soybean homogenate to rats

Based on the annually consumed amount of soybean in Korea, the soybean homogenates were sensitized subcutaneously; 2 mg/kg for low dose and 20 mg/kg for high dose in the protein basis. After 9 times of challenges (3 times a week for 3 weeks), the weight change of rats was determined (Table I). Any significant effect of GM soybean homogenate on rat growth was not detected, compared to the case of non-GM soybean homogenate. Any other apparent symptoms did not appear, either. One week after the final sensitization, the antisera were obtained from the individual rats.

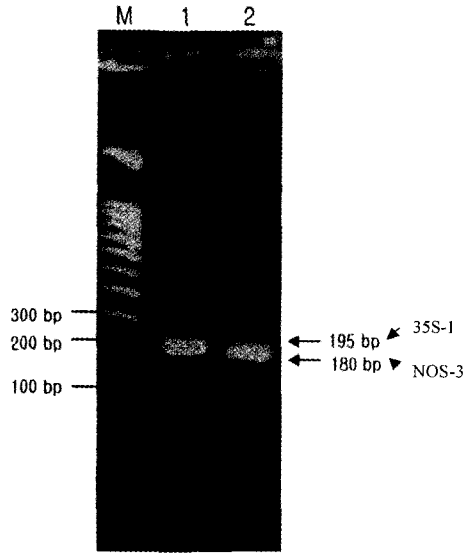

Fig. 1. A polymerase chain reaction to confirm the genetically modified soybean. Lane M, 100bp DNA ladder, lane 1, PCR product of 35S promoter region; lane 2, PCR products of NOS5 terminator region.

In vivo allergenicity test of GM soybean homogenate

In order to identify the presence of specific antibody in the sensitized sera *in vivo*, the passive cutaneous anaphylaxis reaction was tested by injecting the sensitized serum intradermally on the clipped back of the unsensitized rats. As seen in Table II, any positive release of Evans blue by IgE-specific reaction was not observed in either case of GM soybean homogenate or non-GM soybean homogenate. It shows that both soybean homogenates did not induce the passive cutaneous anaphylaxis reaction in rats, and implies that either GM or non-GM soybean did not have any severe allergenicity in the range of Korean soybean consumption.

In vitro allergenicity test of GM soybean homogenate

In order to identify *in vitro* allergenicity of GM soybean homogenate, the mast cells isolated from the unsensitized rats were treated with 5% sensitized sera and 1 μg/ml of sensitized antigen. Even though a great degree of histamine release was observed when the mast cells were exposed to 0.5 μg/ml of A23187, a calcium ionophore, as a control system (Fig. 2A), any significant increase of histamine release in mast cells by anti-(GM soybean homogenate) sera was not observed, compared to the case by anti-(non-GM soybean homogenate) sera (Fig. 2B). It implies that GM soybean is not so much allergenic than non-GM soybean.

DISCUSSION

In some agricultural countries including USA, UK, Dutch,

Table I. Body weight changes in the passive cutaneous anaphylaxis test

Groups	Day 0	1 Week	2 Week	3 Week	4 Week
Saline control	164 ± 7	218 ± 5	267 ± 7	310 ± 7	348 ± 7
GM soybean, 2 mg/kg	162 ± 4	220 ± 5	262 ± 5	314 ± 7	352 ± 5
GM soybean, 20 mg/kg	164 ± 5	225 ± 5	275 ± 6	314 ± 6	320 ± 5
non-GM soybean, 2 mg/kg	154 ± 1	199 ± 1	258 ± 2	297 ± 2	338 ± 5
non-GM soybean, 20 mg/kg	164 ± 3	234 ± 3	287 ± 5	338 ± 7	381 ± 9

GM and normal (non-GM) soybean homogenates in saline were sensitized subcutaneously as the protein basis. The test materials were injected subcutaneously 3 times a week for 3 weeks. The body weights of animals were weighed every week. A week after the last sensitization with test materials, the blood samples were collected from the inferior vena cava of individual animals for the passive cutaneous anaphylaxis test. Each value represents the mean body weight ± S.E. of four animals.

Table II. The Passive cutaneous anaphylaxis test in male Sprague Dawley rats

Groups	Challenged antigen	Body weights of recipient rats (g)	Positive ratio
Saline control	GM soybean, 20 mg/kg	300 ± 8	0/4
GM soybean, 2 mg/kg	GM soybean, 20 mg/kg	299 ± 4	0/4
GM soybean, 20 mg/kg	GM soybean, 20 mg/kg	296 ± 4	0/4
non-GM soybean, 2 mg/kg	non-GM soybean, 20 mg/kg	305 ± 12	0/4
non-GM soybean, 20 mg/kg	non-GM soybean, 20 mg/kg	311 ± 11	0/4

The antisera from the sensitized animals were diluted to 2-, 4-, 8-, 16-, 32-, 64-, 128-, and 256-folds. Each diluted serum (100 μl) was injected intradermally on the clipped back of unsensitized recipient animal with saline control. Two days after the treatment, each animal was challenged intravenously with 20 mg/kg of antigen solution containing 1% Evans blue.

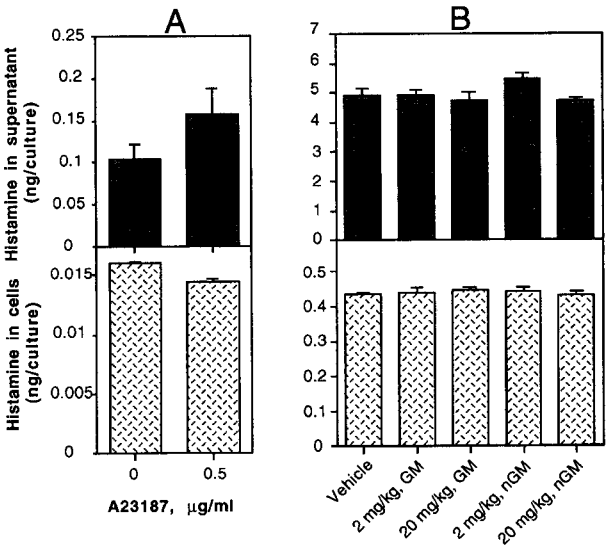

Fig. 2. Histamine release in peritoneal mast cells isolated from unsensitized, male Sprague Dawley rats. Mast cells were isolated from unsensitized animals and the cell numbers were adjusted to be 2×10^5 cells/culture (A) or 8×10^5 cells/culture (B). A23187 (0 or 0.5 μg/ml) in 0.1% DMSO (A) or 5% antisera from sensitized animals (2 or 20 mg/kg of GM or non-GM soybean homogenate) and 1 μg/ml of antigen (B) was treated to the mast cell cultures. Thirty min after the treatment, histamine concentration in the cell pellet and the culture supernatant was determined after centrifugation of culture. Each bar represents the mean histamine ± S.E. of four wells.

Canada, Australia and Switzerland, 28 GM crops including corn, cotton, soybean, papaya, chicory, potato, squash, tobacco and tomato have already been approved for their mass production in 1996 (Metcalfe *et al.*, 1996). In addition, it has been known that more than 2,000 farms in USA and 750 farms in EU are involved in the field study of GM crops for the purpose of quality improvement, easy cultivation and pest-tolerance (Hemmer, 1997).

In Korea, there has been no GM crops or vegetables officially approved by government till now. Recently Korean agricultural scientists have realized the necessity of GMO in the state of globalization, and began to study the development of GM grains and vegetables. Thus the massive production of GM crops will come true in near future in Korea. Already it has been pressed that GM crops are contaminated in some imported agricultural products, especially soybean and corn which are mainly used as animal feedstuffs. Consequently, it became necessary to formulate some regulations or guidelines for GM crops and foods in order to keep the consumers safe, because consumer societies insist that they cannot trust GM crops and foods because of their potential hazardness.

Till now, a lot of allergens in food has been described. In soybean, 11S globulins (mostly glycinin), 7S gobulins (β -con-glycinin) and oil-body linked to Gly m BD 30K (Gly m 1) were known to act as major allergens, and lectin and Kunitz trypsin inhibitor (SKT1) as minor allergens. The 65-kD Ara h1 and 10-kD Ara h2 were also reported as major allergens in peanut, and peanut agglutinin (lectin) as minor allergen. In case of mustard, allergenic 2S albumins were reported (Metcalfe *et al.*, 1996). Those food allergens have common features; they neither are destructed during food processing and cooking, nor be degraded in gastrointestinal fluids, to maintain the allergenicity, even though they are major protein components in food. Thus, in USA and Japan, they have guidelines for the safety test of GMO, including the digestibility in the simulated gastric or intestinal fluids, the degree of inactivation by heat treatment and the possibility of allergenicity.

In spite of the consumers' fear, some reports on the safety of GMO have been made by Monsanto Co. (MO, USA) (Harrison *et al.*, 1996; Hammond *et al.*, 1996; Padgette *et al.*, 1996; Taylor *et al.*, 1999; Sidhu *et al.*, 2000). They reported that GM soybean and GM corn are equivalent to the traditional non-GM cultivar in composition and nutrition. Further, they insisted that GM soybean is comparable in safety and allergenicity because the recombinant proteins were degraded in simulated gastric or intestinal fluids when administered orally.

In order to relieve them from fear, more scientific processes for the assurance of GMO safety have to be suggested. In this viewpoint, the allergenicity of GM soybean on Sprague Dawley rats was tested by employing the passive cutaneous anaphylaxis reaction and the histamine release test in mast cells. Based on the annual

consumption of soybean as food in Korea, the exposing amount of soybean protein was determined as 2 mg/kg for low dose and 20 mg/kg for high dose. Even though soybean is ingested orally and digested in gastrointestinal tract, the soybean homogenate was sensitized subcutaneously in this study, in order to confirm its allergenicity more clearly. Using the antisera recovered after 9 times of sensitization of GM soybean homogenate, the passive cutaneous anaphylaxis reaction on the clipped back of unsensitized rats was tested, but any positive results were not observed in GM soybean. Furthermore, the antisera against GM soybean did not promote the induction of histamine release in mast cells from unsensitized rats, compared to the antiserum against non-GM soybean. These results are much consistent with the previous report that the glycophosate-tolerant soybean is safe as the conventional soybeans in gavaged mice system (Harrison *et al.*, 1996). Although the results implies that GM soybean homogenate does not induce any severe allergenicity in Sprague Dawley rats, these do not directly reflect that GM soybean is non-allergenic in human.

It is not clear why the GM soybean homogenate did not present higher IgE-specific immune responses than non-GM soybean homogenate. One possibility is the coexistence of certain immune-suppressing substances like lectins in GM soybean homogenate. If not, the recombinant proteins in GMO are not so strong immunogens. In order to reveal this, the test of allergenicity induced by recombinant protein only has to be done.

Throughout this kind of efforts, the more scientific and reliable test procedures will be established for GMO safety. Especially, the development of more convenient and time-saving *in vitro* and *in vivo* test method for GMO safety is anticipated in order to evaluate a massive amount of GMO in food market.

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REFERENCES

Bradford, M. M., A rapid and sensitive method for quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.*, 72, 248-254 (1976).

Gaskell, G., Bauer, M. W., Durant, J., and Allum, N. C., Worlds apart? The reception of genetically modified foods in Europe and the US. *Science*, 285, 384-387 (1999).

Gendel, S. M., The use of amino acid sequence alignments to assess potential allergenicity of proteins used in genetically modified foods. *Adv. Food Nutr. Res.*, 42, 45-62 (1998a).

Gendel, S. M., Sequence databases for assessing the potential allergenicity of proteins used in transgenic foods. *Adv. Food Nutr. Res.*, 42, 63-92 (1998b).

Hammond, B. G., Vicini, J. L., Hartnell, G. F., Naylor, M. W., Knight, C. D., Robinson, E. H., Fuchs, R. L., and Padgette, S. R., The feeding value of soybeans fed to rats, chickens, catfish and dairy cattle is not altered by genetic incorporation of glyphosate tolerance. *J. Nutr.*, 126, 717-727 (1996).

Harrison, L. A., Bailey, M. R., Naylor, M. W., Ream, J. E., Hammond, B. G., Nida, D. L., Burnette, B. L., Nickson, T. E., Mitsky, T. A., Taylor, M. L., Fuchs, R. L., and Padgette, S. R., The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvylshikimate-3-phosphate synthase from Agrobacterium sp. strain CP4, is rapidly digested in vitro and is not toxic to acutely gavaged mice. *J. Nutr.*, 126, 728-740 (1996).

Hemmer, W., *Foods derived from genetically modified organisms and detection methods*, BATS, Basel, Switzerland (1997).

Jippo-Kanemoto, T., Kasagai, T., Yamatodani, A., Ushio, H., Tohya, K., Kimura, M., Nishimura, M., and Kitamura, Y., Supernormal histamine release and normal cytotoxic activity of Beige (Chediak-Higashi syndrome) rat mast cells with giant granules. *Int. Arch. Allergy Immunol.*, 100, 99-106 (1993).

Kim. C. S., Kamiya, S., Sato, T., Utsumi, S., and Kito, M., Improvement of nutritional value and functional properties of soybean glycinin by protein engineering. *Protein Eng.*, 3, 725-731 (1990).

Korea Food and Drug Adminstration, *The Guideline for Toxicity Tests of Drugs* (1999)

Lehrer, S. B. and Reese, G., Recombinant proteins in newly developed foods: identification of allergenic activity. *Int. Arch. Allergy Immunol.*, 113, 122-124 (1997).

Lipp, M., Brodmann, P., Pietsch, K., Pauwels, J., and Anklam, E., IUPAC collaborative trial study of a method to detect genetically modified soy beans and maize in dried powder. *J. AOAC Int.*, 82, 923-928 (1999).

Metcalfe, D. D., Astwood, J. D., Townsend, R., Sampson, H. A., Taylor, S. L., and Fuchs, R. L., Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Crit. Rev. Food Sci. Nutr.*, 36, S165-S186 (1996).

Moneret-Vautrin, D. A., Modifications of allergenicity linked to food technologies. *Allerg. Immunol.*, 30, 9-13 (1998).

Moseley, B. E., The safety and social acceptance of novel foods. *Int. J. Food Microbiol.*, 50, 25-31 (1999).

Padgette, S. R., Taylor, N. B., Nida, D. L., Bailey, M. R., MacDonald, J., Holden, L. R., and Fuchs, R. L., The composition of glyphosate-tolerant soybean seeds is equivalent to that of conventional soybeans. *J. Nutr.*, 126, 702-716 (1996).

Sidhu, R. S., Hammond, B. G., Fuchs, R. L., Mutz, J. N.,

Holden, L. R., George, B., Olson, T., Glyphosate-tolerant corn: the composition and feeding value of grain from glyphosate-tolerant corn is equivalent to that of conventional corn (*Zea mays* L.). *J. Agric. Food. Chem.*, 48, 2305-2312 (2000).

Shore, P. A., Burkhalter, A., and Cohn, V. H. Jr., A method for the fluorometric assay of histamine in tissues. *J. Pharmacol. Exp. Ther.*, 127, 182-186 (1959).

Taylor, N. B., Fuchs, R. L., MacDonald, J., Shariff, A. R., and Padgette, S. R., Compositional analysis of glyphosate-tolerant soybeans treated with glyphosate. *J. Agric. Food Chem.*, 47, 4469-4473 (1999).