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Allergenicity Assessment of Cry Proteins in Insect-resistant Genetically Modified Maize Bt11, MON810, and MON863

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Abstract This study aimed to evaluate the potential allergenicity of Cry proteins in insect-resistant genetically modified (GM) maizes (Bt11, MON810, and MON863) using serum screening tests. Serum samples were obtained from Korean children (0-15 years old) with allergic symptoms who had positive maize-specific IgE. The levels of serum specific IgE was measured by the Phadia ImmunoCAP system and considered as positive when they are 0.35 kU/L or higher. Cry proteins (Cry1Ab in Bt11, mCry1Ab in MON810, and Cry3Bb1 in MON863) were expressed in *Escherichia coli* and purified for serum screening. The reactivity of purified Cry proteins was confirmed by IgE immunoblots in 50 patients (maize-sensitized patients). There was no reaction between Cry proteins and sera from maize-sensitized patients. Our results suggest that these Cry proteins are not likely to cause allergic reactions. Further studies using more sera from patients with true clinical allergies are needed to evaluate the potential allergenicity of novel proteins in GM maize.

Keywords: genetically modified maize, Cry protein, serum, allergenicity, immunoblot

Introduction

Most commercially available genetically modified organisms (GMOs) are herbicide-tolerant (HT), insect-resistant (IT), or stacked traits (HT and IT). Herbicide-tolerant GMOs include CP4-enolpyruvylshikimate-3-phosphate synthase (*cp4 epsps*) gene derived from *Agrobacterium tumefaciens* strain CP4, *bar* gene derived from *Streptomyces hygroscopicus*, and/or *pat* gene derived from *Streptomyces viridochromogenes*. Both *bar* and *pat* genes encode phosphinothricin *N*-acetyltransferase (PAT). Insect-resistant GMOs include the *cry* gene family derived from *Bacillus thuringiensis* (Bt). Recently, GMOs with complex traits (stacked traits) have become commercially available and widely cultivated. GMOs are mainly cultivated in the U.S., China, Canada, Argentina, Australia, and Mexico. Herbicide-tolerant soybean, insect-resistant cotton, and insect-resistant maize were first cultivated in 1996 (1). In 1996, GMOs were planted on the area of 1.7 million hectares; by 2008, this area had increased by 74 fold to 125 million hectares (2). The cultivation of GMOs has been approved in 25 countries, which include the U.S., Argentina, Brazil, Canada, India, China, Paraguay, South Africa, Uruguay, Philippine, Australia, Spain, Mexico, Columbia, Chile, France, Honduras, Czech, Portugal, Germany, Slovakia, Romania, Poland, Egypt, and Burkina Faso (2). According to the 2008 ISAAA report (2), a total of 670 approvals have been granted for 144 events for 24 GMOs. GM soybean accounts for 53% of the total cultivation area, whereas GM maize is cultivated in 37.3%. Throughout the world, 8 events of GM soybean and 44 events of GM maize have been approved (2,3).

In Korea, cultivation of GMOs for commercial purposes is not permitted, but many GMO events have been approved for food and feed through safety assessments. Safety assessment of GM foods in humans is carried out by the Korea Food & Drug Administration (KFDA). As of April 2009, 57 GMOs including 3 soybeans, 29 maizes, 13 cottons, 4 potatoes, 6 canolas, 1 alfalfa, and 1 sugar beet have been approved. On the other hand, environmental risk assessment of GMOs for use in feed is conducted by the Rural Development Administration (RDA) in Korea, and a total of 45 GMOs have been approved (4). In May 2008, GM maize was imported from the U.S. for the first time for use as a food additive in Korea. More varieties of GMOs are likely to be imported into Korea for use in food; efficient and systematic safety assessments are therefore essential. Currently, safety assessments of GMOs as food evaluate the novelty, allergenic potential, antibiotic resistance, and toxicity of the GMOs. An assessment of the potential allergenicity of the proteins expressed by the transgenic genes introduced into GMOs may also be useful when determining the safety of a GMO as a food source. However, because it is not simple to examine allergenic potential and risk of GM food, debate on this issue continues between researches (5-10).

Prior to this study, there is no report on the potential allergenicity of Cry proteins in Korea. In this study, the potential allergenicity of recombinant proteins (Cry1Ab from Bt11, mCry1Ab from MON810, and Cry3Bb1 from MON863) was examined using serum from Korean children with allergic diseases.

Materials and Methods

GM maize and sera GM maize Bt11, MON810, and MON863 were provided by the BioFood team of the

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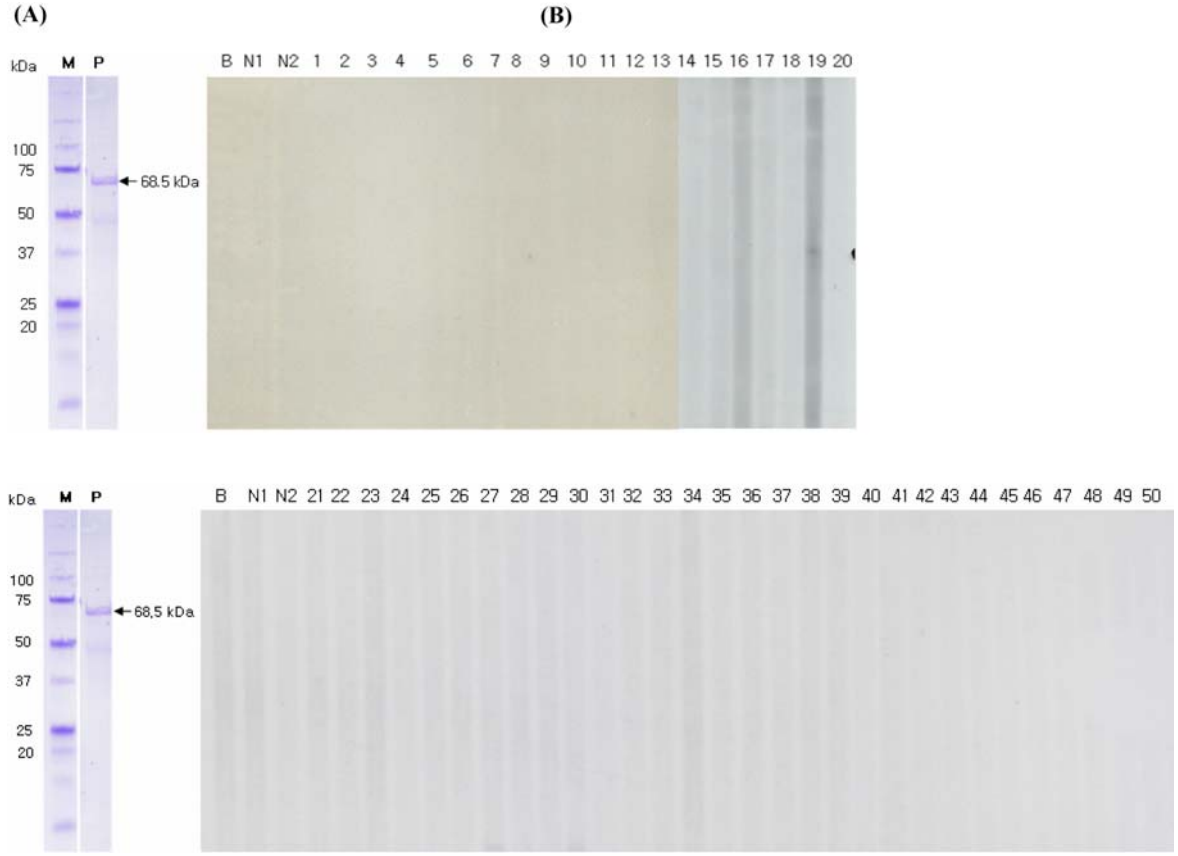

Fig. 1. (A) SDS-PAGE of Cry1Ab (GM maize Bt11), (B) immunoblot analysis with sera from 50 patients (lane 1-50) sensitized to maize. M, marker; P, recombinant Cry1Ab protein; B, blank; N1-2, negative control.

KFDA. All sera were obtained in 2007-2008. Ninety allergic patients sensitized to maize (age 1-15 years; mean age 3.1 years) were included in this study. The maize-specific IgE level of the patients was 3.64 kU/L on average. Most of the patients were diagnosed with atopic dermatitis, while some of them had asthma, urticaria, or other allergic diseases. Sera from 5 non-atopic volunteers were used as negative controls. After informed consent was received, blood samples were obtained and sera were frozen at −80°C until used. Food-specific IgE antibodies in sera were measured using the ImmunoCAP (Phadia, Uppsala, Sweden) according to the manufacturer's instructions. Antigen-specific IgE levels greater than 0.35 kU/L were considered to be positive. This study was approved by the institutional review board (IRB) of Samsung Medical Center, Seoul, Korea, and written informed consent was obtained from each patient prior to participation in this study.

Cloning of the *cry* genes from GM maize Bt11, MON810, and MON863 Three *cry* genes (*cry1Ab* in GM maize Bt11, m*cry1Ab* in GM maize MON810, and *cry3Bb1* in GM maize MON863) were separately cloned into expression vector pET15b (Novagen, Madison, WI, USA), which contains a 6-histidine tag at the amino terminus. Briefly, genomic DNA was extracted from GM maize Bt11, MON810, and MON863. Polymerase chain reactions (PCR) were carried out with the following primer pairs: Bt11 (cry)-F: ggaattccatatggacaacaacccaaacatcaacgaat, Bt11 (cry)-R gcgcggatcctcagtactcagcctcgaaggtaacttcg, MON810 (cry)-F: ggaattccatatggacaacaacccaaacatcaacgagt, MON810 (cry)-R: gcgcggatccgtactcggcttcgaaggtgacctcggct, MON863 (cry)-F: ggaattccatatggccaaccccaacaatcgctccgagc, and MON863 (cry)-R: gcgcggatcctcacagctggacggggat gaactcgatc. The underlined letters indicate the restriction enzyme sites. The amplification of the *cry* genes was performed under the following conditions: pre-incubation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 2 min, and terminal elongation at 72°C for 8 min. PCR was carried out on a Mastercycler (Eppendorf, Hamburg, Germany). PCR products and pET15b vector were digested with *Nde*I (New England Biolabs, Beverly, MA, USA) and *Bam*HI (New England Biolabs), and then ligation was performed using T4 DNA ligase (Takara, Shiga, Japan) at 16°C for 1 hr. *E. coli* DH5α competent cells (Novagen) were transformed with the ligation reaction solution. The recombinant plasmids were sequenced using an ABIPRISM 3700 DNA analyzer (Perkin Elmer, Boston, MA, USA).

Expression and purification of the Cry proteins The recombinant plasmids were transformed into *E. coli* BL21 (DE3) (Novagen) and transformants were selected on a LB agar plate containing ampicillin (50 μg/mL). A single colony was picked and transferred into 5 mL of LB medium containing ampicillin (50 μg/mL) and incubated at 37°C for 18 hr. About 500 μL culture was transferred into 50 mL of LB medium containing ampicillin (50 μg/mL)

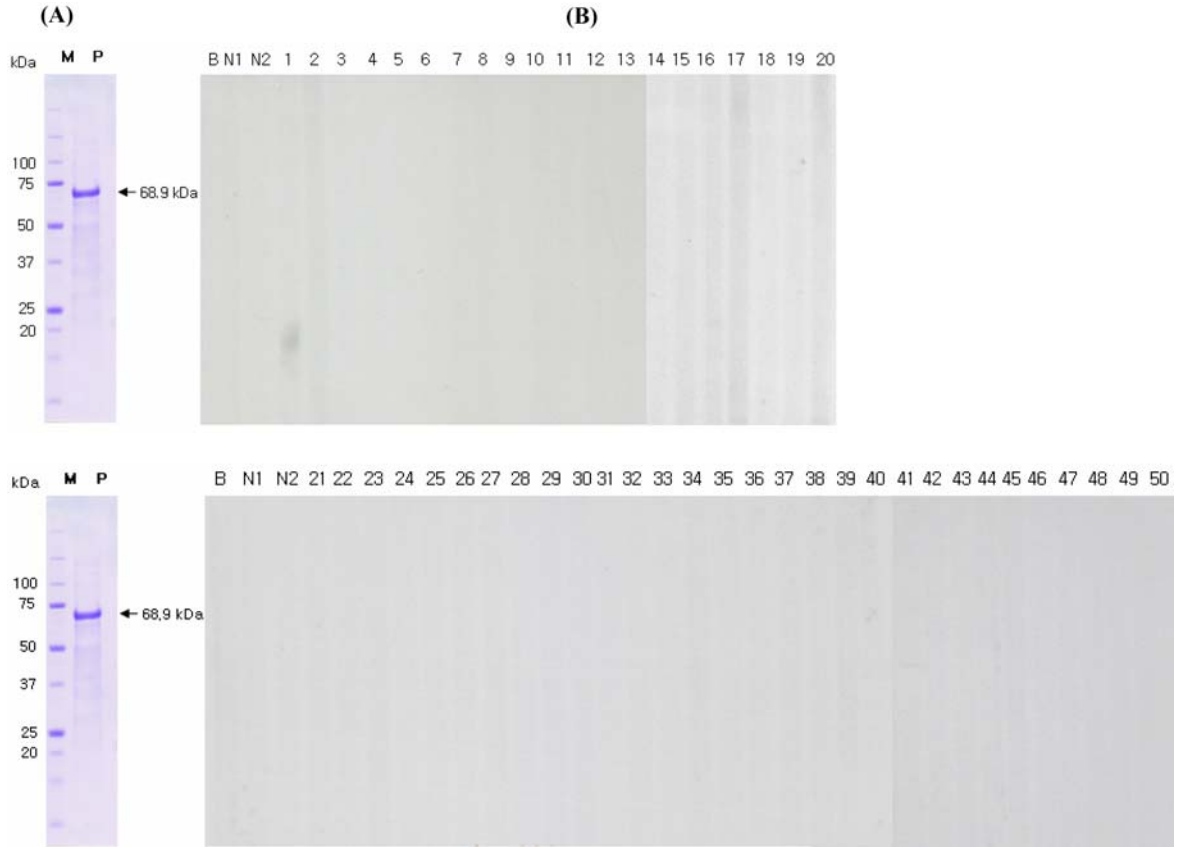

Fig. 2. (A) SDS-PAGE of mCry1Ab (GM maize MON810), (B) immunoblot analysis with sera from 50 patients (lane 1-50) sensitized to maize. M, marker; P, recombinant mCry1Ab protein; B, blank; N1-2, negative control.

and incubated at 37°C until the optical density (OD) was between 0.4 and 0.6. At this time, isopropyl-β-D-thiogalactopyranoside (0.4 mM IPTG) was added to induce protein expression and incubation continued at 37°C for 4 hr. Cell lysates were taken before and after IPTG induction to measure the protein expression and were applied to a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel, which was stained with Coomassie blue brilliant R-250. The cells were harvested and resuspended in 10 mL of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). After sonification and centrifugation at 5,000×g for 15 min, the supernatants containing recombinant proteins were applied to a 2.5 mL Ni^{2+}-chelated nitrilotriacetic acid (NTA) column. The column was washed with 10 volumes of a binding buffer and 6 volumes of a washing buffer [60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9], and the proteins were eluted with an elution buffer [1 M imidazole, 0.5 M NaCl, 10 mM Tris-HCl, pH 7.9]. The purified proteins were analyzed using SDS-polyacrylamide gel electrophosis (PAGE).

Immunoblot analysis Purified Cry proteins were transferred to nitrocellulose membrane, which were then cut into strips. The strips were blocked with 2% non-fat dried milk (NFDM) for 2 hr at room temperature, and were then incubated with sera diluted 1:10 in phosphate buffered saline (PBS)-Tween plus 2% NFDM overnight at room temperature. The strips were then washed with 3 times with PBS for 15 sec each and incubated with rabbit anti-human IgE (KPL, Gaithersburg, MD, USA) diluted in PBS-Tween plus 2% NFDM for 1 hr at room temperature. Membranes were washed with PBS 3 times as above, and incubated with biotinylated goat anti-rabbit IgG (KPL) diluted in PBS-Tween plus 2% NFDM for 1 hr at room temperature. Membranes were washed with PBS 3 times, and were then incubated with NeutrAvidin-HRP (Pierce Chemical Co., Rockford, IL, USA) for 25 min at room temperature, followed by a further 3 washes with PBS. Membranes were reacted with Amersham ECL reagent (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Blotted membranes were exposed to Kodak Imaging Film and the film was then developed. According to the above procedure, the inhibition assay was also performed using PBS buffer, Cry3Bb1, dust mite (Der f), rice, and egg white antigens. All antigens were used at a concentration of 10 μg/mL.

Results and Discussion

Expression, identification, and purification of recombinant proteins Each recombinant protein had 6 N-terminal histidines because the pET vector expression system was used. The His-tagged proteins were isolated and purified using a Ni^{2+}-chelated NTA column and analyzed using SDS-PAGE (lane P, Fig. 1-3). Cry1Ab from GM maize Bt11 had an apparent molecular weight of 68.5 kDa, while the molecular weight of mCry1Ab from GM maize

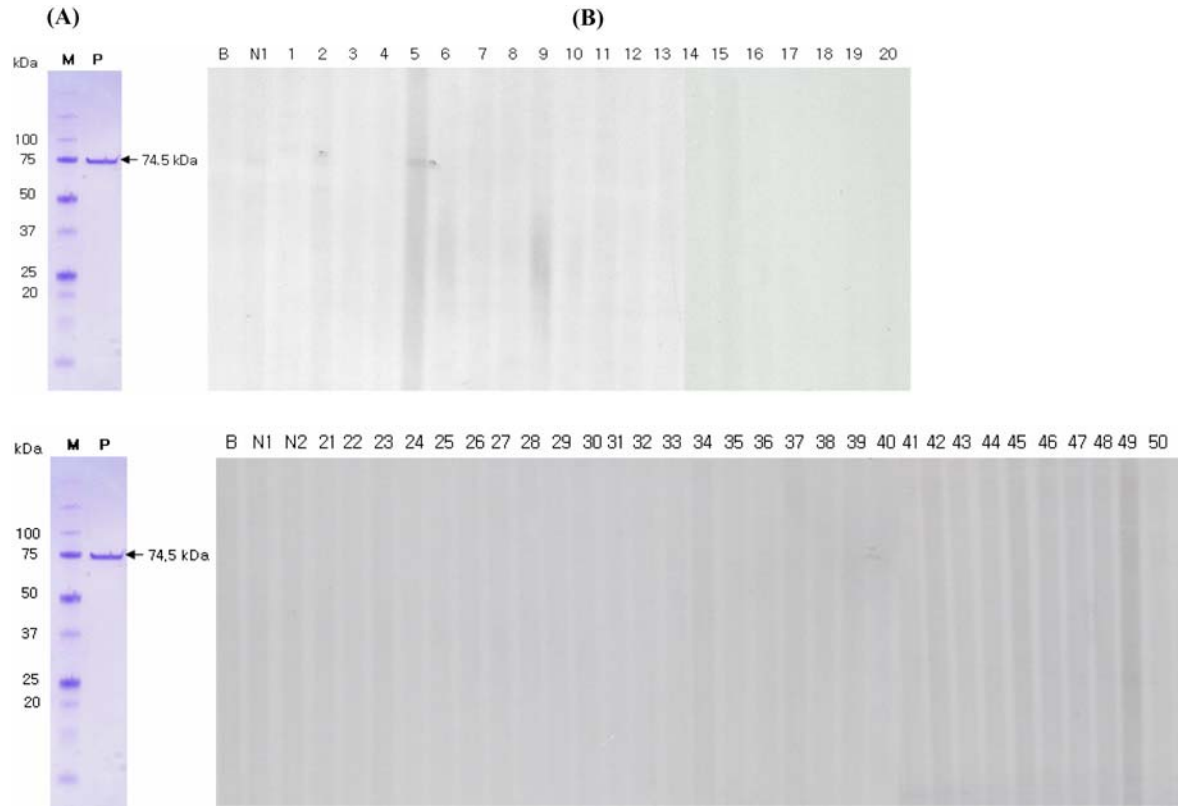

Fig. 3. (A) SDS-PAGE of Cry3Bb1 (GM maize MON863), (B) immunoblot analysis with sera from 50 patients (lane 1-50) sensitized to maize. M, marker; P, recombinant Cry3Bb1 protein; B, blank; N1-2, negative control.

MON810 and Cry3Bb1 from GM maize MON863 were 68.9 and 74.5 kDa, respectively. Western blot analysis was also performed using anti-His_6-peroxidase to confirm the isolation of each protein (data not shown).

Immunoblot analysis of Cry1Ab from GM maize Bt11 Immunoblot analysis of the Cry1Ab protein from GM maize Bt11 was carried out using serum from 50 patients (34 males and 16 females) who had positive maize-specific IgE. Of these patients, 42 had atopic dermatitis, 2 had asthma, 3 had chronic urticaria, and 3 had other allergic diseases. The ages of patients ranged between 0-15 years with an average age of 3.1 years. The total IgE was 2,053.6 kU/L on average and that of maize-specific IgE was 4.7 kU/L. None of the 50 sera (patient 1-50) showed a response to Cry1Ab (Fig. 1).

Immunoblot analysis of mCry1Ab from GM maize MON810 GM maize MON810 is insect resistant due to presence of the mCry1Ab protein, which is also present in GM maize Bt11. However, as the DNA and amino acid sequences of these 2 genes are different in these 2 GM maizes, the IgE response for each recombinant protein was assessed. Immunoblot analysis of the mCry1Ab protein from GM maize MON810 was performed using serum from 50 patients (33 males and 17 females) who tested positive for maize-specific IgE. Of these patients, 43 had atopic dermatitis, 1 had asthma, 4 had chronic urticaria, and 2 had other allergic diseases. Patients' age ranged between 0-15 and their mean age was 3.4 years. The level of total IgE was 1,683.9 kU/L while the maize-specific IgE was 3.4 kU/L on average. As shown in Fig. 2, immunoblot analysis showed no binding between 50 sera from allergic children with positive maize-specific IgE (patient 1-50) and mCry1Ab.

Immunoblot analysis of Cry3Bb1 from GM maize MON863 The immunoblot analysis of Cry3Bb1 protein from GM maize MON863 was performed using serum from 50 patients (35 males and 15 females) who tested positive for maize-specific IgE. Of the patients, 43 had atopic dermatitis, 2 had asthma, 3 had chronic urticaria, 1 had rhinitis, and 1 had other allergic disease. The average age of the patients was 3.2 years. The average total IgE was 1,861.8 kU/L and the average maize- specific IgE was 4.0 kU/L.

In the immunoblot analysis, only patient #5 showed a weak response while the remaining patients did not show any response (Fig. 3). Therefore, we carried out an inhibition assay to examine the specific IgE antibody reaction to Cry3Bb1 protein using PBS buffer, Cry3Bb1, dust mite (Der f), rice, and egg white antigens (Fig. 4). Because inhibition was not observed by Cry3Bb1, the positive response shown in Fig. 3 was concluded to be a non-specific IgE reaction (Fig. 4).

The risk of eliciting allergic reactions has been one of big concerns of GM foods. Several methods have been proposed to assess the potential allergenicity of novel proteins in GM foods, on the basis of evaluating the source of the gene, sequence similarities to known allergens,

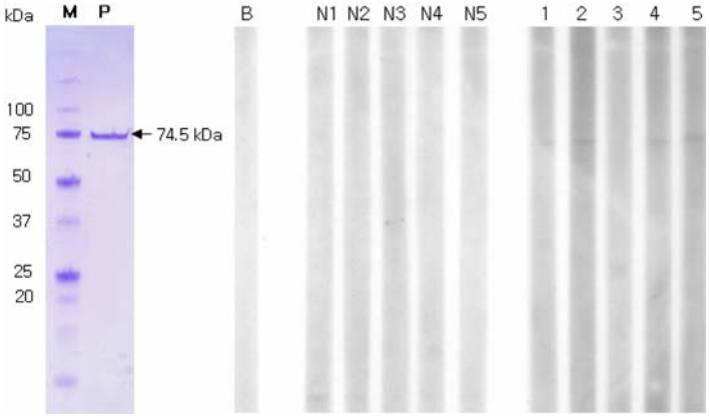

Fig. 4. Immunoblot-inhibition assays of Cry3Bb1 (MON863) with sera using PBS buffer, Cry3Bb1, Der f, rice, and egg white. M, marker; P, recombinant Cry3Bb1 protein; B, blank; N1-5, negative control; Lane 1-5, PBS buffer, Cry3Bb1, Der f, rice, and egg white, respectively.

stability of the protein in pepsin and *in vitro* serum IgE binding (11). However, no single standard approach is determined as yet. In addition, more factors should be considered for allergenicity assessment, because allergic responses are affected by genetic factors, age, dose or duration of exposure, eating habits, and so on. In this regard, several countries, including Korea, have established a serum bank for allergenicity assessment of GM foods (12) in order to collect as many sera as possible from various allergic individuals in each region.

Cry proteins in the insect-resistant GM foods have been assessed. The Cry1Ab protein is digested rapidly in artificial gastric juice (13). Batista *et al*. (14) found no response after performing skin prick test for Cry1Ab to 77 patients and negative IgE immunoblotting results for 54 patients. An IgE-enzyme linked immunosorbant assay (ELISA) for Cry1Ab using the serum from 54 patients and 10 normal people was done in Japanese study, where no difference in IgE responses between these 2 groups was found (15). Herman *et al*. (16) reported digestibility of Cry34Ab1 and Cry35Ab1 in simulated gastric fluid. In the present study, we examined the IgE responses to Cry proteins from 3 different GM maizes (Cry1Ab from Bt11, mCry1Ab from MON810, Cry3Bb1 from MON863) by using sera from allergic children who had positive maize-specific IgE. Immunoblotting analysis showed no detectable IgE antibodies against these novel proteins, suggesting that Cry proteins are not likely to cause allergic reactions.

Although our results support the previous studies in which Cry proteins seem to be safe in terms of allergenic potential (13-16), further studies are needed for conclusion, because of the limitations in our study. First, 50 sera from patients with allergic diseases were used for assessment of each Cry proteins, but this study population is not large enough to reach a definitive conclusion about the potential allergenicity of GM maize. Secondly, we did not perform double-blind, placebo-controlled food challenge tests in our patients to confirm the maize allergy. It is not clear whether they have true clinical allergy to maize. Third, the mean age of our patients was 3.1 years. Although infants and young children are more susceptible to food allergies than adults, duration and amount of exposure to GM maize in our patients could not be assessed. Therefore, no IgE response in our serum screening test might be because our patients are not yet allergic to GM maize due to insufficient exposure, not because Cry proteins are safe.

In conclusion, Cry proteins from insect-resistant GM maize (Cry1Ab, mCry1Ab, and Cry3Bb1) are not likely to have allergenic potential. Postmarket surveillance using more sera from patients with true clinical allergies are needed to evaluate the potential allergenicity of novel proteins in GM maize.

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