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Research on the Allergic Potential of Insecticidal Cry1Ac Proteins of Genetically Modified Rice

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Abstract In Korea, different kinds of genetically modified (GM) crops are under development, including GM-rice expressing insecticidal crystal (Cry) proteins of *Bacillus thuringiensis (Bt)* modified to change a single amino acid. In this study, amino acid (aa) sequences of modified Cry proteins were compared to that of known allergens, and Cry proteins expressed in GM-rice were identified by using Cry protein specific polyclonal antibody. The antigen-antibody reactions were compared between GM and commercial rice to assess the allergic risk of Cry proteins. This analysis showed no known allergen to have more than 35% aa sequence homology with modified Cry proteins in *Bt* rice over an 80 aa window or to have more than 8 consecutive identical aa. Sera from allergic patients showed some IgE reactivity via immunoblotting and enzyme-linked immunosorbent assay (ELISA), although no differences were seen between GM and commercial rice. Based on these results we conclude that GM rice with modified Cry proteins has no differences in its protein composition or allergenicity relative to commercial rice.

Key words: genetically modified food (GMO), *Bt*, allergenicity, rice

Introduction

The incidence of IgE-mediated allergic reactions to foods is increasing as well as the severity of associated symptoms. Numerous foods are now incriminated, most likely due to modifications of dietary habits and increased exposure to new or modified food ingredients. Accordingly, there is a great desire to evaluate the allergenic potential of components in our daily food.

It is still not well known why some proteins cause allergic reactions in some people but not in others. In general, it is accepted that altered dietary preferences as well as changes in food manufacturing and food formulation practices can have significant implications for food allergy development (1). As an example, allergy to peanuts occurs at a significant frequency in North America and Western Europe, but not in other countries where peanuts are less commonly eaten (2).

Genetic engineering techniques are developing rapidly, and as a result many people of the world are now exposed to various kinds of new genetically modified foods or organisms (3-5). Genetically modified organisms (GMOs) are a result of genetic engineering involving recombinant DNA technology to transfer genes from one organism to another (6).

In recent years, along with gene transfer to gut microflora (7), food allergy (8) has received considerable attention as one aspect of the safety assessment of new foods. The expression of novel proteins derived from transferred genes in GM foods carries a risk of eliciting allergic reactions in individuals sensitive to the introduced protein and a risk of sensitizing susceptible individuals. Therefore, the introduction on the market of food composed of or derived from GMOs raises the question of their potential allergenicity. Due to this risk, it is necessary to obtain a cumulative body of evidence to minimize any uncertainty.

Bacillus thuringiensis (*Bt*) is a gram-positive soil bacterium characterized by its ability to produce crystalline inclusions during sporulation. These crystal inclusions consist of proteins (Cry proteins) exhibiting a highly specific insecticidal activity (9, 10). *Bt* crystalline inclusions dissolve in the larval midgut, releasing one or more insecticidal Cry proteins of 27 to 140 kDa. The activated toxin interacts with the midgut epithelium cells of susceptible insects and generates pores in the cell membrane. The larva stops feeding and eventually dies. Based on the insecticidal effects of Cry proteins, *Bt* has been used as biopesticide since the 1960s. Recently, insect-resistant transgenic crops such as maize, cotton, and potato expressing the *Bt* cry gene were developed and commercialized.

Bt rice, officially released in Iran in 2004, was grown on approximately four thousand hectares in 2005 by several hundred farmers who initiated commercialization of GM rice in Iran and produced supplies of seed for full commercialization on 2006. Iran and China are the most advanced countries in the commercialization of GM rice, which is the most important food crop in the world.

In this study we investigated the allergic safety of *Bt* rice expressing a modified cry1Ac gene developed by the Korean Rural Development Administration, which has already field tested GM rice in pre-production trials and is expected to approve GM rice in the near-term.

Materials and Methods

Screening of amino acid sequence homology in the allergen DB The Cry1Ac gene (Accession no. DQ 195217) was modified to replace the leucine residue at position 156 with proline. The entire amino acid sequence of the modified cry1Ac protein was analyzed for similarity

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to the amino acid (aa) sequences of all known allergens present in databases using an 80 amino acid window size with the program IDGeno (http://210.219.43.129/align/IDGeno.html) developed by the Korean Research Institute of Bioscience and Biotechnology, and the Allergen DB AllerPredict (http://sdmc.i2r.a-star.edu.sg/Templar/DB/Allergen/). Proteins with more than 35% identity in any 80 amino acid window or more than 8 consecutive identical amino acids with known allergens were considered to have a high possibility of cross reaction with their counterparts in the alignment.

Protein extraction In order to examine the characteristics of each antigen, total protein was isolated from GM and conventional rice with or without heating. The heating process was carried out using an electric rice cooker. Heated or unheated rice samples were extracted as follows:

The rice sample was ground into powder. To 5 g of rice powder, 25 mL of phosphate buffered saline (PBS) was added followed by incubation at 4°C for 2 hr. After centrifugation at 20,400×g for 10 min, the supernatant was filtered and stored at -20°C until use.

Electrophoresis Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of rice extract was performed as described in the method of Son *et al.* (11). To compare the protein contents of GM and non-transgenic parental rice, proteins were extracted and separated by SDS-PAGE as described in the method of Keum *et al.* (12) using a 13% gel. The gel was stained with Coomassie blue (0.025% Coomassie brilliant blue R250, 5% methanol, 7.5% acetic acid) and destained with 25% methanol, 7.5% acetic acid. For immunoblotting, separated proteins are transferred to polyvinylidene fluoride (PVDF) membrane.

Immunoblotting After transferring the separated proteins to PVDF membrane by tank blotting, the membranes were blocked with 2% non-fat dried milk (NFDM) containing 0.3% Tween 20 in PBS (PBST) for 30 min to avoid nonspecific binding. The membranes were cut into 4-5 mm wide strips and probed with 1:3000 dilutions of polyclonal antibody for 6 hr at room temperature. Immunostaining of bound IgG antibodies was performed with biotin coupled to Protein G (1:2000, 1 hr in room temperature; Pierce Biotechnology; Rockford, IL, USA) and horse redox peroxidase conjugated to NeutroAvidin (1:8000, 30 min in room temperature; Pierce Biotechnology) followed by addition of the 3,3',5,5'-tetramethyl-benzidine (TMB) substrate (KPL, Gaithersburg, MA, USA). The strips were washed 3 times with PBST between each step.

Identification of Cry protein in *Bt* rice using Cry1Ac protein specific polyclonal antibody To determine the optimal conditions for specific binding of polyclonal antibody to Cry proteins in *Bt* rice, 5 PVDF strips containing SDS-PAGE separated proteins from GM or non-GM rice were prepared and incubated with various antibody dilutions (Table 1). As a negative control for nonspecific binding, strip 4 and strip 5 were incubated without primary antibody and strip 5 was further incubated without secondary antibody. Strips 1, 2, and 3 were incubated with primary antibody dilutions of 1:3000, 1:6000, or 1:10000.

Enzyme-linked immunosorbent assay (ELISA) Isolated protein (5 ug/mL) was fixed in a 96 well plate (NUNC polysorb, Denmark) and incubated for 16 hr with serum (1:20 dilution in PBST) obtained from an allergic patient. The serum plate was incubated with biotinylated Protein G (1:4000 dilution) and NeutroAvidin (1:8000 dilution) for 1hr in room temperature, followed by incubation with the TMB peroxidase substrate (KPL) for 10 min at 37°C and measurement of color intensity at 450 nm. The plate was washed 3 times between each step with PBST buffer.

For the inhibitions ELISA test, we coated one 96 well plate with GM rice extracts and the other with non-GM rice extracts. GM rice extracts or non-GM rice extracts were used as inhibitors.

Human sera, polyclonal antibody, and specific IgE Sera from 9 patients (Table 2) were investigated in this study. Serum from a non-allergic patient was used as a control. Diagnostic measurements of allergen-specific IgE were performed with the CAP system FEIA (Pharmacia & Upjohn, Uppsala, Sweden). Sera were taken from all patients and stored at -20°C until used. Polyclonal antibody against Cry protein was obtained from Abraxis Bioscience (Schaumburg, IL, USA).

Detection of Cry1Ac protein in GM rice For the detection of Cry proteins, commercial detection strips (Strategic Diagnostic Inc., Newark, DE, USA) were purchased. GM and non-GM rice was ground into a fine powder, dissolved in 1× buffer provided by the manufacturer and incubated until the liquid buffer reached to the top of the strip.

To identify Cry protein in *Bt* rice, commercially available Cry1Ac protein specific rabbit polyclonal antibody (Abraxis Bioscience) was purchased and diluted with PBS to a concentration of 1 mg/mL.

Results and Discussion

Screening of amino acid sequence homology in the allergen database Comparison of amino acid sequence of an introduced protein with known allergens is a useful

Table 1. Immunoblotting conditions for the identification of Cry1Ac protein in *Bt* rice

	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5
Primary antibody (polyclonal antibody)	1:3000	1:6000	1:10000	-	-
Secondary antibody (Biotin-ProteinG)	1:2000	1:2000	1:2000	1:2000	-
Tertiary antibody (strepavidin conjugated alkaliphosphate peroxidase)	1:3000	1:3000	1:3000	1:3000	1:3000

Table 2. Characteristics of 9 patients' sera

Patients sera	Age (year)	Sex	Total IgE	Specific IgE against rice proteins
48	5	F	340	0.55
52	0.5	M	76.4	1.48
86	2	M	2755	0.46
185	4	F	461	1.89
258	1	F	974	11.7
259	8	M	398	Not done
296	2	F	585	16.6
374	9	F	5300	16.9
357	5	F	15.8	Not done

indicator of allergenic potential, particularly for proteins that have not previously been part of the food supply. 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 (13). In addition, the important IgE binding epitopes of many allergenic proteins have been mapped (14), with the optimal peptide length for IgE binding being at least 8 amino acids (15). The Cry1Ac gene in GM rice developed by the Korean Rural Development Administration differs by one aa from the native protein. The leucine at position 156 of the Cry1Ac protein is replaced with proline. Although there is no report that the Cry1Ac protein has a homologous stretch of more than 8 consecutive aa with any known allergen, it is still necessary to check whether the single aa modification in the Cry1Ac protein results in significant homology with known allergens. Comparison of the modified cry1Ac protein with known allergens revealed no biologically and immunologically significant homology, specifically no more than 35% homology 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 modified cry1Ac protein and other known allergens due to amino acid sequence homology, and therefore the modified cry1Ac protein is unlikely to be allergenic.

Comparison of the protein bands of GM and non-GM rice SDS-PAGE results showed almost the same distribution of protein bands among all rice varieties regardless of whether it is GM or not (Fig. 1). Although some protein bands are not identical in rice varieties analyzed in this study, it is clear that the differences are not caused by genetic modification, because there is no difference of specific protein bands between GM rice and commercial non-GM rice. It is well known that crops or fruits have a different protein composition depending the variety or conditions in which it is grown. In the case of apples, Son *et al.* (16) reported that the protein content of apples is different depending on the variety. Based on SDS-PAGE, most rice proteins are between 10 and 100 kDa in size.

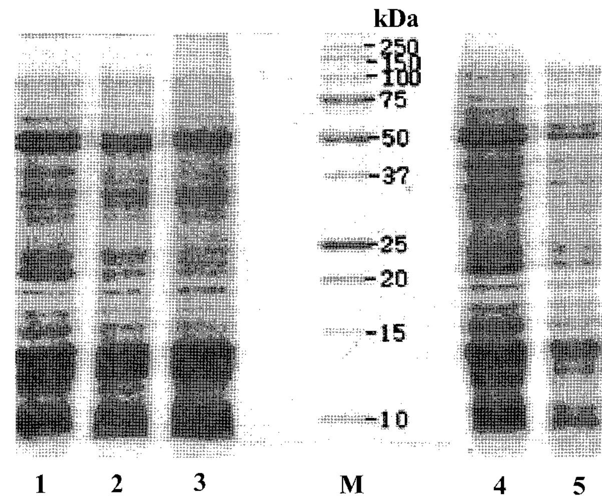

Fig. 1. Comparison of the protein bands of GM rice and a non-transgenic parental rice variety (Nakdong) and other commercial rice varieties. Rice extracts were separated by SDS-PAGE and stained by Coomassie. Lane 1, unpolished rice; lane 2, wild rice; lane 3, unpolished sprout rice; lane 4, cooked non-GM rice (Nakdong); lane 5, GM rice; M, molecular weight marker.

Most protein extracts from foods such as soy, peanuts, or milk show no differences in SDS-PAGE analysis before and after heating. To evaluate rice protein extracts before and after heating, we prepared protein extracts from cooked and uncooked rice. Proteins extracts of different strains of rice prior to heating showed about 10 times higher protein concentration as that of heated rice (Table 3). Two rice products (cooked unpolished rice Hetbahn and cooked unpolished sprout rice Hetbahn) bought in the market show a 2 to 4 times higher protein concentration than rice heated in an electric rice cooker. Most of the protein bands of cooked rice extracts larger than 15 kDa were disappeared by SDS-PAGE analysis. The heating process apparently leads to a change in the structure of rice proteins such that most of the rice proteins become

Table 3. Protein concentration of heated/raw rice extracts

	Rice type	Protein conc. (ug/mL)
Heated	unpolished rice	50
Heated	wild rice	25
Heated	unpolished sprout rice	40
Heated	unpolished rice Hetbahn	283
Heated	unpolished sprout rice Hetbahn	125
Heated	GM rice	50
Heated	Non-GM rice (Nakdong)	75
Unheated	unpolished rice	750
Unheated	wild rice	650
Unheated	unpolished sprout rice	650
Unheated	GM rice	515
Unheated	Non-GM rice (Nakdong)	560

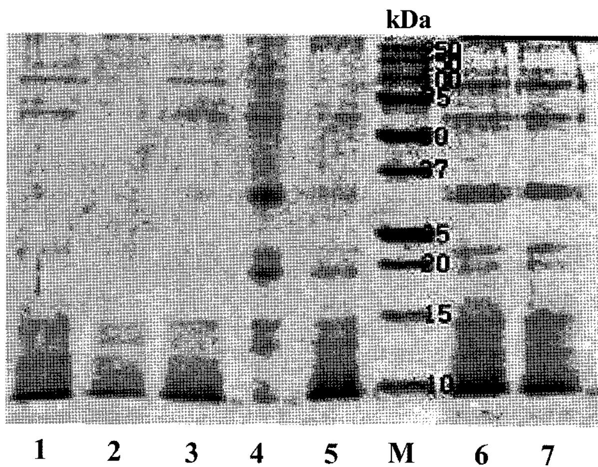

Fig. 2. Coomassie stained SDS-PAGE Gel of cooked rice. Lane 1, cooked unpolished rice; lane 2, cooked wild rice; lane 3, cooked unpolished sprout rice; lane 4, cooked unpolished rice Haetban; lane 5, cooked unpolished sprout rice Haetban; lane 6, cooked GM rice; lane 7, cooked non-GM rice (Nakdong); M, molecular weight marker.

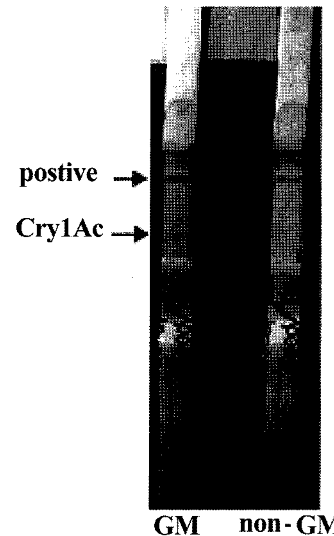

Fig. 3. Identification of GM rice using commercial Cry1Ac protein detection strips.

insoluble. No difference in the protein bands of heat treated GM and non-transgenic rice was observed (Fig. 2).

Identification of Cry1Ac protein in *Bt* rice A positive control band appeared clearly on the upper side of the strip in a few minute regardless if the rice sample is GM or not. On the other hand, the Cry protein band appeared very faint, even after a long incubation time, in the GM rice sample that it was hard to recognize at first (Fig. 3). The faint Cry protein band on the test strip suggests that the amount of expressed Cry proteins in *Bt* rice may be extremely low.

To identify Cry1Ac protein in *Bt* rice, PVDF strips of separated GM and non-GM rice proteins were incubated with Cry1Ac protein specific polyclonal antibody. A specific Cry1Ac protein band (marked with an arrow in Fig. 4) was identified on strips 1, 2, and 3 with the expected molecular size of 69 kDa. It faded out with increasing dilutions of Cry1Ac specific antibody. The 618 aa Cry1Ac protein and the modified cry1Ac protein are expected to have a theoretical molecular weight of 69.3 kDa which correlates very well with the results of our analysis.

One nonspecific band of approximately 70 kDa was present in all 5 strips.

Allergenicity of GM versus non-GM rice by serum

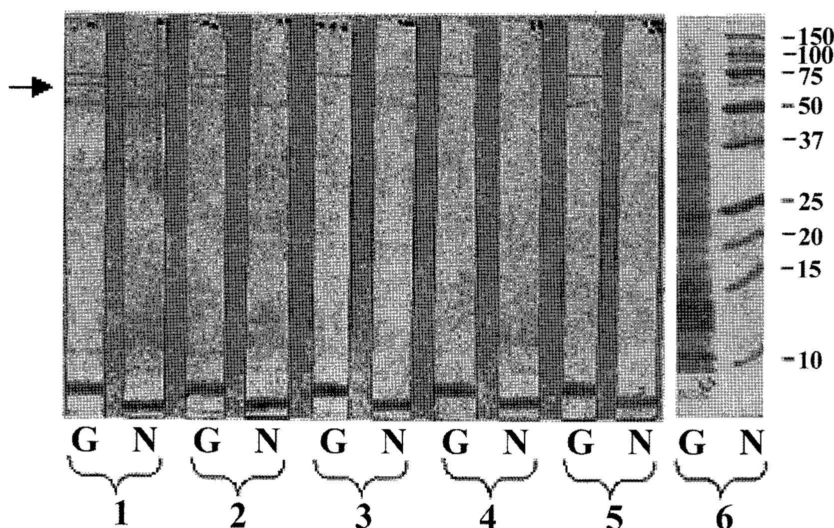

Fig. 4. Identification of Cry1Ac protein in *Bt* rice using Cry1Ac specific polyclonal antibody. Strips were incubated with polyclonal antibody diluted to 1:3000 (lane 1), 1:6000 (lane 2), 1:10000 (lane 3), without polyclonal antibody (lane 4), or without polyclonal antibody and without biotin protein G (lane 5). Small pieces of membrane containing GM rice and molecular weight marker were stained with Coomassie (lane 6). G, genetically modified rice; N, non-genetically modified rice (Nakdong); M, molecular weight marker.

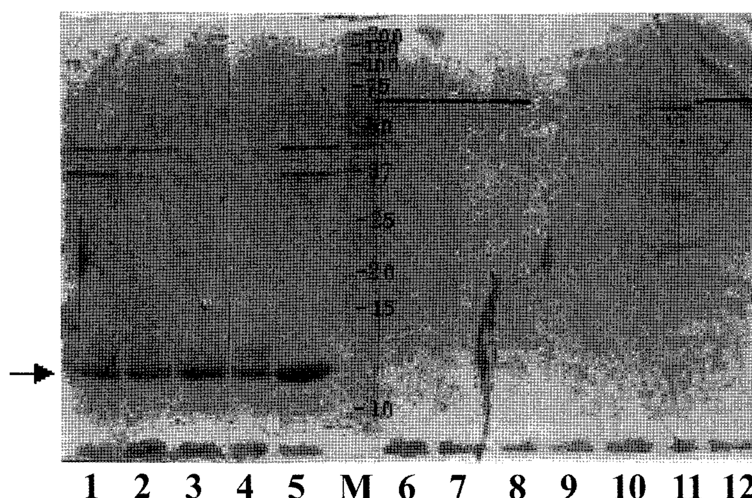

Fig. 5. Immunoblotting of raw (lane 1-5) / cooked (lane 6-12) rice protein with serum from an allergic patient. Lane 1, unpolished rice; lane 2, wild rice; lane 3, unpolished sprout rice; lane 4, non-GM rice (Nakdong); lane 5, GM rice;lane 6, cooked unpolished rice; lane 7, cooked wild rice; lane 8, cooked unpolished sprout rice; lane 9, cooked non-GM rice (Nakdong); lane 10, cooked GM rice; lane 11, cooked unpolished rice Haetban; lane 12, cooked unpolished sprout rice Haetban; M, protein molecular weight marker.

test To confirm whether heating influences the antigen-antibody reactions of rice, a rice allergic patient's serum was incubated with proteins isolated from 5 different rice varieties (heated or raw) and 2 commercially available cooked rice products. As shown in the Fig. 5, this patient's serum IgE bound to the protein from raw rice with molecular weight size between 10 and 15 kDa (Fig. 5, lane 1 to 5, marked with an arrow). Heating caused the loss of antigen-antibody reactivity and showed no IgE bound protein band from heated rice (Fig. 5, lane 6 to 12). This result agreed well with a report that the heating of rice for 30 min at 100°C reduces up to 60% of its binding activity for IgE compared with that of raw rice (17).

For the comparison of allergenicity of GM rice and non-GM rice, blots prepared from GM rice and non-GM rice were incubated with 9 allergic patients' sera individually. One blot was incubated with non-allergic serum and one without serum as a control for nonspecific binding.

IgE reactivity to rice proteins was different among the patients, however all 9 patients' sera showed exactly the same reaction pattern with GM and non-GM rice. Some patients' sera (Fig. 6, no. 5, 7, and 9) showed more antigen-antibody reaction bands than others.

This result suggests that there were no differences in allergenicity between GM and non-GM rice due to the introduction of cry gene into rice and the Cry1Ac protein itself.

Major allergens are defined as the antigens which react with IgE from more than 50% of tested patients (18). According to several publications about rice allergy (19-24), a multigene family of 14-16 kDa proteins presents the major allergens from rice seeds/grain which showed significant homology to the α-amylase/trypsin inhibitor family from wheat and barley. Other rice seed/grain allergens are a recently described 33 kDa major and a 60 kDa minor allergen. In our study most of the patients showed antigen-antibody reactions between 10-80 kDa.

Serum screening and inhibition ELISA The reactivity of rice antigens and allergic patients sera was also tested using ELISA. The ELISA test itself involves coating a 96 well plate with food antigens, adding a patient's sera and looking for a classic antigen/antibody interaction. For the test, 9 allergic patients' sera were used and one non-allergic serum and one serum-free sample were tested as a negative control. Three patients' sera (258, 296, and 374) of the 9 investigated showed relatively high reactivity (Fig. 7). The rest of the sera revealed reactivity similar to the negative controls (non-allergic serum or without serum). There is no difference in antigen-antibody reactivity between GM and non-GM rice. Based on this result it can be concluded that there were no changes in allergenicity caused by introducing cry genes except perhaps to the expressed Cry proteins themselves in *Bt* rice.

To confirm the specificity of these reactions between rice antigens and patients' serum IgE, we have chosen 2 patients sera (258 and 296), which showed relative high reactivity by ELISA screening and carried out an inhibitions ELISA test.

The inhibition rate increased depending on the inhibitor

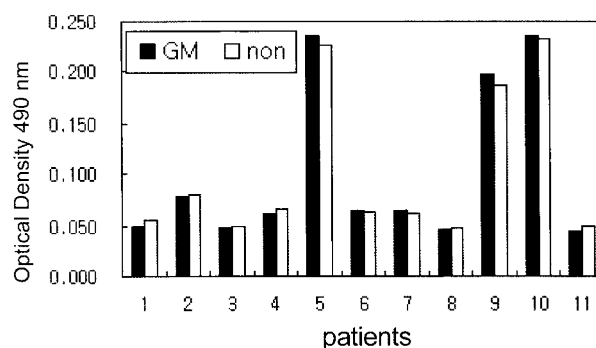

Fig. 7. Screening of sera from rice allergic patients with GM/ non-GM rice protein by ELISA. Lane 1, no serum; lane 2, 48; lane 3, 52; lane 4, 86; lane 5, 258; lane 6, 185; lane 7, 259; lane 8, 357; lane 9, 374; lane 10, 296; lane 11, non-allergic.

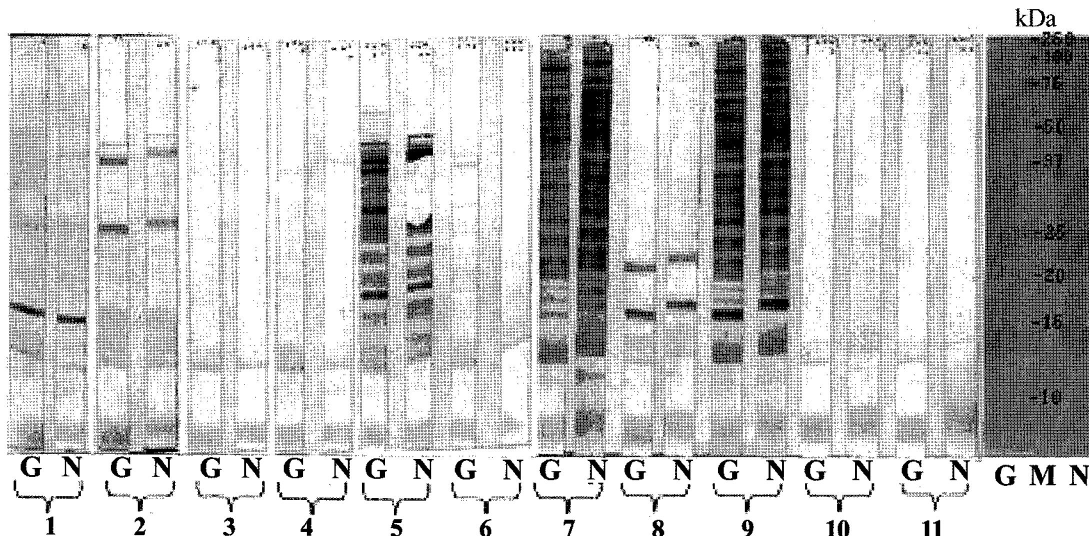

Fig. 6. Detection of GM/non-GM rice proteins with sera from rice allergic patients. Lane 1, 48; lane 2, 52; lane 3, 86; lane 4, 185; lane 5, 258; lane 6, 259; lane 7, 296; lane 8, 357; lane 9, 374; lane 10, non-allergic serum; lane 11, no serum; M, molecular weight marker; G, GM rice; N, non-GM rice.

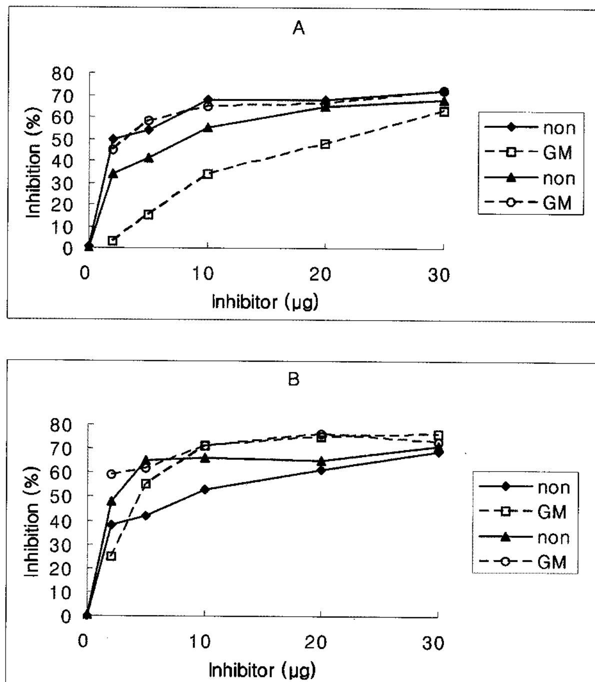

Fig. 8. Inhibiton ELISA of sera from two rice allergic patients. Plate A, coated with non-GM rice protein; plate B, coated with GM rice protein.

concentration indicating that antigen-antibody reactions between serum IgE and rice proteins are specific (Fig. 8). The maximum inhibition rate was about 70% when the plate was coated with non-GM rice extracts and about 75% when the plate was coated with GM rice extracts.

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