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# Allergenicity of Hot Peppers Cultivated in Korea

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**Abstract** The proteins from 15 types of cultivar of hot peppers cultivated in Korea were extracted and its allergenicity was investigated by immunoblotting and enzyme-linked immunosorbent assay (ELISA). The immunoblotting of hot pepper proteins extracts (HPEs) against serum of hot pepper sensitized patients revealed dominant IgE binding to 14, 37, and 40 kDa molecules. The specific levels of IgE to HPEs sample No. 1, 3, and 7 were much higher than the other samples in patients. Also, IgE binding capacity of HPEs were not reduced by thermal processing and digestion in ELISA using human IgE antibody acquired from hot pepper sensitized patients. By means of Western blotting using anti-thaumatin IgY, thaumatin-like protein (TLP) acting as allergen in several plants and fruits was detected in tested hot peppers. This study demonstrates that the antigenic protein in hot peppers are present but are differently contained according to cultivars.

**Keywords:** hot pepper, *Capsicum annuum*, food allergy, thaumatin-like protein, allergenicity

## Introduction

Hot peppers, the genus of *Capsicum* belong to the tropical plant of Solanaceae family, are cultivated in the Mediterranean countries, America and Asia. They have been known to contain the antioxidants of neutral and acidic phenolic compounds as well as a large amount of vitamin A and C, which are widely used in world as medicine, natural colorant, and spicy. Hot pepper is one of the important agricultural products in Korea and is cultivated in the area of 75,574 ha which accounts for about 50% of overall seasoning vegetable cultivation area. The powdered hot pepper was used as a major basic ingredient of various foods such as pepper paste sauce (*kochujang*) and *kimchi* which are very popular food in Korea (1). Although hot pepper is highly consumed in Korea, little knowledge about allergnicity derived from hot pepper has been known.

Food allergy is a hypersensitive immune response that occurs as food passes through gastrointestinal tract. It occurs in 2% of world population and 0.3-7.5% of the cases develop in children with decreasing its incidence as they age (2,3). Food allergies occur in 4 to 6% of children, and in 1.5% of adults in Korea (4). The prevalence of allergies and respiratory diseases and the mortality of these diseases are increasing throughout the world due to air pollution caused with economic and industrial development, westernized life style, and drastic environmental changes. The number of allergy patients is increasing in Korea as well as western countries (8).

Most of known allergens are soluble glycoproteins with the molecular weights of 10-70 kDa. The allergens are relatively stable in heat and promote immune response due to the preservation of allergenicity even during digestion process (6). As the representative foods provoking allergy, soybean, milk, wheat, fishes, crustaceans, nuts, meats, and eggs can be listed. More than 90% of the food allergies have been known to be induced by these types of foods (7,8). However, the prevalent type of food allergy varies with nations or races, so researches on food allergy have to consider the unique situation for each nation or race.

This study was attempted to screen allergens derived from hot peppers, to compare the difference in allergenicity of 15 types of cultivars of hot peppers, and to investigate the change in the allergenicity by the treatment of heat and digestive-enzyme.

## Materials and Methods

**Patients' sera** The tested sera were obtained from 16 patients with hot pepper allergy, who visited the pediatrics clinic in Samsung Medical Center (Seoul, Korea). Specific IgE levels were measured by the CAP IgE PEIA method (Pharmacia, Uppsala, Sweden). The results of CAP test showed that the IgE level of serum from patient No. 3, 9, 10, and 16 were higher than other patients as between 9.3 and 24.1 KUA/L (Table 1). Therefore, their sera were used for immunoreactivity test in hot pepper proteins.

**Isolation of hot pepper protein extracts (HPEs)** The 15 types of various cultivar of hot peppers produced in Korea were provided from the Hyundai seed company. These were cultivated in green house and harvested in August, 2006. The hot pepper protein extracts (HPEs) was prepared by a low temperature method (9,10). Each hot pepper sample was initially grinded in liquid nitrogen to make a fine powder. The pre-chilled diacetone alcohol (4-hydroxy-4-methyl-2-pentanone; Kanto, Tokyo, Japan) and acetone (Kanto) were added and mixed onto the fine hot pepper powder. The mixtures were kept overnight at −20

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**Table 1. Biological characteristics of hot pepper sensitized patients' sera**

| Patient No. | Age | Sex | CAP IgE FEIA |
|---|---|---|---|
| | | | Hot pepper IgE (kU/L) |
| 1 | 0 | F | 1.5 |
| 2 | 0 | M | 2.6 |
| 3 | 0 | F | 18.0 |
| 4 | 3 | M | 0.7 |
| 5 | 3 | F | 0.4 |
| 6 | 3 | M | 2.0 |
| 7 | 5 | F | 1.4 |
| 8 | 1 | F | 1.0 |
| 9 | 1 | F | 21.8 |
| 10 | 1 | M | 24.2 |
| 11 | 0 | F | 0.7 |
| 12 | 2 | M | 2.0 |
| 13 | 15 | M | 1.7 |
| 14 | 1 | F | 0.7 |
| 15 | 3 | F | 0.8 |
| 16 | 1 | M | 9.3 |
| 17 | 2 | M | 3.5 |

°C. After being filtered with 3 M filter paper (Advantec, Tokyo, Japan) they were washed once with pre-chilled acetone/diethyl ether (Junsei, Tokyo, Japan) (1:1) and the remaining materials were lyophilized. Each lyophilized extract was mixed with phosphate buffered saline (PBS) in 1:15 (w/w) ratio, and agitated at 4°C for 1 hr. After it was centrifuged at 17,640×g for 20 min, the supernatant was filtered through 0.45-μm membrane (Millipore, County Cork, Ireland) filter to remove bacteria and other contaminants, lyophilized and stored at −70°C until used.

**Heat- and simulated gastric fluid (SGF)-treatment of the HPEs** HPEs samples for each cultivars were prepared at the concentration of 1 μg/μL by adding PBS to lyophilized HPEs. Two-hundred μL of the HPEs samples were heated for 30 min at 40, 60, 80, and 100°C in e-tubes and the reaction was stopped by directly dipping them into ice water.

The change of the HPEs samples against SGF was examined, as described by Astwood *et al.* (11). Shortly, the protein concentration of HPEs sample was dissolved by 1 μg/μL in SGF containing 0.32%(w/v) of pepsin A (Sigma-Aldrich, St. Louis, MO, USA) and shaken at 37°C and samples were collected at intervals of 0, 0.5, 5, 10, 15, and 30 min after treatment. These aliquots were mixed with 60 μL of 200 mM $Na_2CO_3$ solution and stored at -20°C until further analyses. The immunoreactivity change of HPEs after the heat- and SGF- treatment was checked by using enzyme-linked immunosorbent assay (ELISA) reader (Expert 96 UV; Asys Hitech, Eugendorf, Austria).

**Electrophoresis and immunoblotting of HPEs** The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (12), using 15% separating gel and 5% stacking gel. A 30 μg of sample protein was loaded on each well and electrophorized at 100 V. The gel was stained with Coomassie blue (0.0025% Coomassie brilliant blue R-250, 5% methanol, and 7.5% acetic acid) and destained with 25% methanol, and 7.5% acetic acid. For immunoblotting, separated proteins are transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Milford, MA, USA). After transferring the separated proteins to PVDF membrane by tank blotting, the membranes were blocked with 5% skimmed milk dissolved in Tris-buffered saline (20 mM, pH 8.2) containing 0.1% Tween-20 for overnight at 4°C to avoid nonspecific binding. Thereafter, the transferred proteins were reacted 3 hr at room temperature with one of the pooled sera diluted (1:100) in the blocking buffer. After washing with Tris-buffered saline containing 0.1% Tween-20 (3 times, 15 min for each), the membrane was incubated for 3 hr at room temperature with peroxidase-conjugated anti-human IgE antibodies (Sigma-Aldrich) diluted 1:1,000 in the blocking buffer. After repeated washings, the chemiluminescence was observed using an appropriate volume of ECL Plus solution (Pierce, IL, USA) (12).

For detection of thaumatin-like protein, blots were treated with 5% skimmed milk dissolved in Tris-buffered saline (20 mM, pH 8.2) containing 0.1% Tween-20. Immuno-blotting was performed by using chicken anti-thaumatin IgY (Genway Biotech., San Diego, CA, USA) in a 1:1,000 dilution. Binding antibodies were detected by peroxidase-conjugated anti-chicken immunoglobulin Y diluted 1:1,000.

**ELISA for specific IgE to HPEs** For ELISA analysis, a 96-well microplate was coated at 4°C for overnight with 100 μL HPEs (10 ng protein/μL) diluted in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6, Sigma-Aldrich). After washing (0.05% Tween in PBS), the plate was incubated at 37°C for 1 hr with 100 μL/well of patient's sera diluted 1:3,000 in PBS, washed again, and incubated with peroxidase-labeled goat anti-human IgE (diluted 1:3,000, Sigma-Aldrich) or biotinylated goat anti-human IgE (diluted 1:1,000, Vector, Burlingame, CA, USA) in PBS for 1 hr. The plate was then developed by the addition of 100 μL/well of a solution of 3,3',5,5'-tetraethylbenzidine (TMB, Sigma-Aldrich) or avidin peroxidase (diluted 1:400, Sigma-Aldrich) in 1% fetal bovine serum and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS, Sigma-Aldrich) containing $H_2O_2$. The reaction was stopped after 30 min by the addition of 2 M $H_2SO_4$. Optical density was measured at 450 or 405 nm using an ELISA reader (Asys Hitech).

**Sandwich ELISA for thaumatin-like protein (TLP) in HPEs** Microtitre plates were coated overnight at 4°C with 100 μL anti-thaumatin IgY antibody (diluted 10 ng protein/μL, Genway Biotech.) diluted in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6). After washing (0.05% Tween in PBS), the plate was incubated at 37°C for 1 hr with 100 μL HPEs protein (10 ng protein/μL) diluted in PBS, washed again, and 100 μL/well of patient's sera diluted 1:3,000 in PBS, washed again, and incubated with peroxidase-labeled goat anti-human IgE (diluted 1:3,000, Sigma-Aldrich) in PBS for 1 hr. The plate was then developed by the addition of 100 μL/well of a solution of TMB. The reaction was stopped after 30 min by the

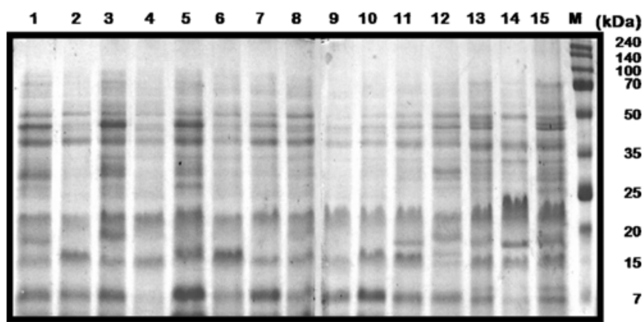

**Fig. 1. Coomassie stain of SDS-PAGE separated extracts of the 15 cultivars of hot pepper.** Lane 1, green unripe hot pepper; lane 2, 'Nok-kwang'; lane 3, 'Wi-poong-dang-dang'; lane 4, 'Acda'; lane 5, 'Am-hang-ea-sa'; lane 6, 'No. 9'; lane 7, 'No. 10'; lane 8, 'No. 3-1'; lane 9, 'Bangbang-tunnel'; lane 10, 'Tunnel-hong'; lane 11, 'Mi-lak-hong'; lane 12,' Dok-ya-chung-chung'; lane 13, 'Chun-ha-jang-sa'; lane 14, 'Chung-top'; lane 15, 'Dae-jeon'; M, molecular weight marker.

addition of 2 M $H_2SO_4$. Optical density was measured at 450 nm using an ELISA reader (Asys Hitech).

## Results and Discussion

**Electrophoresis and immunoblotting profile of HPEs** The protein of 15 different cultivars of hot peppers were extracted and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 1). The molecular weight of proteins extracted from the 15 cultivars of Korean hot peppers ranged from 7 to 69 kDa, and especially the bands of 7, 15, 23, 30, 32, 37, 40, and 46 kDa were prevalent in most of samples. They had a similar pattern of protein band on SDS-PAGE, but there were some differences in the amounts of the proteins among the cultivars.

The sera of 4 patients suffering from hot pepper allergy were used on immunoblotting to detect the allergenic protein in hot pepper (Fig. 2). The 40, 37, and 15 kDa proteins (marked with the arrow) reacted with all of the tested sensitized patients' sera. The 46 kDa protein in HPEs which showed a strong band on Coomassie stain of the SDS-PAGE was not visualized on most of the immunoblots with the patients' sera. This result suggested that 46 kDa proteins in HPEs might not be allergens in the most of the patients with hot pepper allergy and the intensity of band binding with the patients' sera was various according to the hot pepper cultivars and characteristic of patients.

**Specific IgE levels to HPEs in the sera of hot pepper-sensitized patients** Food allergy is classified as IgE mediated reaction or non-IgE mediated reaction. The levels of specific IgE in food were correlated with the likelihood of clinical reaction. Using ELISA, we therefore measured the levels of specific IgE antibody reacting to 15 HPEs samples in the sera from 4 patients sensitized to hot pepper (Fig. 3). The levels of IgE to HPEs sample No. 1, 3, and 7 were much higher than the other samples in all of the 4 patients. Furthermore, sample No. 15, 12, and 4 also displayed significant IgE binding capacities. However, specific IgE levels to sample No. 2 and 9 in tested sera were very weakly detected. These patterns were not obviously identified by immunoblotting using tested sera and HPEs samples.

This finding suggested that the use of different cultivars of hot pepper could avoid or reduce the allergic responses to hot pepper. The HPEs sample No. 1, 3, and 7 with high levels of specific IgE were used to investigate the IgE binding capacity of hot pepper protein against heat- and SGF-treatment.

**IgE binding capacity of heat- and SGF- treated HPEs** To determine whether treatment of heat can change the

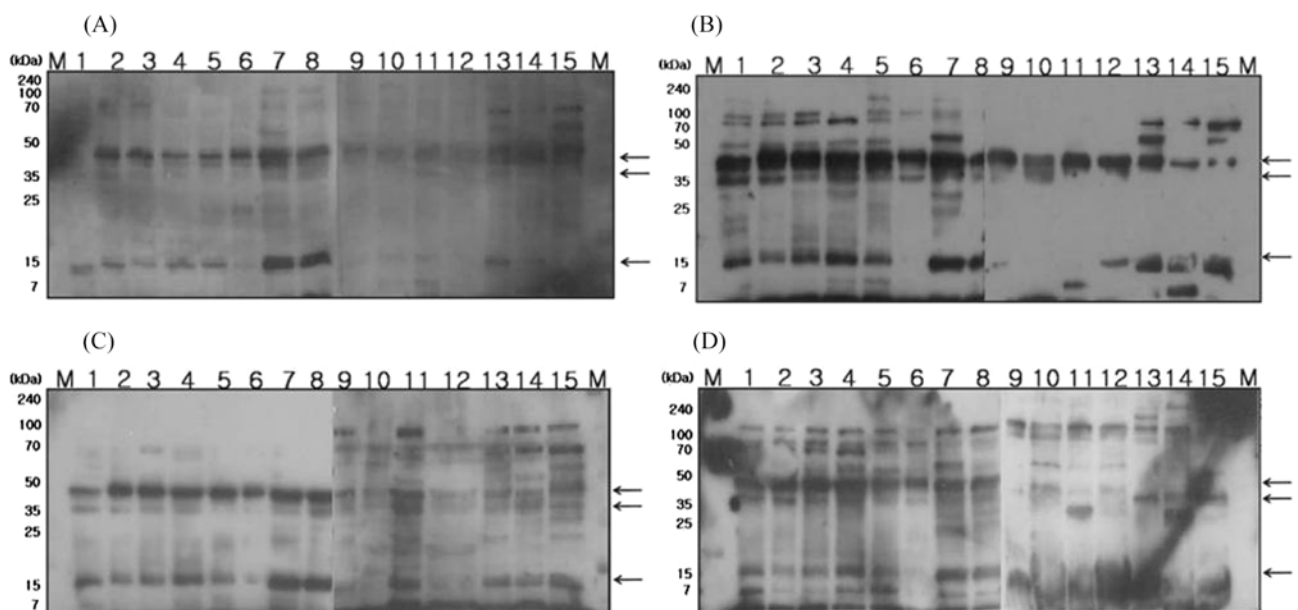

**Fig. 2. Immunoblotting of hot peppers protein extracts with sera of 4 hot pepper sensitized patients' sera.** For description of lane 1 to M, see Fig. 1. A-D: immunoblotting against 15 HPEs in patient No. 3, 9, 10, and 16, respectively. Patients' sera were diluted by 1: 100.

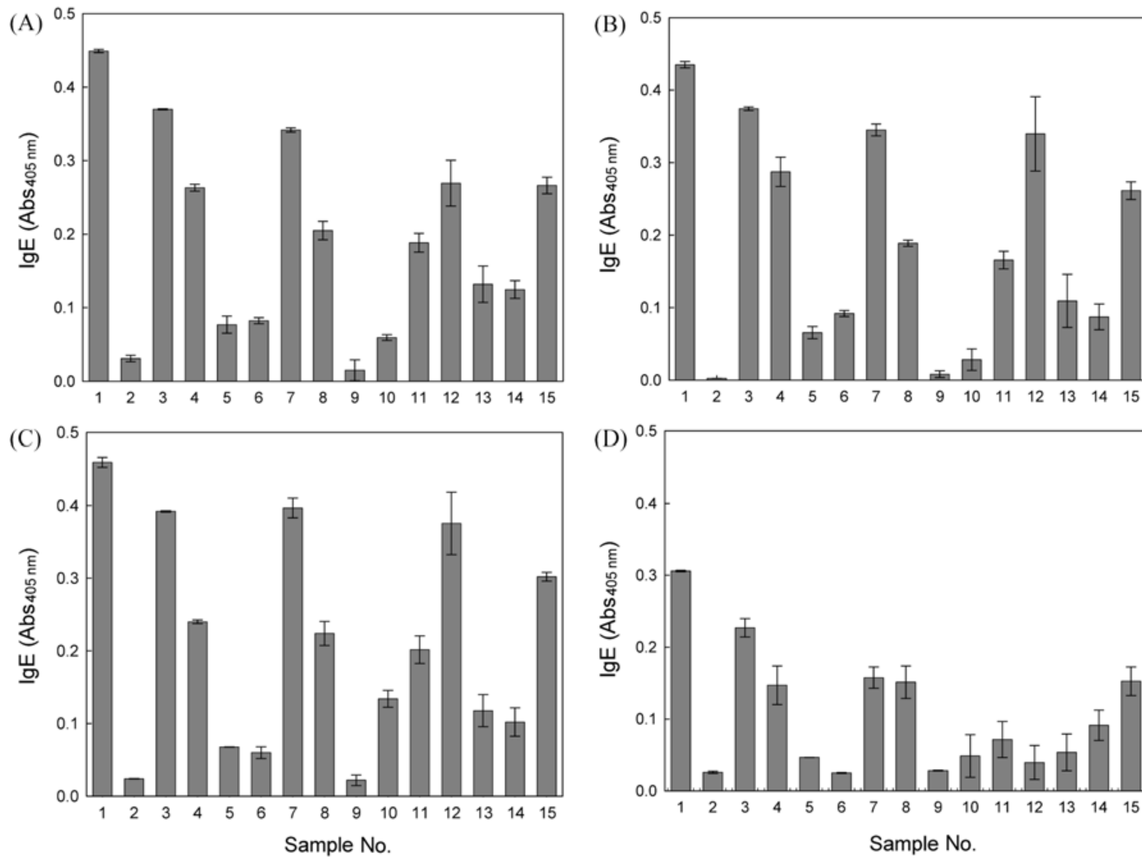


**Fig. 3. Specific IgE levels of hot pepper sensitized patients' sera to HPEs.** For description of sample No. 1-15 see lane No. of Fig. 1. A-D: specific IgE antibody levels against 15 HPEs in patient No. 3, 9, 10, and 16, respectively. Patients' serum was diluted by 1:100.

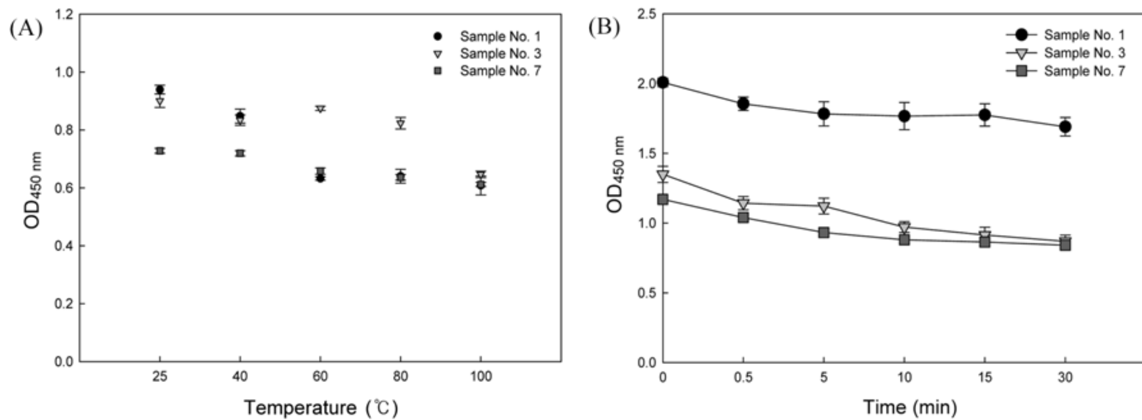


**Fig. 4. ELISA analysis of heat (A) or digestion-enzyme (B) treated hot pepper proteins with hot pepper sensitized patients' serum No. 3.** (A) Heat-treated sample, (B) SGF-treated sample. Patients' serum No. 3 was diluted by 1: 3,000. After heat or SGF treatment, reactivity of treated-samples with hot pepper sensitized patients' serum was measured by ELISA as described previously. The control was not heat treated (25°C) or SGF treated (0 min).

allergenicity of hot pepper proteins, the selected HPEs samples were heated at various temperatures (40, 60, 80, and 100°C) for 30 min. ELISA analysis for heat treated HPEs (Fig. 4A) showed that the reaction of heat-treated HPEs to the patients' serum No. 3 was not reduced as compared with that of untreated HPEs (25°C). These patterns were observed in ELISA results using other patients' sera and other cultivars of hot pepper (data not shown). In addition, no significant changes in protein patterns of heat-treated HPEs were observed in SDS-PAGE and immunoblotting analysis (data not shown).

Son *et al.* (14) described that allergenicity of soybean proteins were preserved when they were treated at 100°C for 1 hr. Food causing allergy, such as peanuts, shrimps, milk, and fishes, have been reported to have heat tolerance (15).

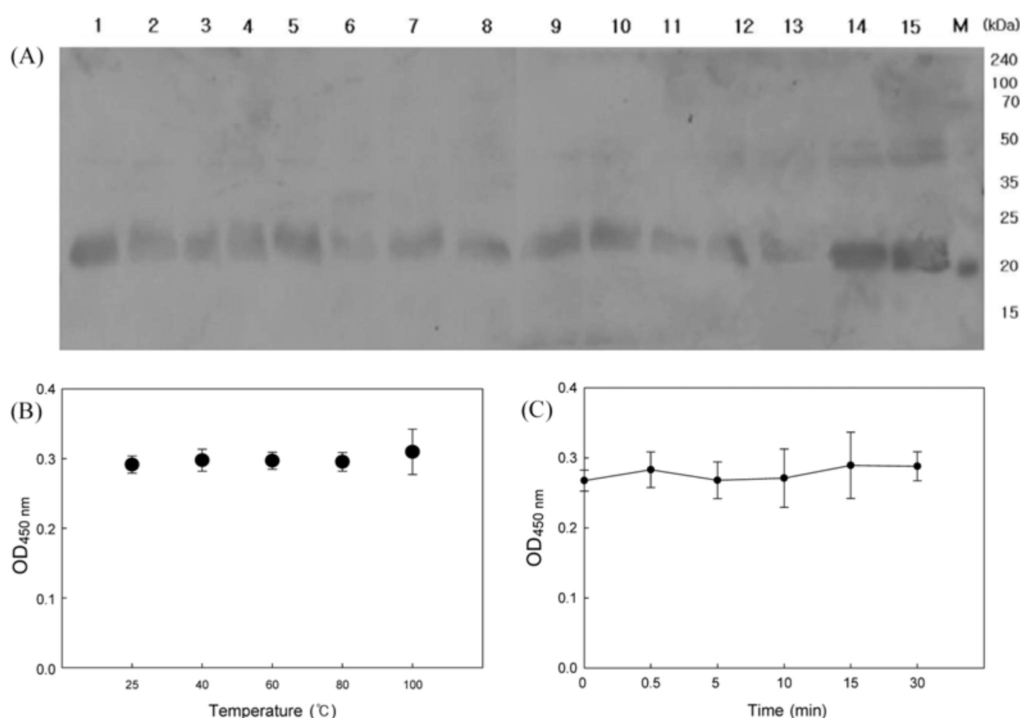


**Fig. 5. Western blotting of extracted proteins of the 15 cultivars of hot pepper with chicken anti-thaumatin polyclonal antibody (A) and ELISA analysis of immunoreactivity of thaumatin (or TLP) in heat- and SGF-treated HPEs against hot pepper sensitized patients' serum No. 3 (B,C).** (A), For description of lane 1 to M, see Fig. 1; (B, C), Immunoreactivity of thaumatin (or TLP) in heat- or SGF-treated HPEs was measured by sandwich ELISA as described previously. The hot pepper sensitized patients' serum No. 3 was tested.

Generally the allergens show stability even after heat treatment, and enzymatic treatment and the fact is well known that major food allergens have to arrive at the gastrointestinal tract with an immunologically activated form (16). We accordingly carried out an ELISA in order to examine the reactivity of sera from 4 patients with HPEs hydrolyzed by SGF. As shown in Fig. 4B, immunoreactivity of hot pepper allergy patient sera against SGF-treated HPEs were slightly digested in the time-dependent tendency. The immunoreactivity of SGF-treated HPEs was the highest in sample No. 1 than other samples. It showed that allergen from hot pepper may result in the proteolytic destruction of the conformational epitopes of major allergens.

The study of Yagami *et al.* (13) and Vieths *et al.* (17) reported the allergenicity of peanut proteins had resistance to the digestion. Some allergens or, more properly, some allergenic foods, are described as heat stable (e.g., milk, egg, fish, peanuts, and products, thereof), while others are considered partially stable (e.g., soybean, cereals, celery, tree nuts, and their products) or labile (fruits of the Rosaceae family and carrots) (18).

**Detection of TLP using anti-thaumatin polyclonal antibody** Many important plant food allergens are homologues to proteins that are members of pathogenesis-related proteins (PRs) families. The family 5 of PRs comprises unique proteins with diverse functions. Because of the sequence homologies between PR-5 proteins and thaumatin, an intensely sweet tasting protein (22 kDa) isolated from the fruits of the West African rain forest shrub *Thaumatococcus daniellii*, members of this family of proteins are referred to as TLPs (19). Several TLPs are known to act as an allergen in cherry (20), grape (21-23), tomato (24), and bell pepper (25), etc.

We checked that whether TLP acting as an allergen in various fruits or plants was included in hot pepper and whether immunoreactivity of TLP against sera of hot pepper sensitive patients was stable by heat- and SGF-treatment or not. By means of Western blotting analysis with anti-thaumatin IgY (Fig. 5A), we confirmed that tested hot peppers included thaumatin or TLP and its contents were different on certain cultivars of hot pepper. Also, the sera of hot pepper sensitized patients were incubated with thaumatin (or TLP) of heat- and SGF-treated HPEs resulted in immunoreactivity against IgE was not changed (Fig. 5B and 5C). These findings suggest that the thaumatin (or TLP) in hot pepper are resistant to heat- and SGF-treatment.

The allergens from various plants or fruits have been defining but it was rarely reports about allergy of hot pepper until the recent. Therefore, this study confirmed hot pepper had some different profiles of allergenic proteins according to the cultivars and some proteins such in the hot peppers were stable in the treatment of heat or digestive enzymes. Further studies to confirm the sequence and structure of hot pepper allergens and to determine epitope regions are warranted to produce alternative foods derived from hot peppers which are free for allergy.

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