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# An Evaluation of Changes in the Allergenicity of Kochujang upon Preparation Using Aloe Extract*

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**Soybeans are well-known as allergenic foods. Koreans consume large amounts of soybean foods, such as kochujang, which have gone through the fermentation process. To lower the allergenicity of these foods, we prepared hypoallergenic kochujang with aloe extract (AK). A sensory evaluation was conducted along with a clinical evaluation that used a double-blind, placebo-controlled food challenge (DBPCFC) test. These tests were designed to evaluate the acceptability of the fermented foods. In comparison to normal kochujang (NK), AK elicited a higher sensory test score, and the rate of positive reactions in atopic dermatitis patients during the DBPCFC test was reduced. Methods of protein extraction, protein quantitation with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and protein identification using two-dimensional (2D) gel electrophoresis were performed for both NK and AK to compare the functional factors. We found a reduction in the levels of high molecular proteins even though the bands of the proteins had not entirely disappeared, indicating that the boiling and fermentation process changed the soybean protein patterns. The rate of the reduction of high molecular proteins was more effective in the AK. In conclusion, AK can be recognized as a food with hypoallergenic effect.**

**Key words:** Kochujang, Allergenicity, Atopic dermatitis, Aloe, DBPCFC, SDS-PAGE, 2D electrophoresis

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## INTRODUCTION

Allergy is a hypersensitivity reaction where symptoms appear following the exposure to macromolecules (generally proteins).[1)] The prevalence of atopic disease, such as asthma and food allergy, has increased and dietary changes and modifications in consumer habits influence to infection. Malnutrition, such as malnutrition caused by genetic disorders, protein-calorie-associated malnutrition, or secondary malnutrition syndromes of childhood cause immune deficiencies.[2)] This malnutrition makes allergy to become aggravated.

Kochujang, a traditional Korean hot pepper soybean paste, is a fermented mixture of meju (thick soypaste), red pepper powder, glutinous rice, malt flour, and salt. It contains more vitamin $B_1$, $B_2$, C, and folic acid than doenjang or ganjang.[3-6)] Meju, cooked soybean with Aspergillus oryzae, is used for enzymatic reaction with proteins in soybean.[7)]

Soybeans are one of the well-recognized allergenic foods and recent studies have identified the major allergens in soybeans.[8-11)] The prevalence of soy allergy has increased with greater consumption of soybean products, including fermented soybean foods, in many countries.[8-11)] In previous studies, Gly m Bd 28K was found to be a major allergen in soybeans, along with Gly m Bd 30K and the $\alpha$ subunit of $\beta$ -conglycinin.[12)] However, Gly mBd30K was not detected in fermented soybean food (miso) in Japan. In addition, miso has an antioxidant effect.[13,14)]

Aloe is a tropical cactus that belongs to the lily family. It has been used both topically and orally as a purgative and as an antiviral remedy for burns, radiation wounds, and skin infections.[15)] Aloe also helps regulate immune system function. The glycoprotein, NY 945, found in aloe, has an antiallergenic effect that represses the secretion of mediators, such as histamine and eosinophils, into the tissue.[16,17)] Yun et al.[18)] showed that chungkukjang with aloe extract had a lower allergenicity.

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The purpose of this study was to determine whether the antigen level of hypoallergenic kochujang could be lowered by adding aloe during its production. To evaluate the allergenicity of kochujang with aloe (AK), in comparison to normal kochujang (NK), Western blotting using a soybean allergy-positive serum, SDS-PAGE, two-dimensional gel electrophoresis (2D-PAGE), and a clinical severity test were performed.

## MATERIALS AND METHODS

### 1. Preparation of NK

Soybeans were purchased at a local market in Seoul, Korea, and soaked for 18 hours in water that was three times the volume of the soybeans. The soaked soybeans were cooked for three hours and, afterwards, placed in a mold for one month. At this stage the soybeans, or meju, were ground. Seeds were removed from the dried hot peppers, and the peppers were ground into powder. Water was boiled, cooled, and mixed with powdered malt for 12 hours. After the malt was removed, the supernatant and glutinous rice powder were mixed and boiled. After cooling, ground meju and red pepper powder were mixed in the water with the glutinous rice.

### 2. Preparation of AK

Aloe was washed and heated for 3 hours in water five times its volume (20 min. at 121℃, followed by 2 hours 40 min at 60 ℃). After boiling, the supernatants were filtered, and the extract was collected. The process of making AK was similar to that of making NK, except that the amount of water added to the aloe extract was 2: 1 by volume when boiling with soybeans. The fermentation method used to make NK and AK was otherwise the same.

### 3. Selection of Patients

Skin prick tests (SPT) and antigen-specific IgE tests were used to screen atopic dermatitis patients who visited the Seoul Allergy Clinic in Seoul, Korea. The 11 patients who had a positive reaction to soybeans by the SPT and IgE test were examined from April 2005 through July 2005 using double-blind, placebo-controlled food challenge (DBPCFC) tests. After blood sampling, immunized serum was obtained. Patients who were in the process of drug treatments lasting more than six months or who had problems with their blood glucose, serum lipid, or hepatic enzyme levels were excluded. Smokers or participants who consumed alcohol at least three times per week were also excluded from the study.

### 4. Sensory Evaluation

Twenty participants who were graduate students studying food and nutrition were randomly selected for sensory evaluation. The subjects, who were given kochujang with a cucumber, evaluated the kochujang (NK and AK) for the criteria of taste, color, smell, and overall acceptance.

These evaluations were rated, using a 9-point Hedonic Scale rating where a score of 9 (like extremely), 8 (like very much), 7 (like moderately), 6 (like slightly), 5 (neither like nor dislike), 4 (dislike slightly), 3 (dislike moderately), 2 (dislike very much), and 1 (dislike extremely).

### 5. Evaluation of Clinical Severity

Patients consumed carrots with NK and AK for 3 days and had a rest period for 3 days between the 2 DBPCFC experimental periods.[19] The carrot was chosen because it did not produce any allergic responses. Patient's allergic symptoms were evaluated by the clinical severity and improvement system (Table 1), developed by Noh and Lee.[20] When the clinical severity score was more than 10%, allergic symptoms were diagnosed as positive reactions.

### 6. Protein Extraction and Western Blotting

#### *Preparation of Soybean Protein Extracts*

A constant weight (100 g) of kochujang was lyophilized over 72 hours and then made into powder. The powdered kochujang was mixed with hexane at a ratio of 1:5 (w/v) to remove the lipids. The solution was stirred and left for 30 minutes, and then the supernatant was removed. This process was repeated two times, and the hexane residue was removed by air drying for 12 hours. Samples were stirred in PBS (phosphate buffered saline) at a ratio of 1:3 (w/v) for 8 hours at 4 ℃. Samples were separated by centrifuge (OprHDV/5008, Operon, Korea, 12,000 rpm, 30 minutes, 4 ℃). The supernatant was collected and filtered through a 0.45-㎛ membrane filter, followed by dialysis in PBS at 4 ℃. The protein concentrations were measured at a wavelength of 595 nm, using a Multiscan Reader (Multiscan-EX, Labsystem, Finland), according to the method described by Zor and Selinger.[21]

#### *Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed, according to the

**Table 1.** Clinical Severity Scoring System

**A Pruritis Scoring**
A-1 Day
<Duration>
100: All day
75: More than two-thirds of the daytime but less than all day
50: More than one third of the daytime but less than two-thirds
25: Occasionally (less than one third of the daytime)
0: None
<Grade>
0 No itching or no scratching
1 Feeling of itching to tolerable itching or unconsciousness scratching
2 Mild interference in day work by scratching or itching
3 Moderate interference in day work by scratching or itching
4 Severe interference in day work
5 Complete interference
A-2 Night
<Duration>
100: All night
75: More than two-thirds of the sleep duration but less than all night
50: More than one third of the sleep duration but less than two-thirds
25: Occasionally (less than one third of the sleep duration)
0: None
<Grade>
0 No itching or no scratching (good sleep)
1 Feel of itching to tolerable itching or unconsciousness scratching during sleep
2 Some sleep disturbance by scratching or itching (Mild)
3 Sleep disturbance by scratching or itching (Moderate)
4 Some awakening during sleep almost all by scratching or itching (Severe)
5 Complete awakening by scratching or itching

**B Skin lesion scoring**
<Grade>
0 No lesion or no scar or no pigmentation
1 Pigmentation or depigmentation or scar- only
2 Redness (erythema) or coarseness of skin
3 Scaly change or scratch wound
4 Papular or vesicular eruption, desquamation
5 Bleeding, oozing, infection, exudation of pus, lichenification

**C Clinical severity score**
Day pruritis score = Day duration × Grade
Night pruritis score = Night duration × Grade
Pruritis score = (Day pruritis score + Night pruritis score) / 2
Skin lesion score = Sum of (Skin lesion % × Lesion grade)
Clinical severity score = Pruritis score + Skin lesion score

method used by Lammli[22] on Ammersharm Midiprotein II system gels (12% separation gel and 5% stacking gel). Samples were diluted 5:1 with loading buffer (0.06 M Tris-HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 0.02% bromophenol blue) for electrophoresis. The samples were denatured at 100 ℃ for 10 minutes. Thirty ㎍ each of the denatured protein was loaded, and the SDS-PAGE was run at 110 V and 35 mA for 2 hours, using a Protein Ⅲ vertical electrophoresis kit (Novex, San Diego, CA, USA). After separation, the gel was fixed and stained for 3 hours, using a Coomassie blue stain solution (BioRad, Hercules, CA, USA). The gel was then de-stained with 40% methanol and 10% acetic acid. Another gel was used for the immunoblotting study.

***Western Blotting***

Sera of patients who were allergic to soybeans was used for immunoblotting.[23,24] Samples were separated, using SDS-PAGE, and the gel was electroblotted onto a nitrocellulose membrane (0.45 ㎛, Amersham Pharmacia Biotech, Uppsala, Sweden) with the use of a transblot cell (Novex) at 250 mA and 25 V for 3 hours at 4 ℃. Nonspecific protein binding to the blot was blocked by the incubation of the membrane in 5% skim milk protein and PBS for 2 hours at room temperature, which was followed by washing the solution in 0.1% PBST (PBS/Tween-20, v/v). Each incubation step was followed by 3 washes with 0.1% PBST. The nitrocellulose membrane was incubated for 2 hours with mouse biotinylated anti-human IgE (DAKO, Copenhagen, Denmark), which was diluted 1:500 with PBS. After washing, the nitrocellulose membrane was incubated for 45 minutes with 1:1000 diluted streptoavidin-peroxidase conjugate (Medac, Hamburg, Germany). Purified human IgE (Biom Wittaker, Inc., Walkersville, MD, USA) and human serum albumin (Sigma, St. Louis, MO, USA) were used as positive and negative controls, respectively, for immunoblotting. For visualization of the IgE-binding protein band, enhanced chemiluminescence (ECL, Amersham-Pharmacia) was performed according to manufacturer's instructions.

## 7. Two-dimensional (2D) Gel Electrophoresis

***Preparation of Protein Samples for 2D Electrophoretic Analyses***

Freeze-dried soybeans and the 2 types of kochujang (NK, AK) were suspended in distilled water and homogenized. The samples were put in hexane (1:5 w/v) and centrifuged. The delipidated powders were suspended in PBS (1:3 w/v) and stirred for several hours at 4℃. Insoluble residues were removed by centrifugation at 12,000 rpm. The pellet was stirred with ammonium sulfate (516 g/1L) for 1 hour and centrifuged at 12,000 rpm. The precipitate was dialyzed with 1 X PBS. The pellet was washed twice in 70% acetone, 7 times its volume, and the precipitate was dissolved in a solution consisting of 7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropy) dimethyammonio]-1-propanesulfonate (CHAPS), 1% (w/v) dithiothreitol (DTT), 1% (v/v) IPG

buffer, 1 mM EDTA, 1 X protease inhibitor cocktail (PIC) for 1 hour. The solution was centrifuged at 14,000 rpm for 10 minutes and the supernatant was used for 2 D gel electrophoresis.

*2D Electrophoresis*

For isoelectric focusing (IEF) the strips were rehydrated overnight in a solution consisting of 8 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, 1% (v/v) IPG buffer and BPB (bromo-phenol blue, a trace). Each 20 μg of samples per strip was used and IEF was performed at 20 ℃. The IEF condition was focused from 150 V to 3,500 V for 3 hours and the function was maintained at 3,500 V for 26 hours. Strips were focused to a total of 96 kVh. Prior to the second dimension, strips were incubated in equilibration buffer (50 mM Tris-HCl, pH 6.8, 6 M urea, 2% SDS, 30% glycerol) with 1% DTT for 10 minutes and then with 2.5% iodoacetamide in the same buffer without DTT for 10 minutes. The separation was performed in 10-16% SDS-PAGE gels using the Hoefer DALT 2 D system (Amersham Biosciences) at 20 ℃. The gels were silver stained according to the method described by Jin et al.[25] The silver stained gel was scanned with a Duoscan T 1200 scanner.

## RESULTS AND DISCUSSION

### 1. Sensory Evaluation

The results of the sensory evaluation are shown in Table 2.

Even though the tests were not statistically significant, AK had better scores than NK. When we evaluated the scores for color, AK had a higher score of 5.10 while NK scored lower at 4.55. In the smell test, AK had a higher score of 4.90 and NK had a lower score of 3.95. According to the taste test, AK had a higher score of 5.10 while NK had a lower taste with a score of 4.50. The AK had a higher overall score of 5.50 and NK had a lower score of 4.65. Although adding aloe extract could improve the sensory value of kochujang, the addition of aloe extract was less effective than expected due to the strong taste of kochujang.

**Table 2.** Sensory evaluations of patients given AK or NK

| | Color | Smell | Taste | Overall evaluation |
|---|---|---|---|---|
| AK[1)] | 5.10±1.33$^{NS}$ | 4.90±1.25$^{NS}$ | 5.10±1.48$^{NS}$ | 5.50±1.32$^{NS}$ |
| NK | 4.55±1.00 | 3.95±1.10 | 4.50±1.24 | 4.65±1.73 |

[1)] AK: kochujang with aloe extract, NK: normal kochujang
Mean ± standard deviation, n=20
NS: not significant by independent t-test
Nine-point Hedonic scale rating : 9=like extremely, 8=like very much, 7=like moderately, 6=like slightly, 5=neither like nor dislike, 4=dislike slightly, 3=dislike moderately, 2=dislike very much, 1=dislike extremely.

**Table 3.** DBPCFC test results of patients given NK or AK

| Subject | Sex | Age | Height (cm) | Weight (kg) | Soybean | NK | AK |
|---|---|---|---|---|---|---|---|
| 1 | M | 4 | 88 | 12 | + | + | - |
| 2 | F | 16 | 162 | 40 | + | + | - |
| 3 | M | 25 | 178 | 63 | + | - | - |
| 4 | M | 3 | 91.5 | 14 | + | - | - |
| 5 | F | 4 | 106 | 17 | + | - | - |
| 6 | F | 33 | 168 | 50 | + | - | - |
| 7 | F | 4 | 106 | 16 | + | - | - |
| 8 | M | 87 | 178 | 70 | + | - | - |
| 9 | F | 4 | 100.6 | 16.5 | + | + | + |
| 10 | M | 16 | 175 | 57 | + | - | - |
| 11 | F | 7 | 115 | 21 | + | - | - |

M: male, F: female, + Positive, - Negative

### 2. Evaluation of Clinical Severity

Among atopic dermatitis patients who visited the Seoul Allergy Clinic, 11 patients (5 males (M) and 6 females (F)) were diagnosed with soybean allergy by the SPT and IgE test. They had a DBPCFC test for NK and AK. The results are shown in Table 3.

Three out of 11 patients (1 M and 2 F) had a positive reaction to NK and 8 out of 11 patients (4 M and 4 F) had a negative reaction to NK. Ten out of 11 patients (5 M and 5 F) had a negative reaction to AK and 1 out of 11 patients (0 M and 1 F) showed a positive reaction to AK. In a previous study[26], we investigated changes in allergenicity of doenjang with aloe extract and observed that the effect of aloe extract reduced the allergic reaction of atopic dermatitis patients in the DBPCFC test. Results of the clinical severity evaluation showed the allergy aggravation rate decreased with AK as compared to NK. This demonstrates that adding aloe to food is beneficial to allergy patients.

### 3. SDS-PAGE and Western Blotting

The results of the SDS-PAGE are shown in Figure 1 A. AK protein bands at 21.5 kDa were fainter as compared to NK. In Figure 1 B of the western blotting, the IgE binding band at 21.5 kDa was observed in NK but not in AK. The ability of IgE binding was lost when adding aloe extract to kochujang. The process of heating and fermentation in kochujang, doenjang and chungkukjang, is well known to reduce the reactogenicity of allergens with IgE, as cooked foods are usually well or better tolerated by allergic individuals.[27-29] Kataoka[30] reported that some harmful

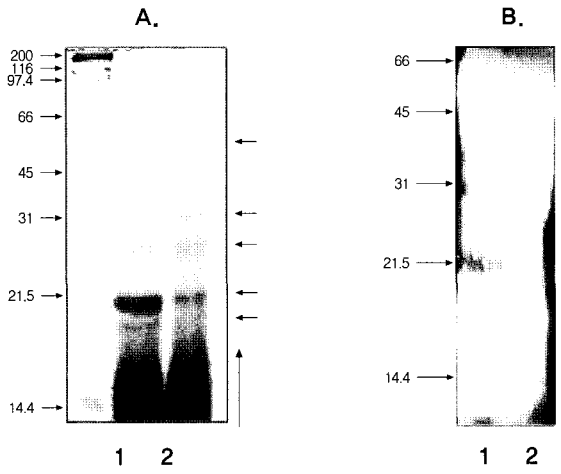


**Fig. 1.** SDS-PAGE gel (A) and western blot (B) of kochujang
Replica gels were stained with Coomassie blue (A) or electrotransferred to nitrocellulose membranes and immunoblotted with a pool of sera from patients with a soybean allergy (B)
Lane 1: NK, Lane 2: AK

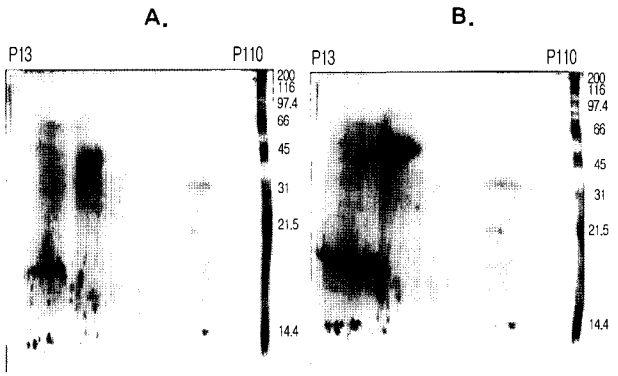


**Fig. 2.** Protein spots of the two types of kochujang (NK and AK), obtained with 2D-PAGE
A: Normal Kochujang (NK) B: Kochujang with Aloe Extract (AK)

ingredients, such as allergens, are eliminated during the fermentation process of soy sauce and miso. First of all, the ability of IgE binding was lost during fermentation, and then affected upon adding aloe extract.

## 4. Two-dimensional (2D) Gel Electrophoresis

The results of the 2 D gel electrophoresis are shown in Figure 2. In the NK, 106 protein spots ranging between pI 3-5.5 were observed. Of the pI 3-5.5 spots, high concentrations appeared at a high molecular weight range of 66-45 kDa and at the 21.5-14.4 kDa range. In the AK, 99 protein spots ranging between pI 3-5.5 were observed. Compared to NK, the concentration of spots in the 66-45 kDa range was reduced significantly in AK. The concentration of some spots in the molecular weight range of 21.5-14.4 kDa was increased. The concentration of IgE binding bands at the 66-45 kDa range was lower in AK as compared with NK, indicating the adding of aloe extract could be effective in reducing the allergen concentration. We used the silver staining because it offers the greatest sensitivity in the realm of nonradioactive protein detection.[25]

The results showed that the antigen level of hypoallergenic kochujang could be lowered by adding aloe during its production through the Western blotting using a soybean allergy-positive serum, SDS-PAGE, two-dimensional gel electrophoresis (2 D-PAGE), and a clinical severity test.

It has been estimated that around 1-2% of the population and up to 8% of children suffer from some type of IgE-mediated food allergy.[1] Among them, many have IgE-mediated food allergy and the foods most commonly responsible for allergic reactions include peanuts, tree nuts, wheat, soy, cow's milk, egg, fish and shellfish.[1,31] Genetic approaches towards allergen-free foods may represent a casual strategy to avoid contact with these allergens. However, a small qauntity of allergen suffice to trigger allergic reactions.[31] Therefore an adequate diet as the denaturation of protein and nutrition strategies might not completely prevent, but could hopefully attenuate allergenicity for allergy patients.[2] The principle of reducing the allergeniciy of food proteins by enzymatic hydrolysis and heat processing has been effectively demonstrated.[31] Kochujang seems to be able to reduce allergenicity because kochujang is made with meju which is prepared by the methods of heating and fermentation. Pal reported that this very simple strategy to reduce the reactogenicity of allergens might be useful for allergen-specific immunotherapy, by investigating the effect of heat-denaturation.[32] Even though many studies for alleviating allergic reactions with foods have been investigated in Japan[12,30], there are a few studies on the allergenicity of fermented foods in Korea.[11,18,20] The relationship between the Korean fermented foods and allergenicity with respect to immunology, biochemistry, and nutrition needs to be studied further.

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