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Changes in the Allergenicity of *Saeujeot* by Fermentation

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Abstract The aim of this study was to observe the changes in allergenicity of *saeujeot* (salted and fermented shrimp) using a competitive indirect enzyme-linked immunosorbent assay (Ci-ELISA). The fermentation conditions tested for *saeujeot* consisted of various temperatures (25, 15, and 5°C) and salt concentrations (25, 15, and 10%). When *saeujeot* was fermented at a low salt concentration and high temperature, the binding ability of mAb and shrimp-allergic patient serum to allergen was significantly decreased. In particular, the binding ability of mAb to allergen in *saeujeot* fermented with 10% salt at 25°C for 5 days decreased to 5%. Also, the binding ability of shrimp-allergic patient serum to allergen in *saeujeot* fermented for 5 days with 10% salt at 25°C was 8%. In conclusion, the binding of mAb and shrimp-allergic patient serum to tropomyosin in *saeujeot* decreased with longer fermentation periods, lower salt concentrations (10%), and higher temperatures (25°C).

Keywords: *saeujeot* (salted and fermented shrimp), allergenicity, allergen, tropomyosin

Introduction

Saeujeot is a traditional Korean salt-fermented food which inhibits spoilage by fermenting shrimp in high concentrations of salt for several months (1). *Saeujeot* ferments due to self-digestive enzymes or enzyme-producing microorganisms, and is the most common salt-fermented seafood product (2,3). *Saeujeot* is mainly added to *kimchi* to enhance flavor, and is also widely used in pot stews, and seasonings for everyday cooking (4). After harvesting shrimp, *saeujeot* must be promptly preserved with salt because shrimp contain a large number of enzymes in the gut, and decompose more readily than other fish and shellfish. *Saeujeot* also has more added salt than other salted fish (5).

Shrimp can provoke an immediate hypersensitivity response after the ingestion of other crustacean foods such as lobster, prawns, crabs, etc (6-8). Shrimp is known to be one of the more common allergenic foods along with eggs (9), soybeans (10), and milk (11). The major allergen identified in shrimp is tropomyosin, a heat-stable protein (12,13). Symptoms of shrimp allergy include urticaria, abdominal pain, diarrhea, vomiting, and anaphylactic shock (14,15). Studies on *saeujeot* have investigated its taste compounds (16), fatty acid composition (17), and flavor composition (18), and many investigators have reported that salted and fermented seafood has antihypertensive (19) and antioxidant effects (20). Presently, there are few studies addressing the allergenicity of *saeujeot*. Therefore, investigation of the changes in *saeujeot* protein structure and its allergenicity during fermentation will be useful for understanding the pathogenesis, treatment, and prevention of shrimp allergy. In this study, we investigated changes in the binding ability of tropomyosin from *saeujeot* fermented at various temperatures and salinities to mAb or shrimp-allergic patient serum.

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Materials and Methods

Saeujeot For preparing *saeujeot*, shrimp (*Acetes japonicus*) was mixed with various concentrations of salt (25, 15, and 10%) and fermented at various temperatures (25, 15, and 5°C).

Standard antigen and antibody Tropomyosin (*Fenneropenaeus chinensis*) standard antigen, as well as mouse monoclonal IgG (mAb 4.9.5) were supplied by the Radiation Food Science and Biotechnology team at the Korea Atomic Energy Research Institute. Anti-mouse IgG conjugated horseradish peroxidase was purchased from Sigma-Aldrich Corporation (secondary IgE; St. Louis, MO, USA). The serum of shrimp-allergic patients was provided by the medical department of Hokkaido University Hospital in Japan, and jointly used with Saeki Hiroki of the Fisheries Department. Goat anti-human IgE/IgG conjugated peroxidase was purchased from Sigma-Aldrich (secondary IgE).

Competitive indirect enzyme-linked immunosorbent assay (Ci-ELISA) Ci-ELISA, a modified method of Lee *et al.* (21), was conducted to investigate the reactivity of tropomyosin with monoclonal or serum antibodies. Tropomyosin was diluted in coating buffer (0.2 M bicarbonate buffer; pH 9.6) and used to coat a Costar 96-well flat bottom plates (469957; Nunc, Kamstrupvej, Denmark) at 4°C overnight, and blocked with 0.01 M

phosphate buffered saline (PBS) containing 1% gelatin for 2 hr. The antigen and antibody were diluted in 0.01 M PBS (pH 7.3), and 50 μL of each was added to the wells simultaneously. Subsequently, 100 μL of the secondary antibody (goat anti-human IgE/IgG-peroxidase) was added. *O*-Phenylenediamine (OPD, Sigma-Aldrich) solution was added to assess color development for 30 min. Color development was stopped by adding 2 M H_2SO_4, and the absorbance was measured at 490 nm using an ELISA reader (Model 550; Bio-Rad Lab., Hercules, CA, USA). After the completion of each reaction, the wells were washed 4 times with 0.01 M PBST [phosphate buffered saline containing 0.05%(v/v) Tween 20]. All reactions were conducted at 37°C for 2 hr, with the exception of the coating step.

Titration curve of Ci-ELISA Titration curve was made to define the optimum concentrations of standard antigen and primary antibody (monoclonal antibody and the serum of shrimp-allergic patients) using a slightly modified version of the method of Lee *et al.* (22). Tropomyosin was diluted to the proper ratio with coating buffer (pH 9.6), and placed into the wells overnight at 4°C. The mAb and serum from shrimp-allergic patients were then diluted to various concentrations with 0.01 M PBS and 100 μL of each dilution was put into the wells. All subsequent Ci-ELISA experiments were conducted as described above.

Standard curve of Ci-ELISA Tropomyosin was diluted to optimal concentrations in coating buffer (pH 9.6) and put into the wells of a 96-well plate. Fifty μL each of the diluted antigen and pre-titrated antibody in 0.01 M PBS (pH 7.3) were put into the wells simultaneously. All Ci-ELISA conditions were the same as described above. Each 50 μL aliquot of the antibody and 0.01 M PBS (pH 7.3) was added for 100% binding of the standard antigen and antibody. One-hundred μL of 0.01 M PBS (pH 7.3) was added as a blank.

Extracts of *saeujeot* *Saeujeot* extracts were used to determine changes in allergenicity during fermentation. Two g of *saeujeot* was lightly washed with triple-distilled water to remove foreign debris, and homogenized with 20 mL of 0.01 M PBS. The homogenate was stirred overnight at 4°C, and then centrifuged at 9,000×g for 30 min at 4°C. The protein concentration of the supernatant was adjusted to 1 mg/mL using a bicinchoninic acid (BCA) protein assay kit.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) SDS-PAGE was carried out to examine the decomposition of tropomyosin during fermentation using the method of Laemmli (23). A 12% polyacrylamide gel (acrylamide:Bis=30:0.8) was used with a molecular weight marker purchased from New England BioLabs (P7702S; Protein Marker Broad Range, Hitchin, UK). The molecular weight markers were insulin A and B chains (2.3-3.4 kDa), aprotinin (6.5 kDa), lysozyme (14 kDa), trypsin inhibitor (20 kDa), triosephosphate isomerase (26 kDa), lactate dehydrogenase (36 kDa), MBP_2 (maltose-binding protein) (42 kDa), glutamic dehydrogenase (55 kDa), serum albumin (66 kDa), phosphorylase b (97 kDa), β-galactosidase (116 kDa), MBP-β-galactosidase (158 kDa), and myosin (212 kDa). First, *saeujeot* was lightly rinsed to remove foreign materials and was homogenized in PBS (pH 7.3). Next, 40 mg/mL of shrimp muscle protein was loaded on the gel after SDS treatment. SDS-PAGE was conducted using a 12% running gel with a 4% stacking gel, and 10 μL of sample solution was put into each well. To observe the separated bands, the gel was stained with Comassie brilliant blue R250 (CBB) and destained using a 5% methanol and 7% acetic acid solution. A scanner (Power Look III; Amersham Pharmacia Biotech, Piscataway, NJ, USA) was used to visualize the SDS-PAGE gel.

Statistical analysis Analyses of variance were carried out by standard analysis of variance procedures. Significant differences ($p<0.05$) between means were determined by Duncan's multiple range test using SAS software.

Results and Discussion

Titration curve Shrimp tropomyosin as standard antigen and mouse monoclonal IgG (mAb) were diluted to various concentrations to determine the optimal dilutions for antigen and antibody binding. The greatest dilution of antigen to show binding with antibody was 10 μg/mL, and of antibody binding to antigen was from 1 to 2 μg/mL. Thus, the optimal antigen dilution was 10 μg/mL, and the optimal antibody dilution was 2 μg/mL (Fig. 1). To determine the optimal dilution antigen and serum antibody binding, standard antigen was diluted to 10 μg/mL, and shrimp-allergic patient serum was diluted to various concentrations. The titrated dilution of the serum antibody binding to antigen ranged from 0.063 to 0.016 μg/mL. Thus, the coating antigen dilution was 10 μg/mL and the serum antibody dilution was 0.016 μg/mL (Fig. 2).

Standard curve A standard curve was obtained with 10 μg/mL of antigen and 2 μg/mL of mAb. The concentration of heat stable protein (HSP) reacting with mAb was

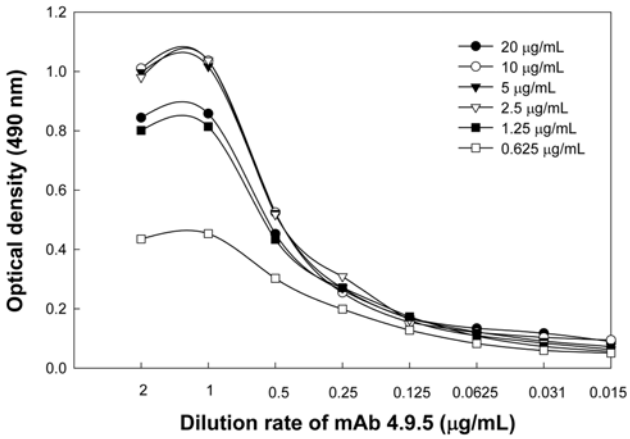

Fig. 1. Titration curves of different concentrations of monoclonal IgG (mAb 4.9.5) binding to tropomyosin using Ci-ELISA. The secondary IgG solution was diluted to 1:5,000 with PBS. The concentration of coated tropomyosin (μg/mL) was 10 μg/mL.

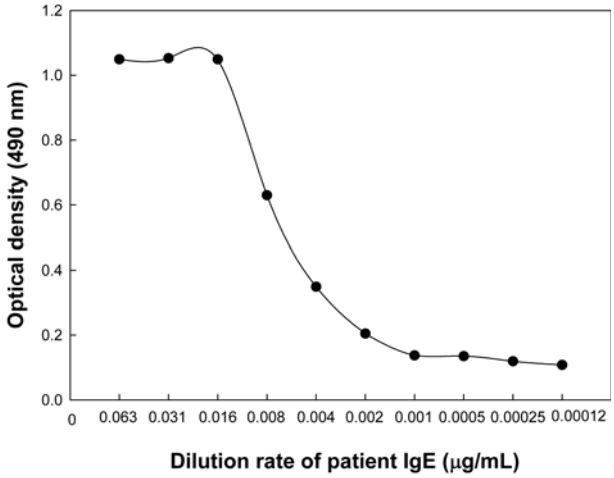

Fig. 2. Titration curve of different concentrations of shrimp-allergic patient serum binding to tropomyosin using Ci-ELISA. The secondary IgE solution was diluted to 1:250 with PBS. The concentration of coated tropomyosin (μg/mL) was 10 μg/mL.

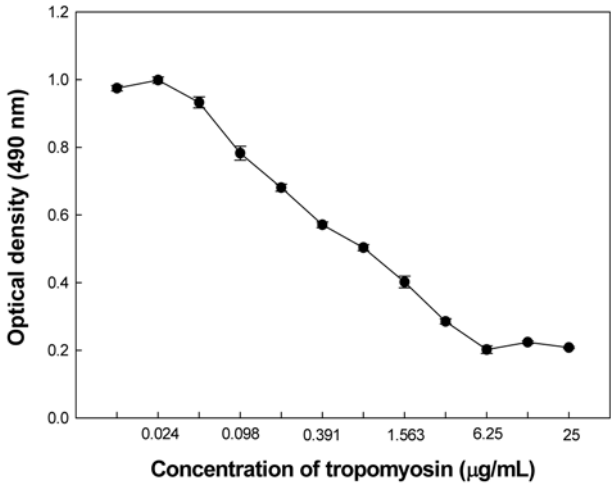

Fig. 3. Standard curve of shrimp-allergic patient serum binding to tropomyosin by Ci-ELISA. HSP was used as a coating Ag. Shrimp-allergic patient serum was used for capturing HSP. HSP was serially diluted from 25 to 0.012 μg/mL.

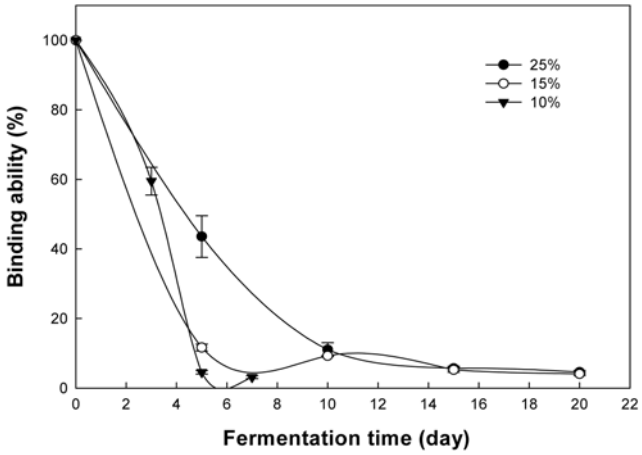

Fig. 4. Binding ability of mAb to different concentrations of salted shrimp meat from *saeujeot* during fermentation at 25°C. Binding ability was measured by Ci-ELISA. Binding ability=Bt/Bo×100. Bt, binding ability of *saeujeot* meat to mAb: Bo, binding ability of raw shrimp extracts to mAb.

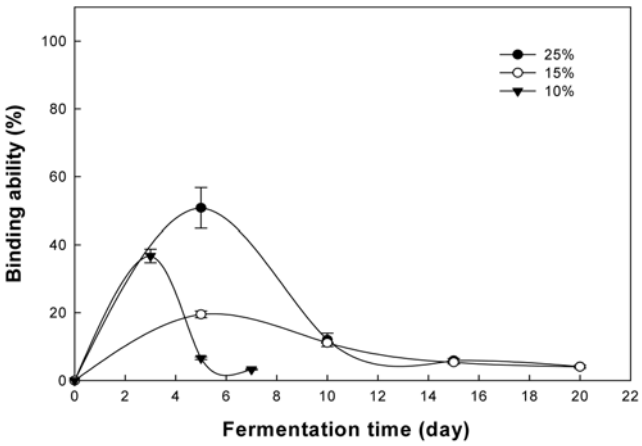

Fig. 5. Binding ability of mAb to different concentrations of salted *saeujeot* sauce during fermentation at 25°C. Binding ability was measured by Ci-ELISA. Binding ability=Bt/Bo×100. Bt, binding ability of *saeujeot* sauce to mAb: Bo, binding ability of raw shrimp extracts to mAb.

calculated by the following equation: $x = e^{[(0.4984 - y)/0.1338]}$, where x indicates the concentration of HSP reacting with mAb and y indicates the optical density (OD) value. The concentration of HSP reacting with mAb was in the range of 0.0487 to 6.25 μg/mL, and the error range was $p \leq 1$ (data not shown). A standard curve was also obtained with 10 μg/mL of antigen and 0.016 μg/mL of shrimp-allergic patient serum. The concentration of HSP reacting with the shrimp-allergic patient serum was calculated by the following equation: $x = e^{[(0.502 - y)/0.1516]}$, where x indicates the concentration of HSP reacting with the shrimp-allergic patient serum and y indicates the OD value. The concentration of HSP reacting with mAb was in the range 0.024 to 6.25 μg/mL, and the error range was $p \leq 1$ (Fig. 3).

The binding ability of allergen and mAb The changes in allergenicity of *saeujeot* that was fermented at various temperatures (25, 15, and 5°C) and salt concentrations (25, 15, and 10%) were investigated. The results show that the allergenicity decreased with longer fermentation periods, and the rate of decrease was faster at higher temperatures and lower salt concentrations. Mok *et al.* (24) reported that the amino nitrogen content in *saeujeot* increased with fermentation time and was higher at low salt concentrations. This result was related to the large decrease in allergenicity at 10% salt concentration conditions. Specifically, the binding ability of mAb to allergen in *saeujeot* fermented for 5 days at 25°C in 25, 15, and 10% salt was approximately 50, 10, and 5%, respectively, and 11, 9, and 3%, respectively, after fermentation for 10 days. The binding ability of allergen in *saeujeot* sauce to antibody increased during the early fermentation period, and then decreased to 5% after fermentation for 10 days (Fig. 4 and 5). Antibody-allergen binding with *saeujeot* fermented at salt concentrations of 25, 15, and 10% at 15°C decreased slowly compared

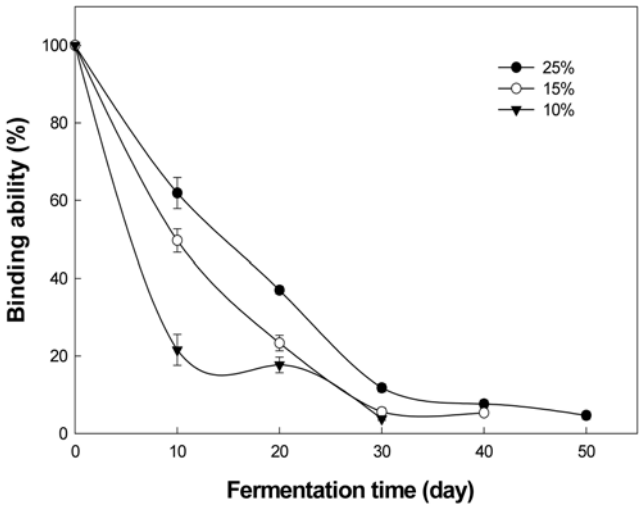

Fig. 6. Binding ability of mAb to different concentrations of salted shrimp meat from *saeujeot* during fermentation at 15°C. Binding ability was measured by Ci-ELISA. Binding ability=Bt/Bo×100. Bt, binding ability of *saeujeot* meat to mAb: Bo, binding ability of raw shrimp extracts to mAb.

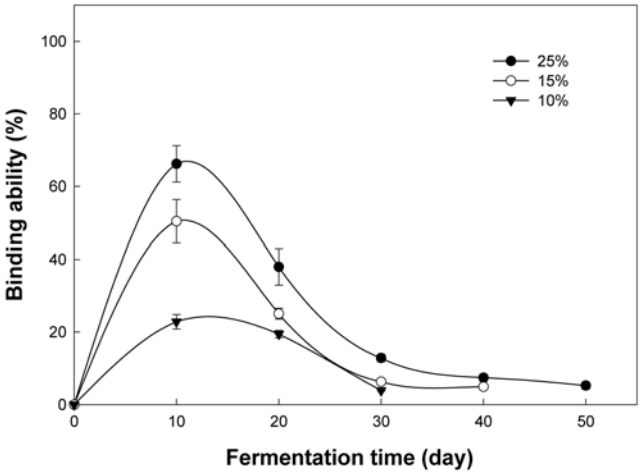

Fig. 7. Binding ability of mAb to different concentrations of salted *saeujeot* sauce during fermentation at 15°C. Binding ability was measured by Ci-ELISA. Binding ability=Bt/Bo×100. Bt, binding ability of *saeujeot* sauce to mAb: Bo, binding ability of raw shrimp extracts to mAb.

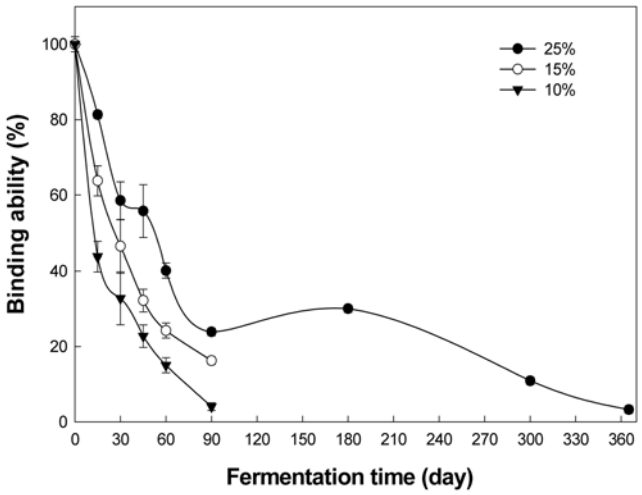

Fig. 8. Binding ability of mAb to different concentrations of salted shrimp meat from *saeujeot* during fermentation at 5°C. Binding ability was measured by Ci-ELISA. Binding ability=Bt/Bo×100. Bt, binding ability of *saeujeot* meat to mAb: Bo, binding ability of raw shrimp extracts to mAb.

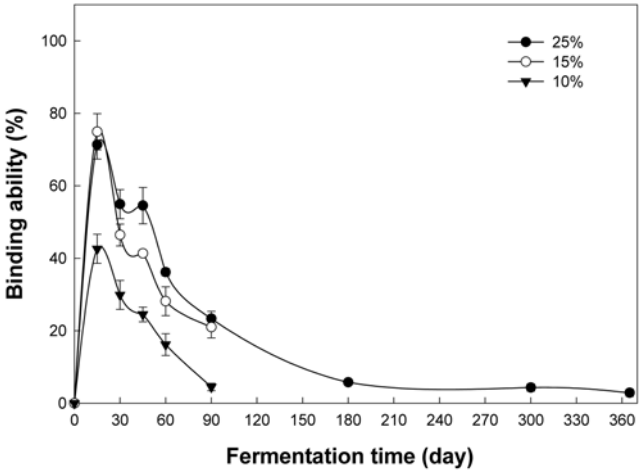

Fig. 9. Binding ability of mAb to different concentrations of salted *saeujeot* sauce during fermentation at 5°C. Binding ability was measured by Ci-ELISA. Binding ability=Bt/Bo×100. Bt, binding ability of *saeujeot* sauce to mAb: Bo, binding ability of raw shrimp extracts to mAb.

to *saeujeot* fermented at 25°C. The binding ability of mAb to allergen in *saeujeot* was approximately 5% after fermentation for 30 days. The binding ability of mAb to allergen in *saeujeot* sauce was greater than 20% after fermentation for 10 days, but below 10% after fermentation for 30 days (Fig. 6 and 7). The binding ability of mAb to allergen in *saeujeot* fermented in 25% salt at 5°C was 40% after 60 days, 30% after 180 days, and below 5% after 360 days. However, the binding ability of *saeujeot* fermented in 15 and 10% salt at 5°C were relatively low (16 and 5%, respectively) after 90 days. The binding ability of mAb to allergen in *saeujeot* sauce fermented in 25, 15, and 10% salt at 5°C increased to over 40% after 15 days, and showed binding ability of 23, 21, and 4%, respectively, after 90 days (Fig. 8 and 9). In conclusion, the binding ability of mAb to allergen in *saeujeot* fermented at 5°C was maintained over extended periods of fermentation. The binding ability of allergen in *saeujeot* sauce to antibody initially increased and then decreased during fermentation because tropomyosin, a salt soluble protein, dissolved in the sauce and thus the binding ability of mAb to allergen increased, after which the salt soluble tropomysin was gradually decomposed by various enzymes. Additionally, the binding ability of mAb to allergen was weak, and changes in binding ability occurred more rapidly at lower salt concentrations. Therefore, the most effective way to reduce *saeujeot* allergenicity is to modulate the salt concentration and fermentation time. Typically, the fermentation of *saeujeot* involves decomposition enzymes from the gut, self-digestive enzymes from the shrimp meat, and enzymes from microorganisms (25,26). The kinds of microorganisms

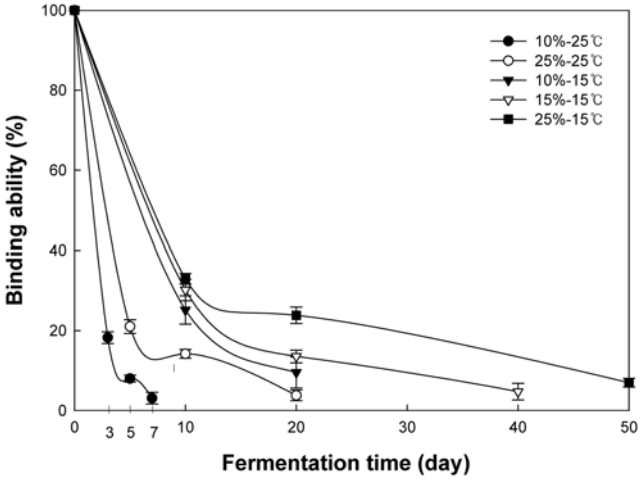

Fig. 10. Binding ability of patient serum to different concentrations of salted shrimp meat from *saeujeot* during fermentation at 25 and 15°C. Binding ability was measured by Ci-ELISA. Binding ability=Bt/Bo×100. Bt, binding ability of *saeujeot* meat to patient serum: Bo, binding ability of raw shrimp extracts to patient serum.

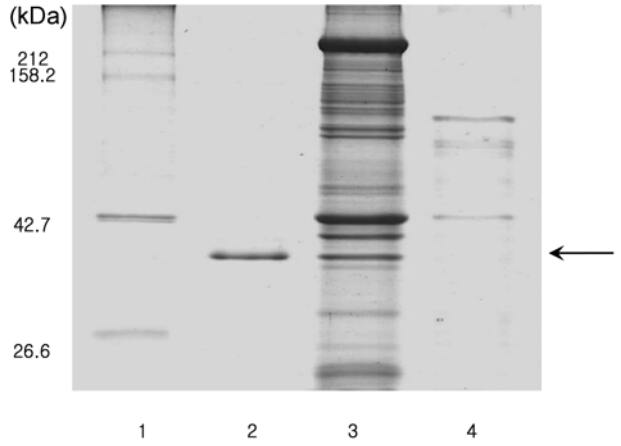

Fig. 11. Changes of the allergen in total muscle protein from 25% salted *saeujeot* at 5°C. Samples are (1) molecular weight marker (2) purified tropomyosin (3) muscle proteins in raw shrimp (4) shrimp muscle proteins after 360 days of fermentation. Arrow indicates the *saeujeot* allergen.

that exist in *saeujeot* include species of *Micrococcus*, *Brevibacterium*, *Sarcina*, *Leuconostoc*, *Bacillus*, *Pseudomonas*, *Flavobacterium*, and various yeasts that produce proteolytic enzymes (25). An alkaline protease isolated from *saeujeot*, a trypsin-like serine protease, exhibits the highest activity (27). Therefore, the binding of mAb to tropomyosin is reduced by decomposition of the allergen.

Binding ability of shrimp-allergic patient serum to *saeujeot* When examining the reactivity between tropomyosin and shrimp-allergic patient serum, the binding ability was reduced with increasing fermentation time. Yet in comparison with mAb, the pattern of binding ability reduction was slightly different. Specifically, the binding ability of shrimp-allergic patient serum to allergen in fermented in 25 and 10% salt at 25°C were 21 and 8%, respectively, after 5 days of fermentation. In the *saeujeot* fermented at a salt concentration of 25%, binding ability was maintained at 3.8% until 20 days of fermentation (Fig. 10). The binding ability of serum antibodies to *saeujeot* allergen fermented in salt concentrations of 25, 15, and 10% at 15°C were 33, 30, and 25%, respectively, after 10 days. For 10% salt *saeujeot* , binding ability was reduced to 9.5% after 20 days of fermentation. For 15% salt *saeujeot*, antigen-antibody binding ability was 5% after 40 days of fermentation, and 7% after fermentation for 50 days in 25% salt *saeujeot*.

In conclusion, the allergenicity of *saeujeot* slowly decreased at higher salt concentrations (Fig. 10). The changes in allergenicity of *saeujeot* fermented in 25% salt at 5°C showed the slowest pattern and binding ability of antigen to antibody was 21% after fermentation for 120 days, and 10% after 300 days (data not shown). Kim (28) and Bae *et al*. (29) reported that *saeujeot* antigenicity was weakened because the allergen was converted to low molecular nitrogen compounds through enzymatic hydrolysis during fermentation. These results agreed with this study. Also, Barkholt *et al*. (30) reported that the antigenicity of pea protein decreased 10% when peas with a moisture content of 70% were fermented with lactic acid bacteria. Based on the results of this study, the immune reactivities of the two antibodies to tropomyosin were slightly different because the epitopes recognized by the antibodies were different. This suggests the possibility for differing results in clinical tests if the mammalian antibody used is obtained during reduced food allergy development. Therefore, to collect data the use of allergic patient serum directly is desirable when using techniques sensitive to structural changes of the allergen and destruction of the epitope (31).

Degradation of tropomyosin in *saeujeot* during fermentation The results of SDS-PAGE of *saeujeot* fermented at a salt concentration of 25% at 5°C indicated that the allergen was almost entirely gone after 360 days of fermentation (Fig. 11).

In this study, we investigated the changes in allergenicity of *saeujeot* fermented at salt concentrations of 25, 15, and 10%, and temperatures of 25, 15, and 5°C. When stored at a low salt concentration and high temperature, the binding of mAb and shrimp-allergic patient serum to the allergen decreased. In particular, the binding ability of mAb to allergen in *saeujeot* fermented in 10% salt at 25°C decreased to 5% after 5 days of fermentation, however the binding ability decreased to 5% with *saeujeot* fermented in 15 and 25% salt after fermentation for 15 days. Also, in *saeujeot* fermented at 25°C, binding was greatly reduced compared to *saeujeot* fermented at 5 and 15°C due to rapid fermentation, thus the allergenicity of *saeujeot* fermented at low salinity and high temperature was rapidly reduced. The binding ability of shrimp-allergic patient serum to allergen in *saeujeot* fermented in 25 and 10% salt was reduced to 21 and 8%, respectively, after 5 days of fermentation. For *saeujeot* fermented in 25, 15, and 10% salt at 15°C, binding ability was 33, 30, and 25%, respectively, after 10 days of fermentation. In particular, antibody-allergen binding ability with *saeujeot* fermented in 10% salt was low at 9.5% after 10 days of fermentation; however binding ability of *saeujeot* with 25% salt was 7% after 50 days of

fermentation. The changes in allergenicity of 25% salt *saeujeot* fermented at 5°C were the lowest since antibody-allergen binding ability after fermentation for 120 days was 21%, and was 10% after 300 days. In conclusion, the binding of *saeujeot* allergen to mAb and shrimp-allergic patient serum was increasingly reduced with fermentation at high temperature and low salinity.

Acknowledgments

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