



Contribution of Structural Reversibility to the Heat Stability of the Tropomyosin Shrimp Allergen

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Tropomyosins are common heat-stable crustacean allergens. However, their heat stability and their effects on antigenicity have not been clarified. We purified tropomyosin in this study from raw kuruma prawns (*Marsupenaeus japonicus*) without heat processing. SDS-PAGE of the purified protein showed a band at approximately 35 kDa that cross-reacted with IgE from the serum of a shrimp-allergic patient, identifying it as Pen j 1. The circular dichroism spectrum of native Pen j 1 revealed the common α -helical structure of tropomyosins which easily collapsed upon heating to 80 °C. However, there were no insoluble aggregates after heating, and the protein regained its native CD spectral pattern after cooling to 25 °C. There was no significant difference in total IgG production between mice sensitized with native and heated Pen j 1. These results suggest that heat-denatured Pen j 1 refolded upon cooling and maintained its antigenicity following the heat treatment.

Key words: shrimp allergen; tropomyosin; heat stability; structural reversibility

Crustaceans are common allergic foods¹⁾ displayed in CODEX and on food labels in many countries. An allergic reaction to shrimps, a typical crustacean allergenic food, causes symptoms as severe as exercise-induced anaphylactic reactions.²⁾ Shrimp-allergic patients are at risk even from a small amount of shrimp in processed foods. Additional preventive information is very important for patients because shrimp and shrimp extract are ingredients in various processed foods; for example, kamaboko (fish cake), imitation crab, fish offal, laver, snack food, and noodles.

Tropomyosin is the major allergen in shrimps and prawns, and it has been characterized as a heat-stable allergen.³⁾ The cDNA sequences, amino acid sequences, molecular weights,⁴⁾ and IgE epitopes⁵⁻⁷⁾ have been reported for some shrimp tropomyosins. These shrimp tropomyosins have highly conserved amino acid sequences and show IgE cross-reactivity in crustaceans and various other invertebrates; for example, squid, octopus,

shellfish, mites, and cockroaches.⁷⁻¹⁰⁾ Tropomyosin is therefore recognized as a pan-allergen in invertebrates.

Although our understanding of shrimp tropomyosin has advanced, the mechanism for its heat stability has not been clarified. Tropomyosin generally has an α -helical structure¹¹⁾ and forms dimers in muscle fiber.¹⁰⁾ α -Helical structures are easily denatured by heat, and this is one of the principal causes of the denaturation and aggregation of proteins with intermolecular disulfide bonds. Many proteins irreversibly lose their solubility, functional properties, and structural features during the formation of high-molecular-weight aggregates as a result of denaturation. Nevertheless, some proteins have higher solubility after heat denaturation, due to the formation of water-soluble aggregates, and the collapse of conformational epitopes and intramolecular burying of sequential epitopes greatly decrease the antigenicity of some proteins; for example, the Japanese cedar pollen allergen Cry j 1.¹²⁾ However, shrimp tropomyosins have been shown, as both boiled meat and in boiling water, to be water-soluble non-aggregated proteins when cooked and to strongly bind with shrimp-allergic patient's sera.³⁾ These data suggest that shrimp tropomyosin is different from other allergenic proteins in its denaturation and collapse of epitopes.

We purified shrimp tropomyosin (Pen j 1) in this study from uncooked kuruma prawns without any heat treatment and such a denaturing agent for shrimp tropomyosins as ethanol, and performed a secondary structural analysis before and after heating. We also investigated the effect of heating on its antigenicity in mice. Understanding the heat-stabilization mechanism for shrimp tropomyosin will contribute to the development of proper preventive methods for the crustacean allergy.

Materials and Methods

Samples. Live kuruma prawns, *Marsupenaeus japonicus* (*Penaeus japonicus*), were purchased from a local fish farmer (Yamazaki Suisan, Yamaguchi, Japan) and stored at -80 °C.

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Abbreviations: CD, circular dichroism; CAP-RAST, capsulated hydrophilic carrier polymer-radioallergosorbent test; CBB, Coomassie brilliant blue; PBS, phosphate-buffered saline; IgE, immunoglobulin E; IgG, immunoglobulin G; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase

Shrimp-allergic human serum. The serum of a shrimp-allergic patient was purchased from PlasmaLab™ International (Everett, WA, USA). The CAP-RAST score for this serum was 6 (>100 kU/L).

Mice. Six-week-old male BALB/c mice were purchased from KBT Oriental Co. (Fukuoka, Japan) and housed under conventional conditions. All experiments were performed according to the Guidelines for Laboratory Animals of the National Fisheries University.

Purification of native Pen j 1 from raw kuruma prawns. Pen j 1 was purified from kuruma prawns according to the method of Kunimoto *et al.* with minor modifications.¹³ Shelled prawns (100 g) were homogenized in twice the volume of a 50 mM KCl/2 mM NaHCO₃ buffer (pH 8.0) containing 10 mM 2-mercaptoethanol at 4 °C, and the soluble protein was removed by centrifugation at 10,000 × g for 20 min at 4 °C. The precipitate was resuspended in the same buffer and homogenized, and washing the precipitate was repeated twice more. After the final centrifugation, the precipitate was extracted overnight at 4 °C with 20 volumes (2 L) of a 20 mM Tris–HCl buffer (pH 7.5) containing 1 M KCl and 10 mM 2-mercaptoethanol. After centrifugation at 10,000 × g for 20 min at 4 °C, the supernatant (crude extract) was adjusted to pH 4.5 with 2 M HCl and stirred for 1 h at room temperature (isoelectric precipitation). After centrifugation under the same conditions, the precipitate was dissolved in 600 mL of 1 mM NaHCO₃. The solution was adjusted to pH 7.5 with 2 M KOH and stirred for 1 h at room temperature. After centrifugation at 10,000 × g for 10 min at 4 °C, 2-mercaptoethanol and KCl were added to the supernatant at respective final concentrations of 10 mM and 1 M. The proteins in the supernatant were fractionated with solid ammonium sulfate after the third isoelectric precipitation. The precipitates forming at 35%, 45%, 55% and 65% ammonium sulfate saturation were collected and dissolved in 1 mM NaHCO₃. The 35% saturation fraction was exchanged by dialysis with a 50 mM Tris–HCl buffer (pH 8.0) after desalting by dialysis against the same solution. After centrifugation at 17,300 × g for 10 min at 4 °C, the supernatant was applied to a Toyopearl DEAE-650M (Tosoh Corporation, Tokyo, Japan) weak anion-exchange column that had been equilibrated with the 50 mM Tris–HCl buffer (pH 8.0). The column was washed with the same buffer, and the adsorbed materials were eluted with a linear gradient of 0–300 mM NaCl in the 50 mM Tris–HCl buffer (pH 8.0). The protein was monitored by measuring the absorbance at 280 nm, and the peak fractions showing a band at 35 kDa by SDS–PAGE were collected. After dialysis against deionized water at 4 °C and lyophilization, this preparation was used as the native shrimp allergen (native Pen j 1).

SDS–PAGE and Western blotting. SDS–PAGE was performed by the method of Laemmli,¹⁴ with e-PAGEL precast polyacrylamide gels (Atto Corporation, Tokyo, Japan) or hand-cast gels. Samples containing ammonium sulfate were purified by the Pierce SDS–PAGE sample preparation kit (Thermo Fisher Scientific, Rockford, IL, USA). The gels were stained after electrophoresis by using a CBB rapid staining kit (Nacalai Tesque, Kyoto, Japan). Western blotting was done by electrotransferring the separated proteins onto a PVDF membrane and incubating with a membrane blocking agent (GE Healthcare Japan Corporation, Tokyo, Japan). The membrane was incubated with the human serum diluted 1:50 in PBS–Tween-20. Bound Pen j 1-specific IgE antibodies were detected by using the HRP-conjugated anti-human IgE antibody (Bethyl Laboratories, Montgomery, USA) and an ECL™ Prime Western blotting detection reagent (GE Healthcare Japan Corporation).

Thermal stability analysis of native Pen j 1. The thermal denaturation of native Pen j 1 was measured by monitoring the change in ellipticity of the CD spectrum at 222 nm according to the method of Harada *et al.* with minor modifications.¹⁵ CD measurements were carried out with a Jasco J-600 spectropolarimeter in a 0.1 cm-path-length cuvette with a temperature increase from 20–80 °C at a heating rate of 1 °C/min. The protein solution was prepared in PBS (pH 7.4) at a protein concentration of 1.0 mg/mL. Ellipticity values at 222 nm were normalized to the fraction of unfolded protein (F_u) by $F_u = (\theta - \theta_N)/(\theta_U - \theta_N)$, where θ is the observed ellipticity, θ_N the native ellipticity, and θ_U the unfolded ellipticity. The thermal

denaturation transition point (T_m) is defined as the temperature at which $F_u = 0.5$.

Heat treatment of native Pen j 1. To investigate the effect of heating on the solubility of shrimp tropomyosin, purified native Pen j 1 was heat treated in PBS at 100 °C for 10 min at a protein concentration of 1 mg/mL (0.1%). After cooling at 25 °C, soluble Pen j 1 was separated by centrifugation at 12,100 × g for 20 min at 25 °C. The supernatant was used for SDS–PAGE and an immunogenicity analysis as the heat-treated shrimp allergen (heated Pen j 1).

CD spectroscopic analysis of Pen j 1. CD spectroscopy was performed as already described with some modifications. The protein solution was prepared in PBS (pH 7.4) at a concentration of 0.5 mg/mL. The temperature of the cuvette was maintained at 25 °C, 45 °C, or 80 °C by a circulating water bath. Wavelength analyses were conducted by scanning native and heated Pen j 1 scanned with a step width of 2 nm. The spectral range was 200–260 nm at 50 nm/min. An average of three consecutive scans was obtained for each sample, the CD spectra being expressed in terms of the mean residual ellipticity (deg cm² dmol^{−1}).

Mouse immunization with Pen j 1. The mice were assigned to groups of native Pen j 1-boosted mice, heated Pen j 1-boosted mice, and PBS-boosted mice ($n = 5$ /group). All the mice were immunized by a subcutaneous injection with 20 µg of native Pen j 1 and 2 mg of alum in PBS. One week later, the mice in the native Pen j 1-boosted group were injected with the same antigen and alum as a positive control. The mice in the heated Pen j 1-boosted group were injected with the same amount of heated Pen j 1 and alum, while the mice in the PBS-boosted group were injected with 2 mg of alum in PBS. The mice were bled from the heart 1 week after the boosters. The collected sera for investigating Pen j 1-specific antibody production were stored −30 °C until needed.

ELISA. The production level of Pen j 1-specific antibodies in each group was measured by Pen j 1-specific ELISA according to the method of Huang and Chu,¹⁶ but with a small modification. Fifty µL of Pen j 1 (10 µg/mL) in a carbonate buffer (pH 9.2) was added to each well of a 96-well ELISA microtiter plate, and the plate was kept overnight at 4 °C. After the solutions had been removed, the wells were incubated with 200 µL of a blocking solution (2.0% BSA in PBS) at 37 °C for 2 h and then washed three times with 300 µL of PBS–Tween-20, using a Biotrak II plate washer (GE Healthcare Japan Corporation). To each well was then added 50 µL of the serum solution (total IgG, 1:5,000; IgG1, 1:5,000; IgG2a, 1:500; respectively diluted in PBS). The plate was gently mixed and incubated at 37 °C for 2 h. After washing the plate three times with 300 µL of PBS–Tween-20, 50 µL of a goat anti-mouse IgG antibody–HRP conjugate (Exalpha Biologicals, Watertown, MA, USA) or goat anti-mouse IgG1 antibody–HRP conjugate (Bethyl Laboratories) or goat anti-mouse IgG2a antibody–HRP conjugate (Bethyl Laboratories) diluted in PBS–Tween-20 containing 0.2% BSA was added to each well, and the plate was incubated at 37 °C for 2 h. The level of each antibody was detected with a TMB microwell peroxidase substrate kit (KPL, Gaithersburg, MD, USA) after washing three times with 300 µL of PBS–Tween-20. After incubating at 25 °C for 15 min, the reaction was stopped by adding the TMB stop solution. The absorbance at 450 nm was measured with an MTP-450Lab microplate reader (Corona Electric Co., Ibaraki, Japan), each result being expressed as the mean ± standard deviation (SD). Statistical evaluations were performed by using Student's *t*-test.

Results and Discussion

Purification and identification of native Pen j 1

SDS–PAGE and Western blotting of each sample obtained during the purification of native Pen j 1 are shown in Fig. 1. The expected Pen j 1 compound of approximately 35 kDa was preferentially concentrated by isoelectric precipitation. This 35-kDa protein was subsequently fractionated by 35% saturated ammonium

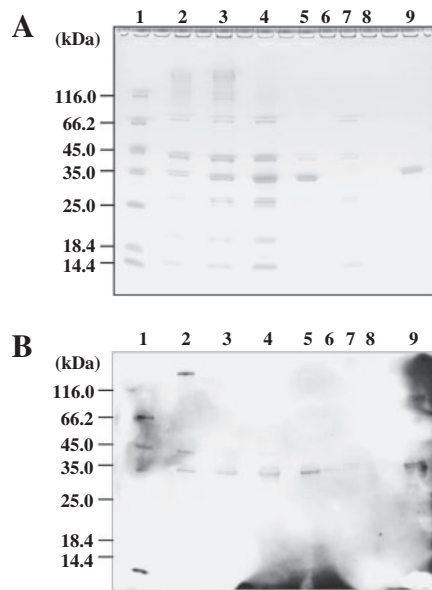


Fig. 1. Purification and Identification of Native Pen j 1.

SDS-PAGE (A) and Western blotting (B) of the purification of native Pen j 1. Lane 1, molecular markers (kDa); lane 2, extract with a 20 mM Tris-HCl buffer (pH 7.5) containing 1 M KCl and 10 mM 2-mercaptoethanol (before isoelectric precipitation); lane 3, after the first isoelectric precipitation; lane 4, after the third isoelectrical precipitation; lane 5, precipitate formed at 35% saturation of ammonium sulfate; lane 6, 45% saturation; lane 7, 55% saturation; lane 8, 65% saturation; lane 9, purified native Pen j 1 (after chromatographic purification).

sulfate. After purification in the anion-exchange column, the 35-kDa protein was a single band by SDS-PAGE and showed specific cross-reactivity with the IgE antibody in the patient's serum. Shrimp tropomyosin has previously been reported to be approximately 35 kDa and to have high cross-reactivity with IgE from shrimp-allergic patients with a band of approximately 35-kDa by more than one research party. Tropomyosin of kuruma prawns has previously been purified by Kunimoto *et al.*, using a similar method, and the N-terminal amino acid sequence and cDNA were determined which encoded 284 amino acid residues.¹³⁾ This amino acid sequence was highly conserved between prawns (99–100%).^{10,13)} In addition, a high concentration of shrimp tropomyosins was present in the crude extract which reacted with IgE in the patient's serum as a major allergen.^{3,4,8,13)} The 35-kDa protein was also detected as a major allergen in this present study in the crude extract and at each purification step. The purified 35-kDa protein showed an α -helical conformation in the CD spectroscopic analysis which is typical for tropomyosin (this will be described later). The purified 35-kDa protein was therefore identified as Pen j 1, a major shrimp allergen tropomyosin derived from kuruma prawns.

Thermal stability of native Pen j 1

The thermal stability analysis revealed that the T_m value for native Pen j 1 was 46 °C and that the α -helix structure had almost completely collapsed at 80 °C (Fig. 2). Shrimp tropomyosins, including Pen j 1, have previously been identified as heat-stable proteins from boiled shrimp.³⁾ However, Pen j 1 was denatured by a heat treatment as easily as the other common proteins,

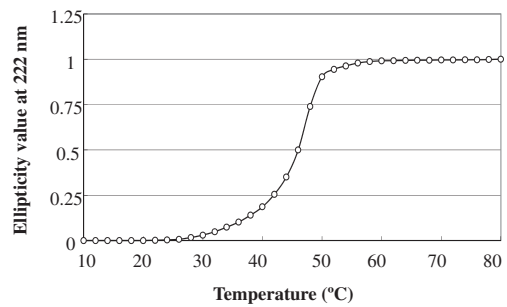


Fig. 2. Thermal Denaturation Curve for Native Pen j 1.

The thermal denaturation of native Pen j 1 was measured by monitoring changes in the ellipticity of the CD spectrum at 222 nm from 10 °C to 80 °C. The average of five individual scans is shown.

Table 1. Absorbance Value for Pen j 1 Before and After the Heat Treatment

	Absorbance at 280 nm
Native Pen j 1	0.208
Heated Pen j 1	0.216

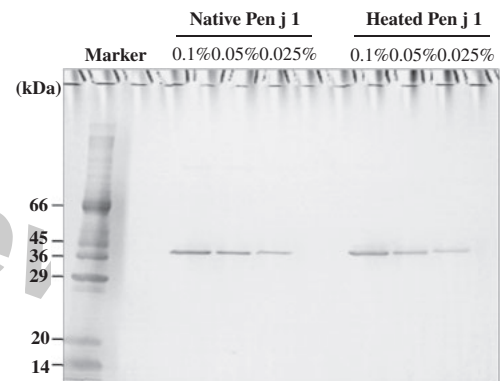


Fig. 3. SDS-PAGE of Heat-Treated Pen j 1.

PBS containing 0.1% native Pen j 1 was divided into equal volumes, and the counterpart was heat treated as described in the Materials and Methods section. SDS-PAGE was conducted with both Pen j 1 samples respectively diluted to a 0.5% and 0.25% protein concentration.

although heated Pen j 1 showed similar absorbance to native Pen j 1 at 280 nm (Table 1) and the same pattern in the SDS-PAGE analysis after the heat treatment (Fig. 3). If Pen j 1 became insoluble and precipitated with heating, the absorbance of the supernatant would be expected to decrease. Moreover, if Pen j 1 had formed an insoluble aggregate, the band on SDS-PAGE should have shifted to that of a higher molecular weight (>70 kDa). Pen j 1 showed neither of these changes, these results suggesting that even if Pen j 1 had heat denatured, it would not form an insoluble aggregate. Amino acid sequencing of Pen j 1 revealed a high content of such hydrophilic amino acids as lysine, glutamine, aspartic acid, and arginine.¹³⁾ Denatured Pen j 1 would therefore be expected to have greater water solubility. Since Pen j 1 had no cysteine residues, it did not form intramolecular and intermolecular disulfide bonds. It thus appears that the hydrophilic amino acid sequence and absence of cysteine residues contributed to the solubility of Pen j 1 at high temperatures.

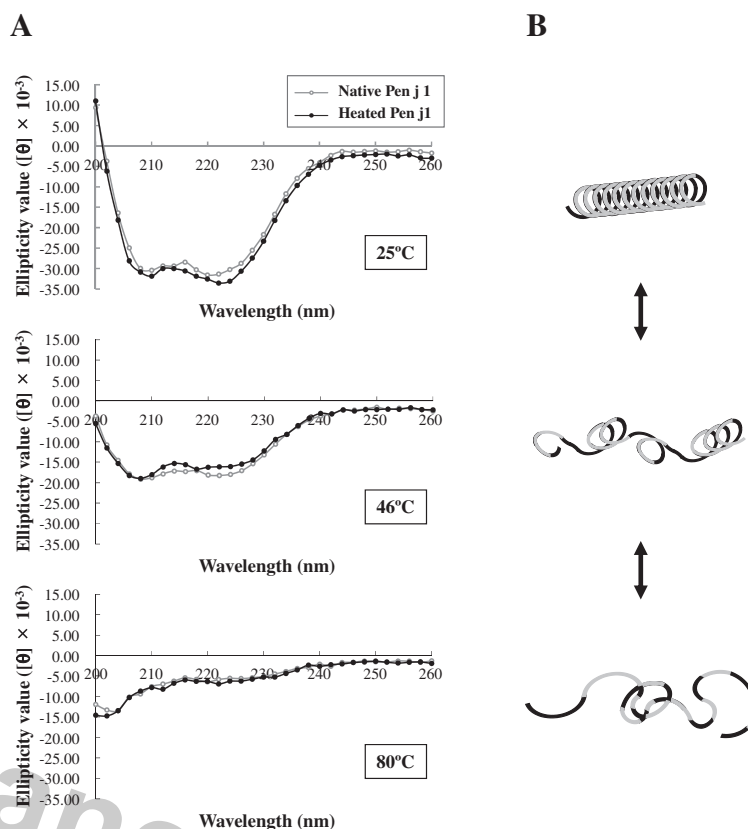


Fig. 4. Far-UV Spectra and Conformation Models for Pen j 1.

A, Far-UV spectra of native and heated Pen j 1 at different temperatures. B, Molecular models for the denaturation and refolding of Pen j 1. Each sample was analyzed at temperatures corresponding to the native state (25 °C), the intermediate state (46 °C), and the denatured state (80 °C). The averages of three consecutive scans are shown.

Structural analysis of Pen j 1 before and after heating

The results of the CD spectroscopic analysis are shown in Fig. 4. Native Pen j 1 showed an α -helical conformation in the CD spectroscopic analysis at 25 °C which is typical for tropomyosin. It has been reported that the CD spectrum of shrimp tropomyosin Pen a 1 was consistent with α -helically folded proteins.¹¹⁾ The result for the native Pen j 1 spectrum was consistent with that for Pen a 1.¹¹⁾ Half of the α -helical conformation of native Pen j 1 collapsed at 46 °C, and nearly all of it had collapsed at 80 °C. These results are consistent with the thermal stability analysis. It is therefore believed that Pen j 1 did not possess structural heat resistance similar to that of a DNA polymerase.¹⁷⁾ It is interesting that, at 25 °C, heated Pen j 1 also showed an α -helical conformation similar to that of native Pen j 1. Its conformation also collapsed with increasing temperature (46 °C and 80 °C), and the shape of the CD spectrum overlapped that of native Pen j 1. These results suggest that, even if Pen j 1 is completely heat denatured, it can refold to the native state by cooling to 25 °C (the denatured and refolding images at each temperature are shown in Fig. 4B). Repeated thermal stability and CD spectroscopic analyses of the same sample after the high-temperature analyses yielded identical results (data not shown). Reese *et al.* have reported that natural and recombinant shrimp tropomyosin (rPen a 1) was overexpressed in *Escherichia coli* [BL21 Star (D3)] and both had an α -helical structure and almost identical curves with respective characteristic minima at approximately 208 nm and 222 nm in the CD

spectroscopic analysis.¹¹⁾ Kunimoto *et al.* have also reported that a GST fusion with recombinant Pen j 1 overexpressed in *E. coli* was found in the soluble fraction of the cell lysate.¹³⁾ Although overexpressed in the common *E. coli* expression system, the recombinant protein with native folding in *E. coli* may indicate that shrimp tropomyosin readily formed its native structure on its own. It appears that the simple structure and high propensity for an α -helix configuration contributed to the reversibility of the structure of Pen j 1 between the native and denatured states.

Effect of the heat treatment on Pen j 1 antigenicity

The production levels of three types of native Pen j 1-specific IgGs (total IgG, IgG1, and IgG2a) in the sera of mice that had received boosters of the respective antigens are shown in Fig. 6. The production levels of all types of native Pen j 1-specific IgG were significantly higher in both the Pen j 1-boosted mouse groups than in the PBS-boosted mice group ($p < 0.05$). The production levels of native Pen j 1-specific total IgG and IgG1 in the heated Pen j 1-boosted mice group were slightly lower than the levels in the native Pen j 1-boosted mice group, although the differences were not significant. In contrast, the production level of native Pen j 1-specific IgG2a was significantly lower than in the native Pen j 1-boosted mice group ($p < 0.05$). A decrease in IgG1 generally suggests suppression of the Th2-type T helper-cell response, while a decrease in IgG2a suggests suppression of the Th1-type response. Both responses were competitively regulated. The

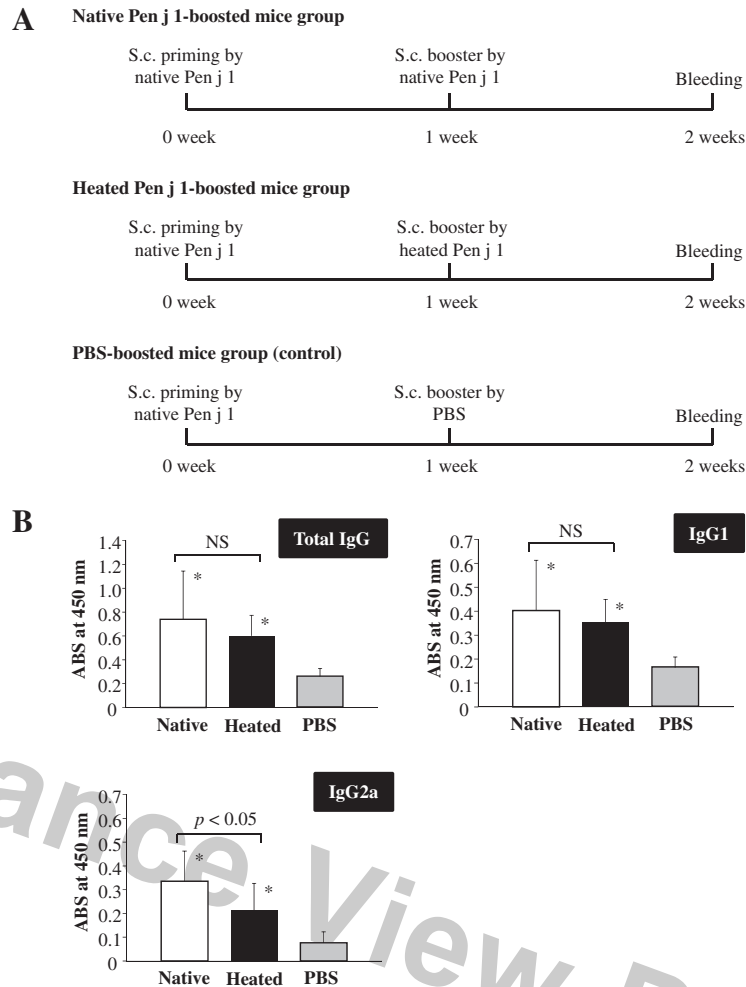


Fig. 5. Production Level of the Anti-Pen j 1 Antibodies in Pen j 1-Immunized Mice.

A, Pen j 1 immunization protocol. The mice belonging to the native Pen j 1-boostered mice group and heated Pen j 1-boostered mice group were boosted by PBS containing 20 μ g of the allergen protein and 2 mg of alum. The mice belonging to the PBS-boostered mice group were boosted by PBS containing 2 mg of alum as a control. B, Production level of the anti-Pen j 1 antibodies. The production level of each antibody was analyzed by Pen j 1-specific ELISA. Each antiserum was added to a Pen j 1-coated plate, and detected by the HRP-labeled antibody to each subclass. Results are indicated as the mean $\pm$ SD. Asterisks indicate a significant difference at $p < 0.05$ compared with the PBS control group. NS, not significant.

decreased IgG2a level in the heated Pen j 1-boostered mice group therefore suggests that the booster injection of heated Pen j 1 suppressed the Th1-type response when compared with native Pen j 1. Although it was a small change, suppression of the Th1-type response would not be useful to controlling the allergy.

The decrease in the production levels of native Pen j 1-specific total IgG and IgG1 in the heated Pen j 1-boostered mice group was not significant compared with that in the native Pen j 1-boostered mice group, suggesting that the antigenic structure of Pen j 1 was sufficient for the cross reaction with the immune system, even after heating. These results suggest that the heat-denatured antigenic structure of Pen j 1 reformed with cooling, and that the antigenicity of Pen j 1 was maintained after the heat treatment. Heat treatment would therefore not be an effective method for reducing the antigenicity of Pen j 1. However, it is also considered that since a decreased production level of native Pen j 1-specific IgG2a was observed, it cannot be discounted that the antigenic structure had changed slightly. Even if that indicates a change in the antigenic structure, since the production levels of total IgG and

IgG1 were not significantly decreased, the change would not effectively reduce the antigenicity and would not affect the safety in food. However, we could not clarify details of the change in antigenic structure in this study. Further understanding of the antigenicity of Pen j 1 will be required to clarify this aspect.

In conclusion, the heat-denatured structure of Pen j 1 was reversible with cooling, probably due to its simple structure and high hydrophilicity. This reversibility also applied to its antigenic structure.

Acknowledgments

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