



Elimination of the major allergen tropomyosin from shrimp muscle by boiling treatment

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Abstract

Tropomyosin (TM) is the major allergen found in invertebrates, including shrimp. To reduce the allergenicity of shrimp muscle, attempts were made to eliminate TM from the muscle by boiling. By boiling cubes (~ 1 g) of shelled shrimp muscle in 10 volumes of water (w/v) for 20 min, 7.1% of TM remained as determined by SDS-PAGE. Increasing the boiling water up to 2000 volumes of water did not result in improved elimination. When the muscle cubes were treated at 121 °C for 20 min in 2000 volumes of water, enzyme-linked immunosorbent assay (ELISA) reactivity against TM was reduced to 2.0×10^{-2} fold. After successive second and third treatments under the same conditions, ELISA reactivity was reduced to 4.2×10^{-5} and 1.6×10^{-5} folds, respectively. In addition, TM content in the muscle after the third treatment was lower than the threshold value for mandatory labeling (10 µg of ingredient proteins per 1 g of food). Thus, boiling treatment, especially high-pressure heating, can be an effective method for providing hypoallergenic shrimp for those who are allergic to shrimp and related invertebrates.

Keywords Shrimp · Tropomyosin · Hypoallergenic · Boiling

Introduction

Marine invertebrates such as shrimp and squid are a frequent cause of food allergic reactions (Reese et al. 1999). In Japanese food-labeling regulations, the labeling of shrimp and crab is mandatory, while that for abalone and squid is recommended (Akiyama et al. 2011; Shoji et al. 2018). The threshold value for the mandatory labeling of allergenic ingredients is 10 µg of proteins, not of the major allergen, per 1 g or ml of food (Akiyama et al. 2011; Shoji et al. 2018), and 1 g of shrimp muscle contains around 0.2 g of

protein (Ministry of Education, Culture, Sports, Science and Technology 2015).

The major allergen in shrimps and crabs has been identified as tropomyosin (TM) (Reese et al. 1999). TM is an actin-binding protein and regulates muscle contraction with the aid of troponin complex in striated muscles (Manning et al. 2011; Williams et al. 2016, 2018; Lehman et al. 2019). TM is a dimeric parallel coiled-coil protein of monomers consisting of 284 amino acid residues (Iwasaki et al. 1997; Inoue et al. 1999; Perry 2001; Motoyama et al. 2006, 2007; Ochiai et al. 2010).

Food allergens are generally resistant to aggregation by heating and thus retain allergenicity even after heating (Shiomi et al. 1998; Offermann et al. 2015). Although TM is unfolded by heating and completely loses its secondary structure, it is rarely aggregated (Huang and Ochiai 2005; Ozawa et al. 2010, 2011, 2019). General food allergens show resistance against digestion in the stomach and/or small intestine and retain the epitopes, which are bound by immunoglobulin E (IgE) (Astwood et al. 1996), although TM shows resistance against digestion only in the stomach and not in the small intestine (Kunimoto et al. 2011). Some proteins which show allergenicity are not homologous to

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human counterparts, while others could have lower amino acid sequence identity irrespective of homologous proteins, compared with the human counterpart proteins (generally less than 62%) and thus are recognized as xenobiotics by the immune system (Jenkins et al. 2007). According to the criteria, TM in many invertebrates shows low identity (around 50%) with human TM (Jenkins et al. 2007). Fish TM shows around 90% amino acid sequence identity with human TM and is rarely allergenic, with some exceptions (Liu et al. 2013).

Shrimp TM was studied intensively and the epitope regions were determined by Ayuso et al. (2002a, b). The epitope regions show higher stability than the non-epitope regions (Ozawa et al. 2018a). To the best of the authors' knowledge, there is so far no satisfactory hypoallergenic method for shrimps, based on the Japanese labeling threshold.

In a previous study, the treatment of shrimp and squid muscle with 10 volumes of boiling water (w/v) for 10 min resulted in a reduction in TM of approximately 89% and 65% in shrimp and squid muscles, respectively (Ozawa et al. 2017). It was also found that the boiling water treatment was very effective compared with treatment in lower-temperature water. In the present study, the dynamics of TM elution from the shrimp muscle were investigated in detail. Treatment at 121 °C was also performed, and the TM content remaining was evaluated according to the criteria set by the Consumer Agency Deputy Director (September 10, 2010).

Materials and methods

Materials

Specimens of Pacific white shrimp *Penaeus vannamei*, which were purchased in a frozen state at a local market, were used. The weight and size of the tail muscle were approximately 8 g and 7 cm, respectively. After shelling, the tail muscle was cut with a cooking knife to obtain cubes of approximately 1 g.

Elution of TM from the shrimp muscle cube by boiling treatment

The experiment was performed as reported previously (Ozawa et al. 2017) with some modifications. For treatment in 10 volumes of water per shrimp muscle cube at 100 °C for up to 20 min, four muscle cubes were used. A centrifugation tube (50 ml) containing 10 volumes of water per shrimp muscle cube (ca. 4 g) was brought to boiling in a stainless-steel pot (H-5871; Pearl Metal, Niigata, Japan) with an induction heating cooker (IHK-T32, 1000 W; Iris Ohyama, Miyagi, Japan). The cubes were

immediately soaked in the boiling water, and aliquots (100 µL) of the soup obtained at 1, 2, 5, 10, 15 and 20 min of boiling treatment were mixed with an equal volume of EzApply (Atto, Tokyo, Japan) as sample pretreatment buffer.

All of the boiled muscle cubes were wiped dry and then homogenized in four volumes of 10 mM potassium phosphate buffer (pH 7.0) containing 150 mM NaCl at 4 °C (Ozawa et al. 2017) using an ULTRA-TURRAX homogenizer with tube drive control with a DT-20 mixer (IKA, Osaka, Japan). The homogenate in the tube (50 ml) was then heated for 20 min in boiling water and subsequently centrifuged for 10 min at 10,000×g at 4 °C, and the supernatant was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The cube without boiling treatment was used as a control which was homogenized and boiled to determine the total TM content.

Other muscle cubes were also treated in a cooker (H-5042; Pearl Metal) with 2000 volumes of water at 100 or 121 °C for 20 min. In addition, the treatment at 121 °C was repeated up to two times. For these treatments, pressurized cooking at 2 atm and 121 °C was performed with a 3 kW induction heating cooker (FICK3430; Fujimak, Tokyo, Japan). The cubes were put in the boiling water, and the lid of the cooker was closed to increase the internal temperature to 121 °C. The temperature reached 121 °C within 90 s after putting the cubes in boiling water. The heating was then lowered with a constant release of steam. After 19.5 min from the start of heating the cubes, the heater was cooled with tap water for around 30 s to reduce the pressure, and the cubes were taken out and used for the following experiments.

SDS-PAGE

SDS-PAGE was performed according to Kimijima and Ozawa (2017). Unlike the ordinary SDS-PAGE gel preparation, stacking gel solution, with neither water nor organic solvent, was poured on top of the separating gel solution to save time. EzGel Ace and EzRun MOPS (Atto) were used as the buffer for gel preparation and running the gels, respectively. The 12.5% polyacrylamide gel was run at 200 V for 23 min, subsequently stained with EzStain Aqua (Atto) overnight, and destained until the background was fully transparent. TM content was estimated by ImageJ (Abramoff et al. 2004), using bovine serum albumin (BSA, Thermo Fisher SCIENTIFIC, Rockford, IL, USA) as a protein standard. Care was taken to consider that the signal intensity of the stained protein bands is not proportional to protein quantity and that the signal intensity per protein quantity was decreased with the increment of protein quantity (Ozawa et al. 2018b). The relationship between the protein content (y) and signal intensity (x) is $y = px^d$, where

p and q are the constants determined by using BSA as a standard.

Enzyme-linked immunosorbent assay (ELISA)

For evaluation of the remaining amount of TM, ELISA was performed for the cubes treated at 121 °C, using a Crustacean Kit II (Maruha Nichiro, Japan), which is specifically designed for crustacean TM detection, according to the manufacturer's protocol. With this treatment, the shrimp muscle was solubilized almost completely. Slight underestimation or overestimation of shrimp TM is possible. Since Japanese food allergen labeling regulations refer to the protein content based on the causal ingredient (10 µg/g), but not on the major allergen content, the content of protein was calculated as the ingredient protein content, but not as TM content, based on the concentration of the protein standard in this kit (Seiki et al. 2007).

Modeling of TM elution from the muscle cube

By treating the cube with a volumes of water per shrimp muscle cube, the concentration of TM could be calculated as follows:

$$-d[\text{TM}]_{\text{solid}}/dt = ad[\text{TM}]_{\text{liquid}}/dt = k\{[\text{TM}]_{\text{solid}} - [\text{TM}]_{\text{liquid}}\} \quad (1)$$

$$[\text{TM}]_{\text{liquid}}/[\text{TM}]_{\text{solid}}^0 = [1 - \exp\{-(a+1)kt/a\}]/(a+1) \quad (2)$$

$$[\text{TM}]_{\text{solid}}/[\text{TM}]_{\text{solid}}^0 = [1 + a\exp\{-(a+1)kt/a\}]/(a+1) \quad (3)$$

$$t = t_{\text{boil}} - t_{\text{lag}} \quad (4)$$

where $[\text{TM}]_{\text{solid}}$ and $[\text{TM}]_{\text{liquid}}$ indicate the concentration of TM in the muscle cube and treated water (soup), respectively. $[\text{TM}]_{\text{solid}}^0$ is the concentration of TM in the muscle before boiling. The k (min^{-1}) is the rate constant of TM elution by boiling, which is dependent on the temperature. From Eqs. (1), (2) and (3) were obtained, where, the t_{boil} and t_{lag} (min) in Eq. (4) are the boiling time and lag time, respectively. The determination of each value was performed by a least-squares method using a solver in Microsoft Excel 2016, and for the treatment of the cubes in 10 and 2000 volumes of water per shrimp muscle cube, Eqs. (2) and (3) were used, respectively. The eluted amount of TM (mg/g of muscle) was divided by the TM content in muscle as determined by the control experiment to obtain the elution ratio.

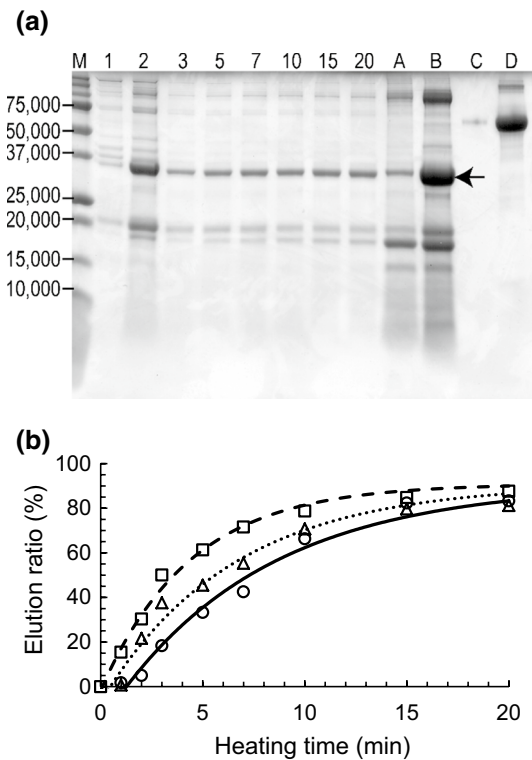


Fig. 1 Elution profiles of tropomyosin (TM) from shrimp muscle cube as analyzed by the SDS-PAGE pattern of the extract (soup). The cubes were treated at 100 °C in 10 volumes (w/v) of water for up to 20 min. **a** M, marker; the numbers on the top indicate the treatment time in boiling water (min); lanes A and B, the 20-min-boiled and control muscles, respectively; lanes C and D, 0.1 and 5 µg of bovine serum albumin, respectively. The applied volume of samples were 20 µl for 1 and 2 min, 2 µl for 3, 5, 7, 10, 15 and 20 min, and 10 µl for lanes A and B. The arrow indicates the band of TM. **b** The elution ratio of TM. The eluted amount of TM (mg/g of muscle) was divided by the TM content in muscle as determined by the control experiment to obtain the elution ratio. The treatments were performed in triplicate successively as the first, second and third ones; the thick line and open circle, the first trial; dotted line and open triangle, the second trial; broken line and open square, the third trial. The SDS-PAGE data in **a** correspond to the second trial in **b**

Statistical analysis

Each treatment was performed in triplicate, and all of the data are shown as the mean $\pm$ standard deviation in the main text. Tukey's test was performed in R version 3.5.2. The level of $p < 0.05$ was considered as statistically significant.

Results

Unlike the other muscle proteins, TM is not precipitated by heating (Ozawa et al. 2011), and its monomer was observed as a protein band of about 33,000 in molecular weight

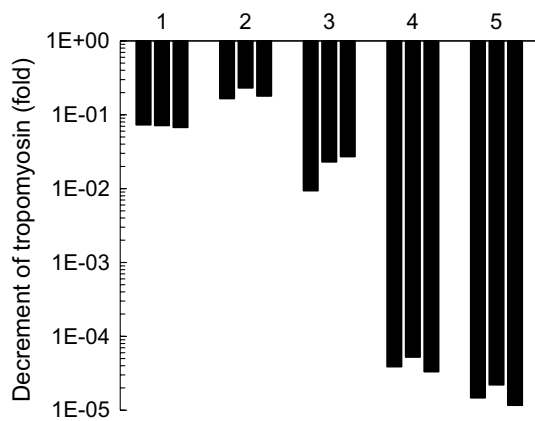


Fig. 2 The decrement of TM in the shrimp muscle cube by boiling for 20 min. 1, 100 °C in 10 volumes of water per shrimp muscle cube; 2, 100 °C in 2000 volumes of water per shrimp muscle cube; 3, 121 °C in 2000 volumes of water per shrimp muscle cube; 4, the second treatments at 121 °C in 2000 volumes of water per shrimp muscle cube; 5, the third treatments at 121 °C in 2000 volumes of water per shrimp muscle cube. For the treatment at 121 °C in 2000 volumes per shrimp muscle cube, consecutive heating was performed. The decrement of TM (fold) shows significant differences among each treatment ($p < 0.05$) by Tukey's test. Protein content was determined by SDS-PAGE and ELISA for 100 °C and 121 °C treatments, respectively, and was divided by the values of untreated raw muscle cube

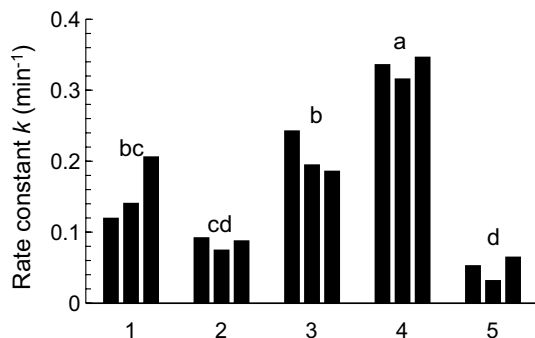


Fig. 3 The rate constant k of TM elution by boiling for the treatment in 10 or 2000 volumes per shrimp muscle cube at 100 °C or 121 °C. Refer to the legend of Fig. 2 for the experimental conditions (1, 2, 3, 4 and 5) and to Eqs. (1–3) in the text for the definition of k . The different lowercase letters indicate significant differences ($p < 0.05$) by Tukey's test

(Fig. 1a). The arrow in Fig. 1a indicates the band of TM, because the protein was precipitated at pH 4.5 (the isoelectric point of TM) or around 40% ammonium sulfate saturation (data not shown), as reported previously (Ozawa et al. 2011). As for the lane B in Fig. 1a, where only 1 μ L of sample was applied, the band was still a single band (data not shown). Thus, there would be little or no overestimation of TM. TM content in the control muscle cube was estimated to be 6.0 ± 0.4 mg/g by SDS-PAGE. Elution of TM by boiling for 1 to 20 min was observed (Fig. 1). After treatment in 10

volumes of boiling water per shrimp muscle cube for 20 min, only $7.1 \pm 0.3\%$ of TM remained in the cube and $85 \pm 2\%$ of the total TM was eluted (Fig. 2). From the TM content in the soup obtained by the treatment for 1 to 20 min (Fig. 1), the rate constant k of elution by boiling (see Eqs. 1–3) at 100 °C and the lag time t_{lag} , which corresponds to the intercept of the horizontal axis in Fig. 1b (see Eq. 4), were calculated to be 0.16 ± 0.05 min⁻¹ (Fig. 3) and 0.61 ± 0.60 min, respectively. By boiling the cube in 2000 volumes of water per shrimp muscle cube at 100 °C, $19 \pm 3\%$ of TM remained in the muscle (Fig. 2), with k being $8.6 \pm 0.9 \times 10^{-2}$ min⁻¹ (Fig. 3), when t_{lag} was assumed to be 0.61 min.

TM content in the treated cube was further determined by ELISA, when the amount of remaining TM was considered to be below the limit of quantification by SDS-PAGE (0.1 μ g). The values of remaining TM were converted to the content of shrimp protein, not as TM content per se. By ELISA, the converted protein content (untreated) in the raw muscle was 0.49 ± 0.33 g/g. By the first treatment at 121 °C for 20 min in 2000 volumes per shrimp muscle cube, the content was reduced to 9.8 ± 4.6 mg/g. In addition, after the consecutive second and third treatments under the same conditions, the content was reduced to 21 ± 5 μ g/g and 8.0 ± 2.7 μ g/g, respectively. Thus, TM content was reduced to $2.0 \pm 0.9 \times 10^{-2}$, $4.2 \pm 1.0 \times 10^{-5}$ and $1.6 \pm 0.5 \times 10^{-5}$ fold after the first, second and third treatments, respectively (Fig. 2). The k values at 121 °C in 2000 volumes per shrimp muscle cube were calculated to be 0.21 ± 0.03 , 0.33 ± 0.02 and 0.051 ± 0.017 min⁻¹ at the first, second and third treatments, respectively (Fig. 3), provided t_{lag} was 0.61 min, and the protein content was 0.49 g/g, 9.8 mg/g and 21 μ g/g before the treatment and after the first and second treatments, respectively.

Discussion

There were significant differences in the remaining TM in the shrimp muscle cube after each treatment (Fig. 2). The increase in boiling water for the treatment from 10 to 2000 volumes per shrimp muscle cube was ineffective for improving TM elimination (Fig. 2) because of theoretical and technical factors. Namely, when k and a were 0.16 and 2000, respectively, 4.5% of the total TM was calculated to remain in the muscle. Theoretically, the increase in water volume was expected to result in a slight improvement, but in the present experiment, it resulted in increased TM content in the muscle cube because of differences in the experimental procedures. The treatment in 2000 volumes of water per shrimp muscle cube was not as effective at removing TM as that in 10 volumes of water per shrimp muscle cube. When heated in 10 volumes of water per shrimp muscle cube, the shrimp muscle cube in a 50 ml tube was placed

in a stainless-steel pot, which was heated by inductive heating at maximum power. On the other hand, when heated in 2000 volumes of water per shrimp muscle cube, the shrimp muscle cube was put in a different stainless-steel pot, which was heated at moderate power to reduce the amount of water loss by evaporation. Treatment in 2000 volumes of water per shrimp muscle cube resulted in a decrease in water by approximately 5%.

The rate constant k for the elution of TM was increased by 2.4 times at the first 121 °C treatment in 2000 volumes per shrimp muscle cube compared with that at 100 °C in 2000 volumes of water per shrimp muscle cube (Fig. 3). Compared with the first treatment at 121 °C, the k value in the second treatment was increased by 1.6 times, although that of the third treatment was decreased by 0.24 times (Fig. 3). Further investigation is needed to fully explain the changes in k values at 121 °C treatment.

For the treatment in 10 volumes of water per shrimp muscle cube, Eqs. (1–4) were validated. In the present treatment, $7.1 \pm 0.3\%$ of the total TM remained in the cube, while $85 \pm 2\%$ of TM was eluted. Thus, $8.9 \pm 2.5\%$ of the total TM was lost. The reason could be the difference in elution efficiency. It should be noted that the above equations were intended for approximation, but it was confirmed that the equations were robust enough in this experiment, as shown in Fig. 1b.

When a and $a \exp\{-(a+1)kt/a\}$ were much larger than 1, the following approximation is possible:

$$[\text{TM}]_{\text{solid}}/[\text{TM}]_{\text{solid}}^0 = 10^{-0.43kt} \quad (5)$$

The difference between the values from Eqs. (3) and (5) was less than 10% when the TM ratio in the muscle, $[\text{TM}]_{\text{solid}}/[\text{TM}]_{\text{solid}}^0$, was larger than 3×10^{-3} and a was equal to 2000. Under such conditions, for every $(0.43 k)^{-1}$ min of treatment, TM content would be reduced tenfold, and the concentration of TM in the water was low enough to ignore the reversion of TM to the muscle cube from water. Thus, for effective TM elimination, the treatment should be performed until Eq. (3) cannot be approximated to Eq. (5). By this criteria, only the second treatment in 2000 volumes of water per shrimp muscle cube was effective, where TM content was reduced to 2.1×10^{-3} fold, compared with the first treatment (Fig. 2). In other words, for effective hypoallergenic treatment in the present study, the first and third treatments should be performed for longer periods. To perform effective hypoallergenic treatment, the volume of water and treatment time should be optimized from the relationship of Eqs. (3) and (5).

In the treatment in 10 volumes of water per shrimp muscle cube, there was a time lag for the water and cube to reach 100 °C, and this was reflected in t_{lag} . In the treatment in 2000 volumes of water per shrimp muscle cube, around 5% of the boiling water was evaporated after 20 min of heating. The

decrement in water was ignored in the parameter determination because it did not affect the parameter ($<1\%$). For the treatment at 121 °C, it took 1.5 min to reach 121 °C from 100 °C and 0.5 min to cool to 100 °C from 121 °C. The temperature could not be maintained at 121 °C throughout the treatment. However, this did not seriously affect the experimental results, because the periods below 121 °C were around 10% of the total treatment time, and the treatment time was fixed to 20 min.

In Japan, when the contamination level of proteins from causal ingredients exceeds 10 µg/g, labeling is mandatory (Akiyama et al. 2011; Shoji et al. 2018). The remnant TM level after the third treatment in 2000 volumes of water per shrimp muscle cube at 121 °C was below the mandatory limit. In addition, it has been reported that shrimp allergy does not occur when only two shrimps are ingested per person (Daul et al. 1988; Lopata and Lehrer 2009), unlike the case of peanut allergy, where only 100 µg of peanut protein results in allergy symptoms (Hourihane et al. 1997). However, since allergen sensitivity depends on individual subjects, further investigations are needed to determine the precise TM limit to prevent any shrimp allergy symptoms.

In the present study, effective hypoallergenic conditions for shrimp based on TM elimination were developed. The texture of shrimp meat was retained, though the substances responsible for taste and flavor were lost by boiling treatment. The boiling treatment deteriorates the palatability of shrimp, but it can be recovered by seasoning. The boiling treatment could be the basis for optimal hypoallergenic treatment of other invertebrates such as squid and abalone, which are allergenic, and could further be applied to insect foods, which will be regarded as candidate protein sources in the future.

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