Allergic Diseases

Chapter 1

Recent Advances in the Structural Studies on Marine Invertebrate Muscle Tropomyosins

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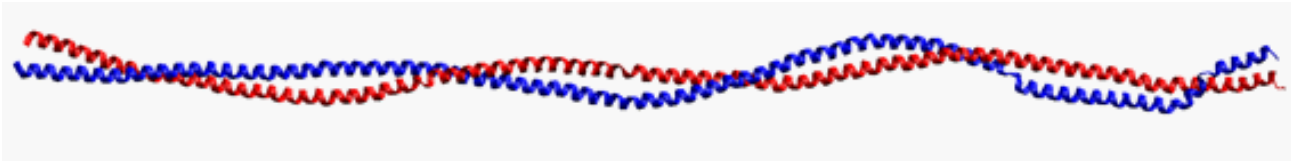
Abstract

The major allergen of marine invertebrate is tropomyosin (TM). Thermodynamic and molecular dynamics approaches have revealed that TMs from strongly allergenic organisms show higher thermal stability than those from less allergenic ones. Especially, the epitope regions show higher stability compared with the non-epitope ones. To reduce allergenicity of invertebrate muscles, partial elimination of TM from shrimp muscle by boiling was successful, resulting in ca. 10% of remnant TM at the maximum. The elimination ratio was high enough for shrimp TM not to induce objective reaction. This method thus could be applied to processing of shrimp for human consumption.

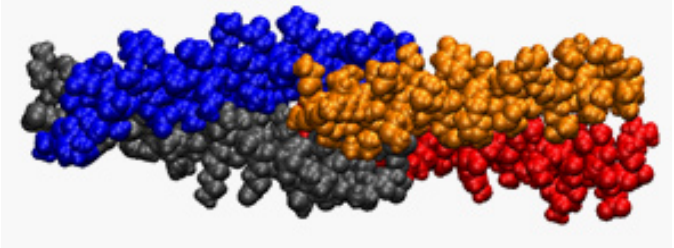
Keywords: Hypoallergen; Molecular dynamics simulation; Shrimp; Squid; Thermal stability; Tropomyosin

1. General Characteristics of Muscle TM

Tropomyosin (TM) is one of the actin binding proteins [1]. The muscle TMs from vertebrates and many marine invertebrates consist of 284 amino acids. TMs exist as a dimer (**Figure 1A**) which can polymerize through the interaction of the C-terminus with the N-terminus of adjacent molecule, in other words, by the head-to-tail interaction (**Figure 1B**) [2,3]. This polymerization is essential for binding to actin filament as a long cable. Dimeric TM forms a parallel coiled-coil structure and its sequence shows heptad repeat represented as *abcdefg* (**Figure 1C**), where *a* and *d* positons are generally occupied by hydrophobic amino acids and are designated 'core residues' [1]. The hydrophobic interaction between the core residues of TM stabilizes the coiled-coil structure. The acidic residues located at the core of the molecule could destabilize TM [4]. In addition, consecutive Ala residues or the other small residues like Ser at the core residue form alanine clusters, which endow TM with the binding ability to actin with flexural flexibility or bending [5,6].



(A)



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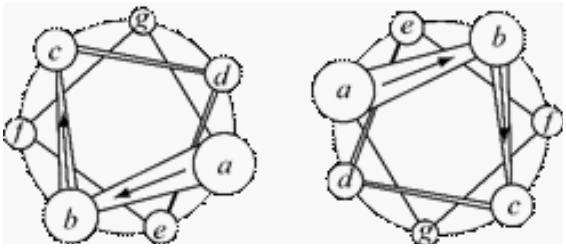

(C)

Figure 1: (A) The tertiary structure (ribbon model) of TM obtained by molecular dynamics simulation at pH 7 [25]. One monomer is indicated in blue and the other in red. (B) The tertiary structure of head-to-tail complex (PDB ID: 2G9J) [3] shown as van der Waals model. C-terminal fragments were represented in blue and black. N-terminal fragments were presented as orange and red. (C) The schematic diagram of heptad repeat.

Marine invertebrates, such as shrimp, squid and abalone, could cause food allergy [7]. The major allergen of marine invertebrates has been proved to be TM [8], while vertebrate TMs do not cause allergy with the rare exceptions [9]. Allergens are generally supposed to be heat stable or be refolded when being cooled down after heating, and are not aggregated by heating [10,11]. In addition, they are known to be resistant to enzymatic digestion in stomach and/or small intestine [12]. TM is unfolded at high temperatures, but fully refolded by cooling, and rarely aggregated [13-15]. TM shows stability against stomach digestion but not so much small intestine [16]. However, it should be pointed out that experiment cannot perfectly reproduce the digestive condition and would rather only mimic as simplified conditions [17,18]. The pH value of stomach may fluctuate due to some factors such as health condition and stomach medicine. Parvalbumin, the major allergen of fish muscle, cannot be digested at higher pH

in stomach [19]. In case of TM, little information is so far available regarding the changes in susceptibility to digestive enzymes by the changes in physiological environment of the digestive tract.

2. Thermal Stability of Invertebrate TMs

The proteins, which are resistant to proteinases, should have stable structures. Proteinases tend to attack the unfolded regions of proteins, which could enter the active center of proteinase [20]. In order to reveal the structural profiles of TMs from several invertebrate species, the thermal stability have been examined by thermodynamic approaches, namely, circular dichroism (CD) and differential scanning calorimetry (DSC) [14,15]. By CD measurement at 222 nm, α-helical content of TMs can be determined and the decrement in the content by heat treatment corresponds to the extent of decay in the higher structure of TMs. By DSC measurement, the enthalpy (ΔH) of unfolding can be directly measured, and, by deconvolution analysis, ΔH of the unfolded domain of TMs can be determined. The numbers of unfolded domains were found to be four to seven for vertebrate and invertebrate TMs [13-15], and these domains were assigned to the amino acid sequence of rabbit TM [21]. Based on the DSC measurement, it is possible to estimate how many domains are unfolded, folded or in the intermediate states. The data obtained at 37°C (mimicking the human body temperature) are summarized in **Table 1**. By the CD measurement, the numbers and thermodynamic parameters of unfolding domains were estimated from the decrement of α-helical content accompanied by increment of temperature. The numbers of the unfolded domains, however, were estimated to be two or three by CD. Thus, CD measurement cannot provide precise information about unfolding, and, thus, true standard Gibbs free energy for unfolding, ΔG^0, but the apparent one, ΔG^0_{app}, could be obtained for the criterion of thermal stability [14,15]. Therefore, DSC measurement is considered to be better for estimating the thermal stability of TMs. In the DSC, heat capacities [J/K] of sample and reference cells were measured and we subtract the latter from the former. The subtracted data contains heat capacities by unfolding, and of folded and unfolded proteins. Thus, the heat capacities of folded and unfolded proteins should be subtracted. In order to correctly subtract these values, unfolding is not supposed to occur both at the initial and last stages of thermal treatment.

The sum of ΔG^0 for each unfolding domain is defined as ΔG^0_{total}, the significance of which is discussed below.

$$\Delta G^0_{total} = \Delta G^0_1 + \Delta G^0_2 + \ldots + \Delta G^0_n = - RT\ln K_1 - RT\ln K_2 - \ldots - RT\ln K_n$$

$$= - RT\ln K_1 K_2 \ldots K_n = - RT\ln K_{total}$$

$$K_i = [D_i^{unfolded}]/[D_i^{folded}]$$

Where $D_i^{unfolded}$ and D_i^{folded} indicate the concentrations of unfolded and folded "i"th do-

main, respectively.

Thus,

$K_{total} = [TM^{fully\ unfolded}]/[TM^{fully\ folded}]$

Where $TM^{fully\ unfolded}$ and $TM^{fully\ folded}$ indicate completely unfolded and folded TMs, respectively.

Here, ΔG^0_{total} is an index for the ratio of fully unfolded TM against fully folded one. In the previous research [14,15], the value was calculated at 293 K (20°C), but in this chapter, at 310 K (37°C) mimicking the human body temperature.

Scallop striated muscle TM is less allergenicity, compared with the shrimp counterpart. Scallop TM consists of two unfolded and three intermediate state domains at 37°C (**Table 1**). On the other hand, in the case of shrimp muscle TM, one unfolded, two intermediate states and four folded domains have been observed. The unfolded and intermediate state domains would be easily digested by proteinases. Therefore, the information about the number of unfolding domains is important for understanding the allergenicity of TMs. The measurement was performed at neutral pH (mimicking the small intestine) and would partially reflect the behavior of TM at acidic pH (expected in the stomach). Thus, the ΔG^0_{total} at 37°C was calculated (**Table 1**). In the previous study, it has been concluded that shrimp TM was more stable than abalone TM [15], but the ΔG^0_{total} at 37°C did not support the tendency, suggesting the difference in the stability is not so much between the shrimp and abalone TMs. Accordingly, the resistance to proteinases should be compared between these TMs.

Table 1: Thermal stability of invertebrate muscle tropomyosin by differential scanning calorimetry

Species	Unfolded domain	Intermediate state domain	Folded domain	ΔG^0_{total} [kJ/mol]
Scallop	3*	2	0	-32
Squid	0	2	2	54
Shrimp	1	2	4	77
Abalone	0	4	3	86

*Measurements were performed at 37°C. The data from the references [14,15] have been edited.

3. Characterization of the Epitopes

Ayuso and coworkers [22,23] determined the epitopes of brown shrimp *Penaeus aztecus* TM by using 15mer peptides and found epitope regions were scattered all over the molecule, which have been confirmed by one-bead-one-compound peptide libraries [24]. The epitope regions are expected to have stable structures against digestion in the stomach and small intestine. The substrate specificity of pepsin, trypsin and α-chymotrypsin, however, did not explain

the structural differences between the epitopes and other regions [25]. Thus, the epitope regions of TM are expected to take stable coiled-coil structures, because the folded region would be resistant to digestion [20].

To understand the behaviors of the epitopes, molecular dynamics (MD) simulation of TM was performed at constant pH [25]. The results showed that the epitope regions had higher α-helical contents and smaller dihedral angle fluctuations, indicating that the structures of the epitope regions are more stable than the non-epitope regions. In addition, the epitope regions were rarely located at the alanine cluster, and showed larger coiled-coil radii. At the alanine cluster, the core packing was not so tight and each α-helix was axially fluctuated. Such flexibility could result in susceptibility to digestion. pK_a values of Glu and Asp are originally 4.4 and 4, respectively, but might be shifted by the environmental effects. The increment of pK_a reflects the stabilizing protonated state or the destabilizing deprotonated state. The attractive ionic interaction (i.e., Glu-Lys, etc.) results in decrease of pK_a, whereas the repulsive ionic interaction (i.e., Glu-Glu, etc.) results in increase of pK_a. When acidic residues are protonated, they are to be neutralized and would form hydrogen bonds. The acidic residues in the epitope regions showed higher ΔpK_a compared with the non-epitope regions. Therefore, the acidic residues in the epitope regions might be much less stabilized by ionic interaction with basic residues, and/or be more destabilized by ionic interaction with acidic residues. Therefore, it is possible that the epitope regions could be stabilized at the stomach by the loss of ionic repulsion between acidic residues and show resistance to pepsin, but could be destabilized at the small intestine and easily digested.

4. Partial Elimination of TM from Allergenic Invertebrate Muscles

Even though the allergens are to be identified, it is difficult to remove them from the allergenic food. In order to reduce the risk of seafood allergy, it is necessary to remove the causative protein(s). TM is known to be water soluble at high temperature unlike the other myofibrillar proteins [13-15]. Based on this unique behavior of TM, attempts were made to remove TMs from shrimp tail and squid mantle muscles by boiling in 10 volumes of water for 10 min (**Figure 2**) [26]. After boiling, TM contents were decreased to 11.2 ± 2.0% and 35.0 ± 1.0% for the shrimp and squid muscles, respectively. It was also demonstrated that seafood broths are as allergenic as the residue (heated muscle). Although the boiling treatment of oyster TM increased IgE reactivity or the allergenicity, the increment was up to tens of percent [27]. Although the 100 μg of peanut proteins can cause allergic symptoms [28], patients of shrimp allergy should ingest more than a few shrimp individuals to elicit objective symptoms of allergy [29]. It follows that boiling treatment could be an effective method for the mild allergy patients. In addition, the boiling pretreatment in food service sites (schools, hospitals, etc.) could prevent patients from accidental ingestion of allergenic food and be economically preferable to preparing elimination diet. For the detection of remaining TM, SDS-PAGE is not

always efficient because the changes in allergenicity of TM based on the structural changes cannot be evaluated. In addition, the detection limit is not high enough (around 0.02 μg by our lab protocols) [30], compared with the commercially available ELISA kit (the detection limit being around 0.29 μg/g total shrimp protein against food sample weight) [31].

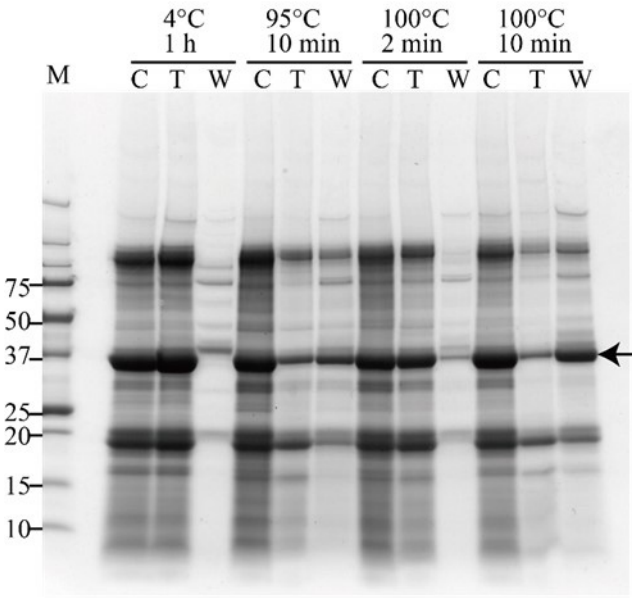

Figure 2: SDS-PAGE patterns for the effect of boiling on the elimination of tropomyosin (TM) from muscle. Shrimp muscle was treated in 10 volumes of distilled water under various conditions as indicated on the top. C, T and W are the control muscle (before treatment), treated muscle (after treatment), and water after treatment, respectively. M, molecular weight markers. The arrow indicates the band of TM. 15% gel.

5. References

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