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Structural and dynamical characteristics of tropomyosin epitopes as the major allergens in shrimp

Authors: Hideo Ozawa¹, Koji Umezawa², Mitsunori Takano³, Shoichiro Ishizaki⁴, Shugo Watabe⁵, Yoshihiro Ochiai⁶

The affiliations and addresses of authors: ¹Faculty of Applied Bioscience, Kanagawa Institute of Technology, 1030, Shimo-Ogino, Atsugi, Kanagawa 243-0292, Japan. ² Biotechnology Division, Department of Biomedical Engineering, Graduate School of Science and Technology, Shinshu University, 8304 Minami-minowa, Kami-ina, Nagano, 399-4598, Japan. ³Department of Pure and Applied Physics, Waseda University, Okubo 3-4-1, Shinjuku-ku, Tokyo, 169-8555, Japan. ⁴Department of Food Science and Technology, Tokyo University of Marine Sciences and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan. ⁵School of Marine Bioscience, Kitasato University, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan. ⁶ Graduate School of Agricultural Science, Tohoku University, Aramaki, Aoba-ku, Sendai, Miyagi 980-0845, Japan

Corresponding author: Hideo Ozawa and Mitsunori Takano

E-mail address: ¹ozawa@bio.kanagawa-it.ac.jp; ²koume@shinshu-u.ac.jp; ³mtkn@waseda.jp; ⁴ishizak@kaiyodai.ac.jp; ⁵swatabe@kitasato-u.ac.jp; ⁶yochiai@tohoku.ac.jp.

Abstract

Ingestion of marine invertebrates often causes food allergy, where the major allergens have been reported to be derived from tropomyosin (TM). Intact or the digestive fragments of food allergens generally show resistance to digestion, which is usually attributable to the structural stability (or rigidity). The difference in the structural and dynamical characteristics between the epitope and the non-epitope regions in TM has not yet been well understood. In the present study, molecular dynamics simulation was performed at constant pHs for shrimp TM. By analyzing the main-chain dihedral angle fluctuations and local α -helix contents, we found that the epitope regions are more stable than the non-epitope counterparts, providing a possible physical reason for the resistance to digestion in the epitopes regions. The difference of the structural stability between the epitope and the non-epitope regions was largest at low pHs, even though pH dependence of the structural stability in itself was not significant in both regions. The lower content of the Ala cluster in the epitope region is considered to cause the higher stability of the epitope region.

Keyword: Allergen; coiled-coil; epitope; molecular dynamics simulation; shrimp; tropomyosin.

Introduction

Tropomyosin (TM) is an actin-binding protein and regulates muscle contraction [1-8]. The monomer of muscle TM contains 284 amino acids in various vertebrates and invertebrates, forming dimeric coiled-coils along the entire molecule [1,9]. TM dimer binds with another dimer in a head-to-tail manner, with the N-terminus of one dimer interacting with the C-terminus of the other, resulting in a four-helix bundle at the junction [2,10]. The structure of the full-length TM was first determined by Phillips et al.[1,11], and later the structures of parts of TM were determined at high resolutions [2,10,12-14]. Recently, the structures of the TM-actin complex have also been elucidated [3,4,7]. However, the TM structures determined so far are only those for the vertebrate muscle TMs.

Coiled-coil proteins show a typical heptad repeat pattern (conventionally seven alphabets, *a* to *g*, are assigned to the seven positions in the heptad repeat) [15]. The *a* and *d* positions, which are located at the interface of the two α -helices, are usually occupied by hydrophobic amino acids. In addition, consecutive Ala (alanine) residues (or other small amino acids such as serine) are often observed at the *a* and *d* positions, which is referred to as “Ala cluster” [12]. Vertebrate TMs contain six Ala clusters [14] (Fig. S1). At the Ala cluster, the two α -helices can be staggered along the coiled-coil axis and the junction between the Ala cluster and the flanking regions are possibly bent [12,13]. The bending [16] and/or flexibility of the coiled-coil [12] are considered to be essential for the binding of TM to actin.

Fig. S1

To investigate the structural and dynamical characteristics of TMs, molecular dynamics (MD) simulations have been performed for the vertebrate TM [9,16,18-24] and TM-actin complex [5,6,8,22]. However, MD study has not yet been carried out for invertebrate TMs. Because the amino acid sequence identity of TM between vertebrate and invertebrate is not so high (e.g. the identity is 56% between shrimp (GenBank ID: BAF47263.1) and rabbit (NP_001099158.1)) and there are many amino acid substitutions even at Ala clusters, the structural and dynamical characteristics of the invertebrate TM may be different from that of the vertebrate TM.

Study of the invertebrate TM has an important role in terms of the food allergy. TMs, as well as casein and parvalbumin, are the major food allergens from animals [25]. Shrimp and other invertebrates TMs cause food allergy, though the vertebrate TMs [26] rarely do. The epitope regions of brown shrimp (*Farfantepenaeus aztecus*) TMs were identified as the major allergens [27,28] (see Fig. S2). Food allergens generally tend to show high stability against heat and high resistance to proteolytic digestion

Fig. S2

[29,30]. Furthermore TMs have high refolding ability [31] and also high resistance against simulated gastric fluid, although they are easily digested by simulated intestine fluid [32]. Larger digested fragments tend to lead to allergy reaction [33]. However, there is little information about the difference between the known epitope regions and the non-epitope regions. In the present study, MD simulation was employed at different pH conditions to characterize the structural difference between the epitope and the non-epitope regions in the shrimp TM and its pH dependence.

Materials and methods

Brown shrimp *F. aztecus* TM (AAZ76743.1) was studied by MD simulation using Amber 14 [17]. Shrimp TM structure was obtained based on porcine TM (PDB ID: 1C1G) [11] by homology modeling using SWISS-MODEL [34]. Then, TM was solvated by 231078 TIP3P water molecules in a rectangular unit cell, in which 464 Na⁺ and 418 Cl⁻ were added to neutralize the system and to adjust the ionic concentration to 0.1 M. The periodic boundary condition was applied to the system and the particle mesh Ewald method [35] was employed for electrostatic energy calculation with a real space cutoff distance of 8 Å. Energy minimization (500 cycles of steepest descent and 500 cycles of conjugated gradient) was performed for water molecules and ions, with heavy atoms in TM being restrained to their initial coordinates with a restoring force constant of 500 kcal/(mol·Å²). Then, further energy minimization was performed for the whole system without restraint. For the following MD calculation, bond lengths involving hydrogen atoms were fixed by SHAKE [36] to enable the time step of 2 fs. Temperature was set to 300 K and Langevin dynamics was adopted with a collision frequency of 2 ps⁻¹. The equilibration of 20 ps under the constant volume condition was performed with heavy atoms in TM restrained to those in the energy-minimized structure with a restoring force constant of 10 kcal/(mol·Å²). Then, to equilibrate the volume of the system, MD simulations were performed under the constant pressure at 1 bar for 10 ns by using the Berendsen barostat [37] with the relaxation time of 2 ps.

Starting from the equilibrated system, constant pH MD simulations [38] at 1 bar were conducted for 10 ns at seven pH conditions ranging from pH 1 to 7. Protonation states were evaluated for 54 Glu, 18 Asp and 1 His, and changed at 1 ps intervals. For the evaluation of the energy change upon the attempt for the protonation state change, the generalized Born implicit solvent model was used at 0.1 M ionic strength. If the attempt for the protonation state change was accepted, MD was performed only for

water molecules and ions [38], with TM atoms fixed for 0.2 ps. For each pH condition, we obtained three independent MD runs. The 2500 snapshots from the last 5 ns were used for the following analyses.

From the ratio of the protonated and the deprotonated states for each residue as a function of pH, denoted by $P_p^i(\text{pH})$ and $P_d^i(\text{pH})$ (here i represents the residue number for Glu, Asp, and His), $\text{p}K_a^i$ and the Hill coefficient n_H^i were calculated with the Hill equation [38],

$$\log(P_p^i(\text{pH})/P_d^i(\text{pH})) = n_H^i (\text{pH} - \text{p}K_a^i) \quad (1).$$

The coiled-coiled diameter for each residue was calculated by TWISTER [39], and the formation of the α -helix for each residue was judged by DSSP [40]. Note that these quantities as a function of the residue number i ($i = 1$ to 284) were calculated for each monomer in the coiled-coil dimer and then the values for the same residue were averaged. The main chain dihedral angles fluctuation was calculated by $\{[(\delta\phi_A^i)^2 + (\delta\psi_A^i)^2 + (\delta\phi_B^i)^2 + (\delta\psi_B^i)^2]/4\}^{1/2}$, where $\delta\phi$ and $\delta\psi$ represent the standard deviations of the distribution of the main chain dihedral angles, ϕ and ψ , respectively, and the indexes A and B distinguish the two monomers in the coiled-coil dimer.

Results

Characteristics of amino acid sequence

The amino acid sequences of TM are shown for shrimp and rabbit (Fig. S1). The amino acid sequence identity between them in the epitope region (59.4%) is not so different from that in the non-epitope region (54.6%). Also, there is no significant difference in the number of the potential cleavage sites for proteases between the epitope and the non-epitope regions (for pepsin, more potential cleavage sites were predicted in the epitope region than in the non-epitope region). Interestingly, we found that the occurrence frequency of the Ala clusters is significantly lower in the epitope region than in the non-epitope region (no significant difference was found for the amino acid contents other than Ala).

$\text{p}K_a$ values

The $\text{p}K_a$ values calculated from constant pH MD simulations were in the range of 3.1 to 6.2 (4.8 ± 0.6 ; the average of 4.8 and the standard deviation of 0.6) for Glu and 1.5 to 5.0 (3.4 ± 0.9) for Asp (Fig. S3). The $\text{p}K_a$ value of His44 was 6.3 ± 0.1 . Thus, in the stomach where pH is about 1, almost all of

Glu, Asp and His are considered to be protonated, while in the small intestine where pH is about 7, all of them are considered to be deprotonated. In addition, the pH dependence of the net charge of TM was examined (Fig. S4). The isoelectric point (pI value) was 5.16 ± 0.02 , which was larger than the theoretical value of 4.72 calculated from the amino acid composition [41].

Fig. S3

Fig. S4

In the present constant pH MD simulations, the intrinsic pK_a values of Glu and Asp were considered to be 4.4 and 4.0, respectively, while the actual pK_a of each amino acid should depend on surrounding environments [38]. The shift of the actual pK_a from the intrinsic value, on average, was $+0.4$ (± 0.8) for the epitope regions and -0.1 (± 0.8) for the non-epitope regions (Fig 1 and Fig. S3). This difference in the average pK_a shift between the epitope and the non-epitope regions was statistically significant ($p < 0.05$ by Student t -test), while that in the Hill coefficient (n_H^i) was not (0.68 ± 0.14 for the epitope region and 0.67 ± 0.15 for the non-epitope region).

Fig. 1

Structural and dynamical characteristics

TM is an α -helical coiled-coil protein, where α -helix may be partially unfolded by thermal fluctuation. As seen in Fig. 2A, partial unfolding of α -helix was actually observed in some regions, including the regions near the acidic residues situated at the coiled-coil core (Asp137, Glu211, Glu218, and Glu263). However, overall α -helical content remained quite high (above 95%) with little pH dependence even though the average α -helical content was slightly lowered at around the isoelectric point as seen in Table 1. Notably, we found that amino acids in the epitope regions showed higher α -helical contents than those in the non-epitope regions at pH 1, 2, 5, 6 and 7 (see Fig. 2B for the data at pH 2 and Table 1): the difference between the epitope and the non-epitope regions was most significant at pH 2 (p value was 0.00024).

Fig. 2

Table 1

The fluctuation of dihedral angles for the main chain should detect the structural stability as well as the α -helical content. The dihedral angle fluctuations were around 10° (Fig. 3A), showing little pH dependence even though the average fluctuation was slightly increased at around the isoelectric point (Table 1). We detected the differences in the dihedral angle fluctuation between the epitope and the non-epitope regions (see Fig. 3B and Table 1): the difference was most clearly seen at pH 2 (p value was 0.0003).

Fig. 3

Coiled-coil radii were around 4.8 Å with almost no significant pH dependence (Fig. 4A). Here

Fig. 4

we can recognize again the difference between the epitope and the non-epitope regions; it was larger in the epitope region than in non-epitope region (see Fig. 4B and Table 1). This result is consistent with the fact that Ala clusters, which show smaller coiled-coil radii due to the small size of the Ala residue, are less in the epitope region than in the non-epitope region. Note, however, that largest coiled-coil radii were observed in the non-epitope region and therefore the non-epitope region exhibited larger fluctuation of the coiled-coil radius, which indicates that the non-epitope region is less stable than the epitope region.

Correlation of parameters

As expected, helical contents and dihedral angle fluctuations were closely and negatively correlated (at pH 2, $n = 281 \times 3$, $r = -0.83$, $p < 0.01$). Coiled-coil radii were positively correlated with dihedral angle fluctuations (at pH 2, $r = 0.46$, $p < 0.01$) and negatively correlated with helical contents (at pH 2, $r = -0.36$, $p < 0.01$). This is because the coiled-coil radius, in general, has a tendency to increase as the structural stability is lowered, as mentioned just above. The pK_a shift showed no correlation with dihedral angle fluctuations (at pH 2, $n = 72 \times 3$, $r = -0.02$, $p = 0.82$), α -helix contents (at pH 2, $r = 0.00$, $p = 0.95$) and coiled-coil radii (at pH 2, $r = 0.09$, $p = 0.18$).

Discussion

Behaviors of peanut allergens [42], birch pollens [43] and other allergens have been analyzed by MD simulations previously. In the present study, by conducting MD simulation of shrimp TM, we found that the epitope region is more stable than the non-epitope region. The higher stability of the epitope region is likely to come from the lower content of the Ala clusters, considering that staggering motion between the two coiled-coil helices along the coiled-coil axis could be enhanced at the Ala cluster regions [12,13]. In addition, pK_a of Glu and Asp in the epitope regions tended to show positive shifts from their intrinsic values. The relationship of these positive pK_a shift with the structural stability remains elusive; at least, attractive electrostatic interactions (i.e. ionic bonds) between those acidic residues and the adjacent basic residues are considered to have no role in the structural stability of the epitope region, because the pK_a shift should be negative if such ionic bonds were to be involved.

Vertebrate TMs contain two acidic cores at Asp137 and Glu218, where the TM structure was

largely fluctuated. The shrimp TM contains two more acidic cores at Glu211 and Glu263. The pK_a values were 3.5 ± 0.7 , 5.1 ± 0.5 , 2.6 ± 0.6 and 3.7 ± 0.3 for Asp137, Glu211, Glu218 and Glu263, respectively. Although all of these are supposed to be protonated and electrically neutralized at pH 2, they were still fluctuated even at this pH (Figs. 2A and 3A). Thus, at pH 2, the fluctuation of these residues would be irrelevant to the instability due to the electrostatic repulsion; instead, it could result from holes (i.e., defects) in the hydrophobic core [14]. The holes have been reported to locate at around Glu218 and Glu263 in the crystal structure of vertebrate TMs [14], which coincide with the acidic core regions of shrimp TM (Fig. S1). The stabilizing mutations in the acidic core of Asp137 in TM (e.g. substitution of Asp to Leu) has been shown to impair the TM-mediated regulation of actomyosin ATPase activity [44], suggesting the role of the holes in the flexibility. The fact that the core residues are substituted to acidic residues (Glu211 and Glu263) in invertebrates such as shrimp, squid and abalone [31] may reflect the importance of maintaining the flexibility in a cold environment.

At the pH conditions apart from the isoelectric point (pH $\sim$ 5), TM possesses large net charge (Fig. S4), which would destabilize the coiled-coil dimer by electrostatic repulsion. In the present simulations, however, the stability judging from dihedral angle fluctuations and α -helical contents was not significantly affected by pH (furthermore, contrary to our expectation, the stability was slightly lowered at around the isoelectric point). The little effect of pH on the TM stability may be due to the electrostatic screening by water and counter ions or due to insufficient sampling of equilibrium fluctuation [45].

We here comment on the computational method to calculate protein fluctuations. To analyze thermal fluctuations of the molecule, the root mean square fluctuation (RMSF) was usually calculated based on the Cartesian coordinates of atoms in the molecule. The Cartesian-coordinate-based RMSF includes the whole spectrum of fluctuations, ranging from global to local ones. Therefore, Cartesian-coordinate-based RMSF is not necessarily suited for the evaluation of local flexibility. In the preceding studies, the flexibility of TM was evaluated by bending fluctuations [16,18], inter-residue distance fluctuations [22] and α -helical contents [18]. The α -helical content and the fluctuation of the main chain dihedral angle that were employed in the present study can detect the local fluctuations better than the bending and inter-residue distance fluctuations.

It is conceivable that the observed structural stability of the epitope regions, especially at lower pHs, might be related to the resistance to digestion and accordingly allergens. One possible scenario

would be that the epitope regions are less digestible in stomach where pH is low ($\text{pH} \approx 1$), which causes a difference in the later digestion in small intestine, leaving larger digested fragments containing the epitope regions. To examine this scenario, it is necessary to conduct more extensive MD simulations to improve the structural sampling. It is also worthwhile to conduct MD simulations of other allergenic and non-allergenic TMs to confirm the difference in the structural and dynamical characteristics between the epitopes and the non-epitopes.

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Table 1 Structural properties of shrimp tropomyosin in epitope and non-epitope regions at different pH.

pH	Type	α -Helical content (%)			Dihedral angle fluctuation (degree)				Coiled-coil radius (Å)				
1	Epitope	97.7	±	7.9	**	9.77	±	2.07	*	5.04	±	0.25	**
	Non-epitope	95.5	±	14.8		10.22	±	3.63		4.75	±	0.47	
2	Epitope	97.7	±	8.3	**	9.65	±	1.96	**	5.03	±	0.21	**
	Non-epitope	94.6	±	15.7		10.57	±	5.58		4.77	±	0.48	
3	Epitope	96.7	±	13.1	N.S.	9.85	±	2.32	**	5.05	±	0.25	**
	Non-epitope	94.7	±	16		10.59	±	5.78		4.71	±	0.42	
4	Epitope	96.8	±	9.5	N.S.	10.36	±	3.27	N.S.	5.04	±	0.27	**
	Non-epitope	95.4	±	14.6		10.32	±	2.96		4.76	±	0.48	
5	Epitope	96.7	±	9.1	*	10.18	±	2.39	N.S.	5.07	±	0.31	**
	Non-epitope	95	±	15.6		10.41	±	4.23		4.75	±	0.45	
6	Epitope	97.3	±	9.1	*	10.11	±	1.86	N.S.	5.06	±	0.29	**
	Non-epitope	95.5	±	15.1		9.99	±	2.83		4.71	±	0.43	
7	Epitope	97.1	±	8.4	*	9.94	±	1.6	N.S.	5.04	±	0.3	**
	Non-epitope	95.4	±	15		10.09	±	3.22		4.71	±	0.43	

N.S., not significant; * $p < 0.05$; ** $p < 0.01$

Fig. 1 The cumulative frequency function of pK_a shift for epitope (solid line) and non-epitope (broken line) regions in shrimp tropomyosin ($n = 3$). Standard deviations are shown as vertical lines.

Fig. 2 α -Helical contents of shrimp tropomyosin at pH 2 ($n = 3$). (A) α -Helical contents calculated for each residue. Epitope regions are shown as horizontal bars. (B) The cumulative frequency function of α -helical contents for epitope (solid line) and non-epitope (broken line) regions. Standard deviations are shown as vertical lines.

Fig. 3 (A) Local flexibility calculated from thermal fluctuations of dihedral angles for the main chain, ϕ (C-N-C $_{\alpha}$ -C) and ψ (N-C $_{\alpha}$ -C-N) at pH 2 ($n = 3$) for shrimp tropomyosin. Epitope regions are shown as horizontal lines. (B) The cumulative frequency function of thermal fluctuations of dihedral angles for the main chain in epitope (solid line) and non-epitope (broken line) regions. Standard deviations are shown as vertical lines.

Fig. 4 (A) The coiled-coil radius at pH 2 ($n = 3$) for each residue in shrimp tropomyosin. Epitope regions are shown as horizontal bars. (B) The cumulative frequency function of the coiled-coil radius for epitope (solid line) and the non-epitope (broken line) regions. Standard deviations are shown as vertical lines.

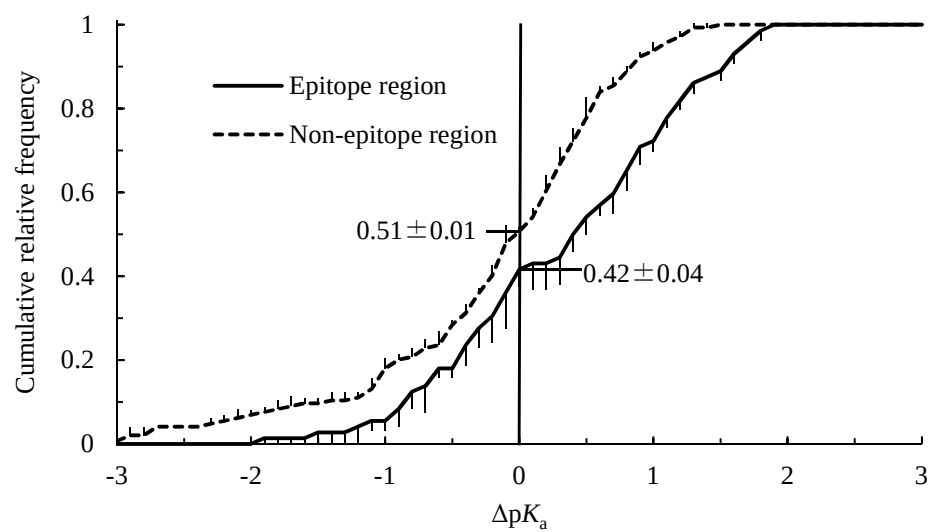
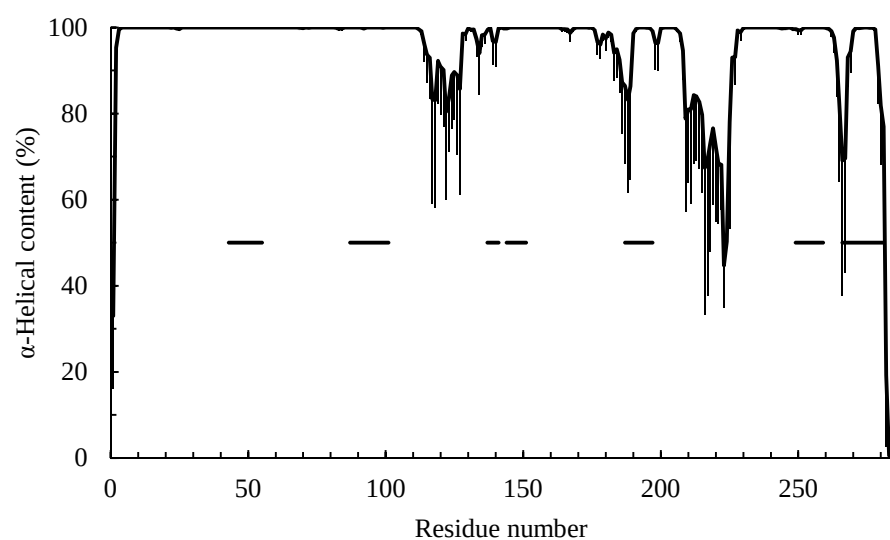


Fig. 1

(A)



(B)

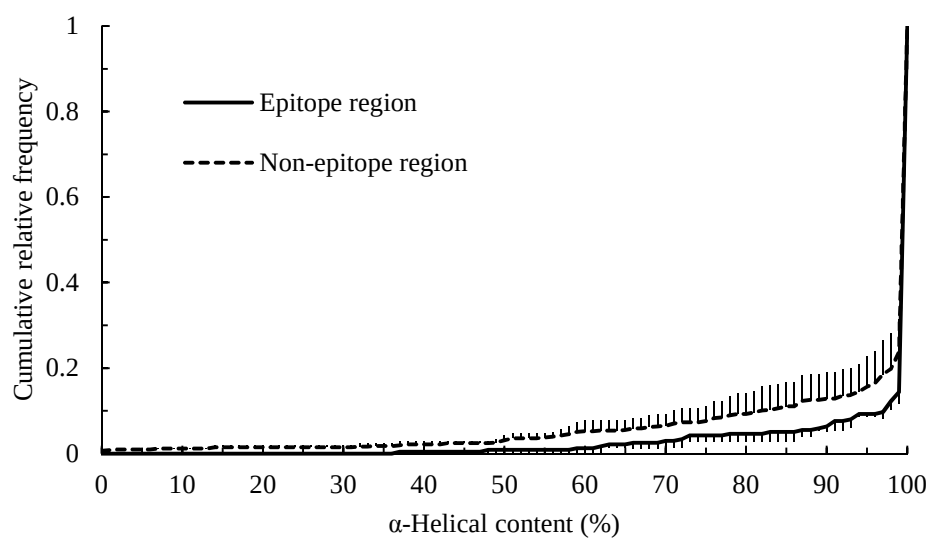


Fig. 2

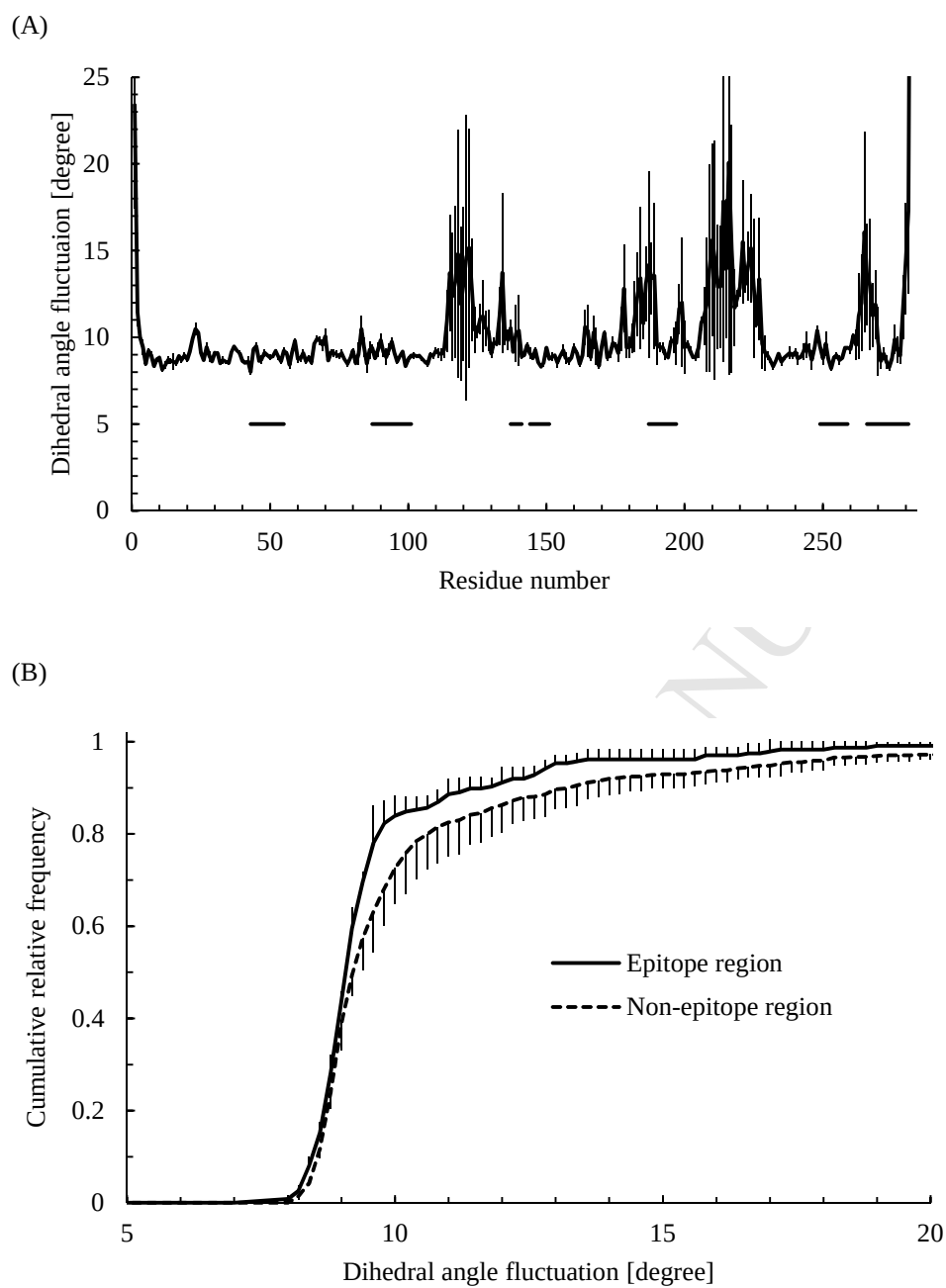


Fig. 3

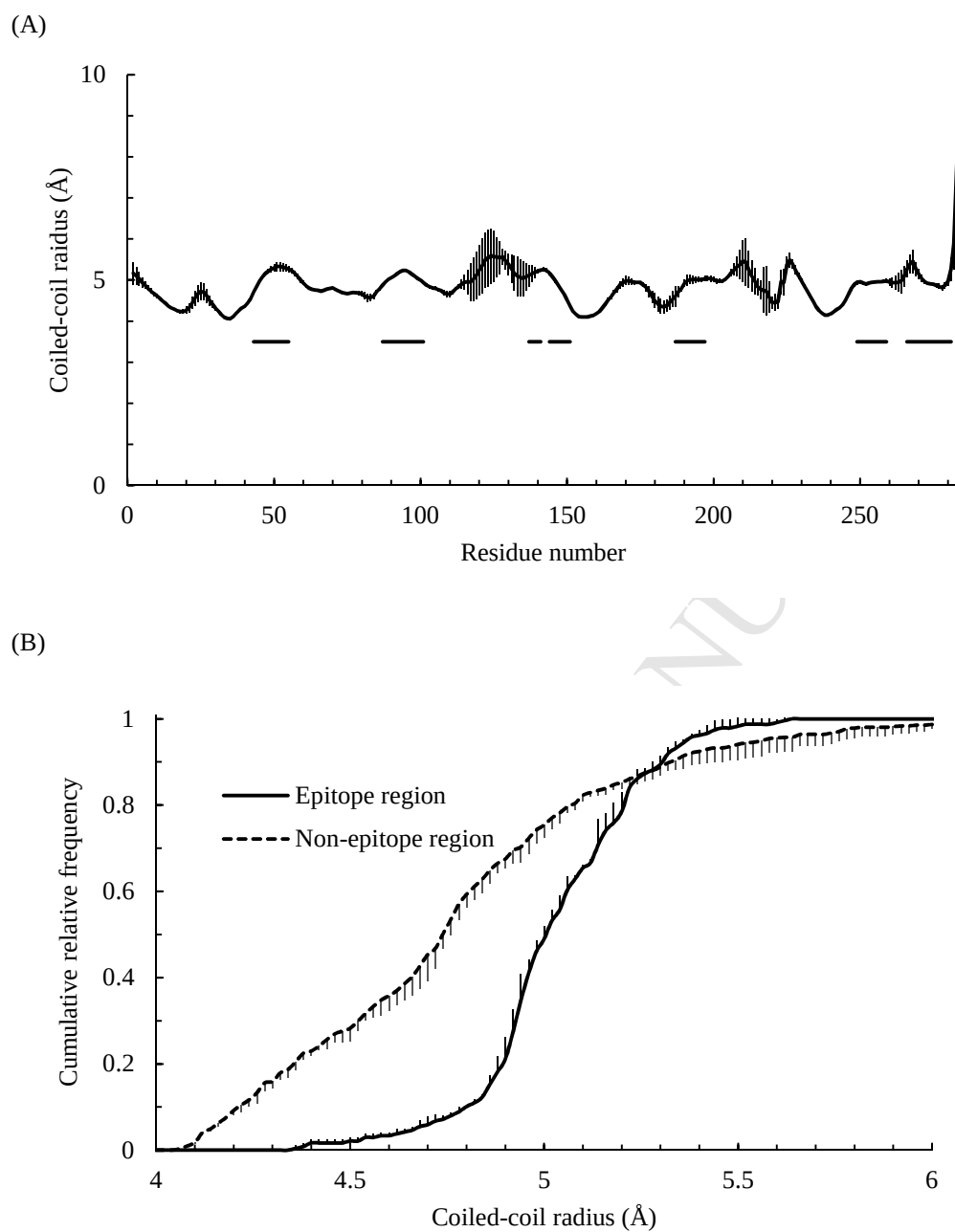


Fig. 4

Epitope region in tropomyosin was more stable than non-epitope region.

The difference between epitope and non-epitope regions was largest at low pHs.

Coiled-coil radii in epitope region of tropomyosin was larger.

The pK_a s of Glu and Asp in epitope region of tropomyosin were positively shifted.

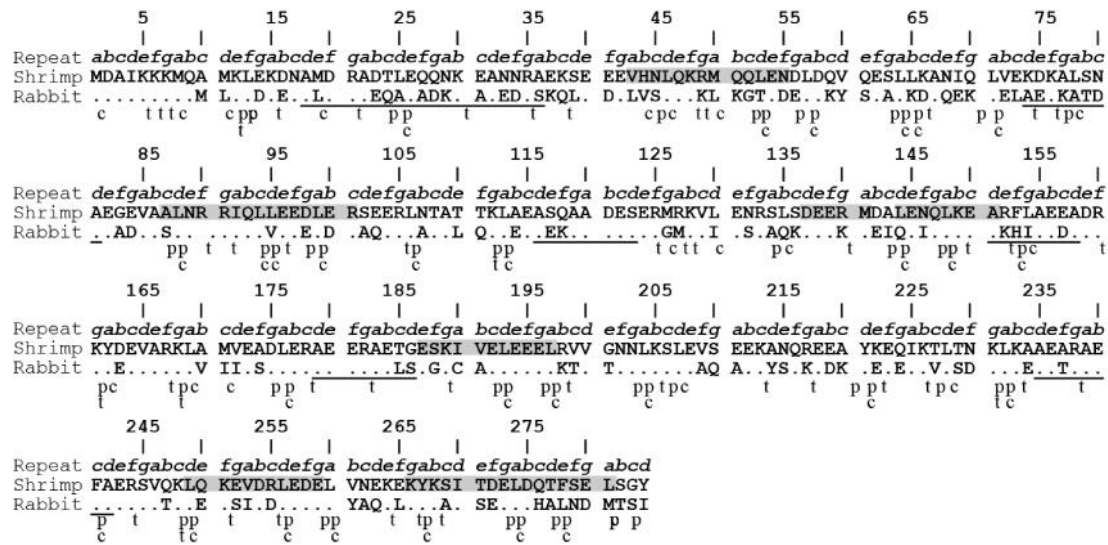


Fig. S1. Alignment of amino acid sequences of shrimp and rabbit TMs. Dots indicate the same amino acid residues as those of shrimp TM. Ala clusters are underlined [14]. Epitope regions are shaded [36]. The heptad repeat is shown as *abcdefg*. Putative cleavage sites of shrimp TM are also shown for pepsin (p), trypsin (t) and chymotrypsin (c) predicted by Peptide Cutter [44]. For the amino acid composition, a significant difference was observed only for Ala; the Ala content was 2.5% (2/79) for epitope regions and 14.6% (30/205) for non-epitope regions ($p = 0.0058$ by χ^2 test). The epitope regions rarely contain the Ala cluster, except for Ala151. Amino acid identities between shrimp and human (NP_001018005.1) TMs were 59.4% (47/79) and 54.6% (112/205) for epitope and non-epitope regions, respectively, and there were no significant differences in the frequency of amino acid substitutions between these regions ($p = 0.46$ by χ^2 test). The fractions of potential cleavage sites by pepsin were 33.3% (23/72), 16.2% (28/197) and 28.6% (4/14) for epitope, non-epitope and linkage regions, respectively, and there were significant sequence differences between these regions ($p < 0.01$). The fractions of potential cleavage sites by trypsin were 15.3% (11/72), 18.8% (37/197) and 7.1% (1/14) for epitope, non-epitope and linkage regions, respectively with no significant difference among the three regions ($p = 0.47$). The fractions of potential cleavage sites by chymotrypsin were 20.8% (15/72), 15.2% (30/197) and 21.4% (3/14) for epitope, non-epitope and the linkage regions, respectively with no significant difference again ($p = 0.50$).



Fig. S2 The positions of epitopes in TM [35,36] are shown in dark gray. The structure of shrimp TM after constant pH simulations for 10 ns was used.

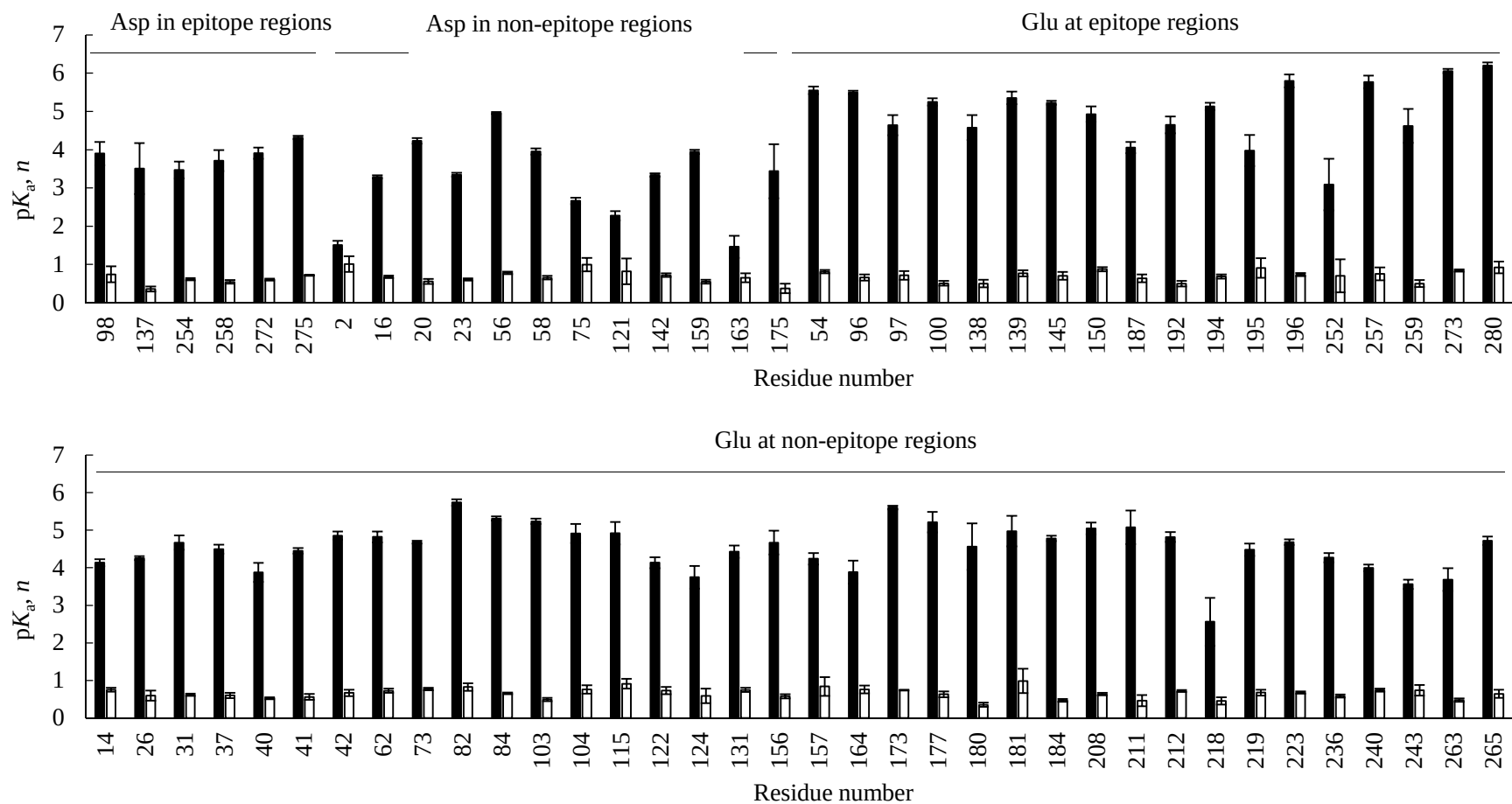


Fig. S3. The values of pK_a (filled bars) and Hill coefficient n_H (open bars) for Asp and Glu in epitope and non-epitope regions of shrimp TM ($n = 3$). Standard deviations are shown as the vertical lines.

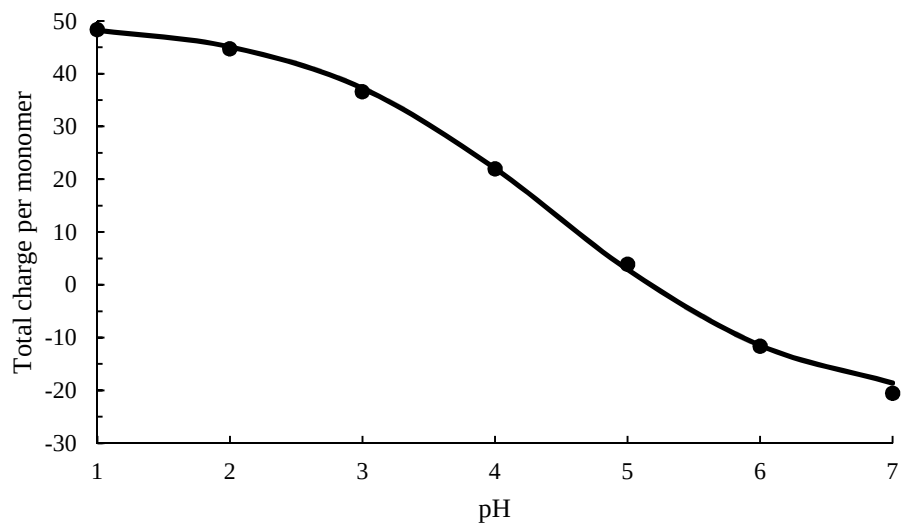


Fig. S4. The pH dependence of net charges in TM. Simulations were performed three times for each pH. The difference of net charges at each pH was small and standard deviations were lower than 0.76. Therefore, error bars are not shown. The line represents $73/(1+10^{0.47 \times (\text{pH}-4.44)}) - 23$, which becomes zero at $\text{pH} = 5.16 \pm 0.02$.