

Analysis of Immunoreactivity of α/α_2 -Tropomyosin from *Haliotis discus hannai*, Based on IgE Epitopes and Structural Characteristics

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ABSTRACT: Tropomyosin (TM) was reported to be a supercoil allergen of shellfish. However, little information is available about its link between structure and allergenicity. In this study, the subunit of TM (α -TM) and supercoil of TM (α_2 -TM) were identified from *Haliotis discus hannai*. α_2 -TM showed higher immunoreactivity than α -TM. Meanwhile, seven linear epitopes in α -TM and α_2 -TM were verified, and two conformational epitopes in α_2 -TM were predicted. The physicochemical properties and chemical bond assays confirmed the existence of the disulfide bond in α_2 -TM. According to spectroscopy and hydrophobicity analysis, α -TM showed higher α -helix features and blueshift of the fluorescence intensity peak compared with those of α_2 -TM. The structure analysis revealed the possibility of conformational epitopes in α_2 -TM, which could explain the immunoreactivity differences between α -TM and α_2 -TM further. These results improved the understanding of *Haliotis discus hannai* TM, which lay the foundation for the food processing of abalone.

KEYWORDS: *Haliotis discus hannai*, IgE epitope, immunoreactivity, structure characteristics, tropomyosin

INTRODUCTION

Shellfish allergies are one of the most commonly encountered food allergies in the Asia-Pacific regions, which are also common causes of immunoglobulin (Ig) E-mediated type I hypersensitivity reactions that continue throughout life.^{1,2} Recently, about 2.5% of the world's population was reported to be affected by shellfish allergy, especially children and adults.³ *Haliotis discus hannai* (*H. discus hannai*) is an economically important shellfish in the coastal area of China.⁴ However, there has been little research on abalone allergies.

At present, shellfish allergens have been widely identified, including tropomyosin (TM),⁵ arginine kinase,⁶ myosin light chain,⁷ sarcoplasmic calcium-binding protein,⁸ paramyosin,⁹ and filamin C,¹⁰ which could cause different degrees of sensitization. TM is a pan-allergen with high allergenicity in shellfish, which consists of two α -helical polypeptide subunits forming a stable coiled coil, whereas it typically exists as one subunit with a molecular weight of 32~40 kDa in invertebrates.^{11,12} However, TM with different subunits was gradually identified in shellfish, but the immunoreactivity differences of these subunits have not been fully elucidated.^{13,14} Thus, a more extensive study of TM in *H. discus hannai* should be explored, especially regarding immunoreactivity.

The allergenicity of an allergen is mainly related to epitopes.¹⁵ As the pan-allergen in shellfish, the analysis of the epitopes of TM has been widely concerned. At present, the study of antigenic epitopes of TM focuses on linear epitopes. One-bead-one-compound peptide libraries and phage peptide display technology were the most common methods of biopanning.^{16,17} Moreover, the method of combining bioinformatics analysis with serological tests was relatively efficient and cost-effective, and its accuracy was confirmed by epitope analysis of oyster and

shrimp.^{18,19} Five main linear epitopes were identified from *Crassostrea gigas* TM-combined bioinformatics analysis and serological tests.¹⁸ Fu et al.¹⁹ localized the TM linear epitopes in *Penaeus chinensis* through bioinformatics analysis, serological experiments, and cell models. The determination of epitopes is an indispensable step in the deep analysis of abalone allergy for the lack of information on IgE epitopes in abalone TM.

Thermal stability, pH stability, and digestibility are important factors to display the allergenicity of allergens.²⁰ Meanwhile, the stability of allergens was influenced by the protein structure.²¹ Preliminary studies have found that β -lactoglobulin aggregated during heat or low pH treatment via redistribution of disulfide bonds.^{22,23} In addition, IgE-binding abilities of two kiwifruit allergens, actinidin and thaumatin-like protein, displayed nearly unchanging behavior via being digested in vitro. Bublin et al.²⁴ found that 40% of Act d 2 remained intact during gastric digestion and were cleaved by duodenal proteases into large fragments covalently linked by disulfide bonds. Previous studies revealed that TM showed high stability in thermal, acid–alkali, and digestion processes.²⁵ Indeed, the spatial structure might influence the allergenicity of allergens to be identified,²¹ whereas few studies have comprehensively researched the relationship between allergens and their spatial structure. Hence, the protein structure and physicochemical properties of abalone TM are important to clarify its immunoreactivity.

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The present study investigated the pan-allergen TM in *H. discus hannai* (HTM). α -TM and α_2 -TM (α/α_2 -TM) were purified from *H. discus hannai* and identified by liquid chromatography mass spectrometry–mass spectrometry (LC–MS/MS). Meanwhile, the immunoreactivity of α/α_2 -TM was analyzed using dot blot and inhibition enzyme-linked immune sorbent assay (iELISA). The linear epitopes were analyzed using bioinformatics tools and serological tests. The conformational epitopes of α_2 -TM were predicted using bioinformatics tools. The physicochemical properties of α/α_2 -TM were analyzed. Besides, the structure of α/α_2 -TM represented by secondary structures, chemical bonds, and surface hydrophobicity was analyzed.

MATERIALS AND METHODS

Materials. Live *H. discus hannai* were purchased at Jimei Market (Xiamen, Fujian, China) and sacrificed immediately. The Q-Sepharose was purchased from GE Healthcare (Waukesha, Wisconsin, USA). The rabbit *Chlamys nobilis* TM polyclonal antibody was prepared in our laboratory.²⁶ Horseradish peroxidase-labeled goat anti-human IgE antibody and horseradish peroxidase-labeled anti-rabbit IgG antibody were obtained from Southern Biotech (Birmingham, Alabama, USA). Pepsin, trypsin, chymotrypsin, and 8-anilino-1-naphthalenesulfonic acid (ANS), and β -mercaptoethanol were purchased from Sigma-Aldrich (St Louis, Missouri, USA). The peptides were synthesized from Genscript Biotech (Nanjing, Jiangsu, China). All the other reagents were of analytical grade.

Human Sera. Sera from 10 abalone-sensitive patients (No. 1–10) and 3 nonallergic individuals (No. 11–13) were provided by the Second Affiliated Hospital of Xiamen Medical College (human ethical approval 2020018, Xiamen, Fujian, China). The IgE level of sera was measured using ImmunoCAP (Phadia AB, Uppsala, Sweden) and ELISA. The levels (Table 1) of specific IgE ≥ 0.35 kUA/L were defined as sensitive sera samples, and optical densities at 450 nm ≥ 0.10 were defined as abalone-sensitive sera samples. Furthermore, the composition of abalone-sensitive patients' serum pool is shown in Table 1 (No. 1–10). Signed informed consent was obtained from all individuals. All sera samples were stored at -80 °C until further use.

Table 1. Specific IgE Levels and Symptoms of the Shellfish-Sensitive Patients

no.	age (years)	sex	specific IgE of f_{22} , f_{23} (kUA/L) ^a	specific IgE of HTM (OD 450 nm) ^b	symptoms
1	55	F	15.53	0.18	eczema
2	8	M	24.17	0.15	acute bronchitis
3	1	F	49.66	0.16	atopic dermatitis
4	12	M	45.61	0.19	nausea and vomiting
5	29	M	22.72	0.14	cough
6	48	F	9.81	0.17	lungs infected
7	67	M	17.17	0.13	urticaria
8	24	F	1.36	0.15	eczema
9	18	F	33.91	0.19	anaphylactic rhinitis
10	31	M	39.19	0.20	cough
11 ^c	24	M	0.15	0.07	^d
12 ^c	23	F	0.09	0.06	^d
13 ^c	17	F	0.13	0.07	^d

^aSera with specific IgE ≥ 0.35 kUA/L are defined as positive. ^bSera with OD 450 nm ≥ 0.10 are defined as abalone-sensitive sera. ^cA person was nonallergic individual. ^dA nonallergic individual was nonallergic symptom. M, male; F, female.

Purification and Identification of Proteins. The myofibrillar protein was prepared as described by Zou et al.²⁷ Ammonium sulfate was added to this myofibrillar protein with the saturation up to 30% and centrifuged at 18,000g for 30 min. The supernatant was adjusted to pH 4.5 with 0.2 M HCl to precipitate protein and dissolved with 20 mM Tris–HCl (pH 7.5). The crude protein was then filtered with a 0.22 μ m membrane filter before loading on the Q-Sepharose column. The elution was carried out with four linear gradients of 0–0.3 M, 0.3 M, 0.3–0.5 M, and 0.5 M NaCl. All operations were performed at 4 °C.

The western blot and dot blot were analyzed using *Chlamys nobilis* TM polyclonal antibody, as described by Bai et al.²⁶ (1:1 $\times 10^4$ dilution), and abalone-sensitive patients' sera (1:3 dilution) as the primary antibody. The *Chlamys nobilis* TM was prepared as positive control described by a previous study.²⁶ Moreover, the target protein bands cut from the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel were sent to Shenzhen Wininnovate BIO (Shenzhen, Guangdong, China) and analyzed by LC–MS/MS. The peptide mass fingerprints were compared with the amino acid sequence in the National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/>).

Immunoreactivity Analysis of α/α_2 -TM. The specific IgG/IgE-binding capacity of α/α_2 -TM was detected as described by Shen et al.⁶ The western blot and dot blot were analyzed using HTM polyclonal antibody (1:1 $\times 10^6$ dilution) and abalone-sensitive patients' sera (1:3 dilution) as the primary antibody. The HTM polyclonal antibody was prepared from a New Zealand white rabbit by immunizing with HTM at Xiamen University (Xiamen, Fujian, China), and the titer evaluation is displayed in Figure S1. Furthermore, an iELISA was analyzed using the HTM polyclonal antibody and abalone-sensitive patients' serum pool (No. 1–10 in Table 1) as the primary antibody, according to the method described by Han et al.⁸ α -TM or α_2 -TM as an inhibitor was diluted into different concentrations and then preincubated with the HTM polyclonal antibody or abalone-sensitive patients' serum pool.

Prediction of Conformational Epitope in α_2 -TM. The three-dimensional (3D) structures of α/α_2 -TM were modeled on the online website (<https://swissmodel.expasy.org/>). Due to the lack of a crystal structure of shellfish TM, α/α_2 -TM was modeled based on *Sus scrofa* (template PDB: 1C1G), which was highly matched with α/α_2 -TM. The conformational epitopes of α_2 -TM were predicted by three bioinformatics tools, including Ellipro, Discotope, and BEpro, based on the primary sequence and 3D structure. The predicted conformational epitopes were mapped on the 3D structure using Discovery Studio software.

Prediction and Verification of Linear Epitopes in α/α_2 -TM.

Allergen epitope sequences were predicted by four bioinformatics tools, including DNASTar Protean, BepiPred 1.0, ABC-pred, and IEDB database, which are server-based computationally different approaches described previously.¹⁸ The repeated peptides were chosen and synthesized (Table 2) and stored at -20 °C until use.

Dot blot and iELISA were performed to analyze the IgE-binding capacity of peptides, as described by Han et al.²⁸ with slight modifications. Various concentrations (0.5, 5, 25, and 50 nmol) of synthesized peptides were preincubated with abalone-sensitive patients'

Table 2. Information of Synthesized Peptides of α/α_2 -TM^a

peptide no.	amino acid sequence	position	verified linear epitope no.
P1	AMKMEKENAVDRAEQ	10–24	L-HTM-1
P2	NEQKLDRDTEEQKAKIEE	25–41	L-HTM-2
P3	QEAMAKLETSEKRV	65–79	L-HTM-3
P4	AQLKEAKYIAED	146–157	L-HTM-4
P5	EAARKLAITEVDLER	164–178	L-HTM-5
P6	EAAEAKILELEEL	184–197	L-HTM-6
P7	VVGNNMKSLEISEQ	199–212	L-HTM-7

^aThe peptides were predicted by four bioinformatics tools (DNASTar Protean, BepiPred 1.0, ABC-pred, and IEDB database).

serum pool as inhibitors to iELISA. The identified epitopes were simulated at the 3D structure by Discovery Studio software eventually.

Physicochemical Properties Analysis of α/α_2 -TM. The thermal and pH stability between α -TM and α_2 -TM was performed as described by Hu et al.²⁹ with slight modifications. α/α_2 -TM was adjusted to 0.1 mg/mL and heated for an hour at different temperatures. The stability of α/α_2 -TM in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) in vitro were performed according to the methods of Zhang et al.⁷ Meanwhile, the enzymatic cleavage sites were predicted by an ExPASy peptide cutter (<https://www.expasy.org/>).

Secondary Structure, Chemical Bonds, and Surface Hydrophobicity Analysis of α/α_2 -TM. The secondary structures of α/α_2 -TM were measured using the circular dichroism (CD) spectrum (Applied Photophysics, Ltd., Surrey, UK), as described by Zhang et al.⁷ Briefly, all the samples were adjusted to 0.1 mg/mL. The results of the secondary structure were calculated by CDNN software. Meanwhile, the secondary structure regions were predicted by Discovery Studio software. The analysis of chemical bonds was performed by Wang et al.³⁰ The contents of ionic bond, hydrogen bond, hydrophobic force, and disulfide bond were measured. The surface hydrophobicity of α/α_2 -TM was analyzed using ANS as a probe, as described by Han et al.⁸

Statistical Analysis. All experiments were repeated at least three times, and the data were analyzed by GraphPad Prism 5 Software. All the data are presented as the mean $\pm$ SD. A difference with $p < 0.05$ was considered statistically significant, and a difference with $p < 0.01$ was considered highly significant.

RESULTS

Purification and Identification of α/α_2 -TM. The target proteins were purified from *H. discus hannai* eluted at 0.5 M NaCl using Q-Sepharose column chromatography, and a flowsheet of the purification process is shown in SDS-PAGE (Figure 1A). The 36 kDa protein and 72 kDa protein were obtained using β -mercaptoethanol addition and ultrafiltration concentrator, respectively. The purified proteins and *Chlamys nobilis* TM showed a positive binding capacity with the rabbit *Chlamys nobilis* TM polyclonal antibody (Figure 1B). In the dot blot analysis (Figure 1C), HTM showed a positive reaction with 10 sera samples from abalone-sensitive patients (No. 1–10) and a negative reaction with 2 sera samples from nonallergic individuals (No. 11–12). Furthermore, the 36 kDa protein and 72 kDa protein were detected by LC–MS/MS, and the amino acid sequence of the two proteins was the same as TM in *Haliotis discus discus* (Genbank accession number: BAH10148.1, Figure 1D,E), indicating that the 36 kDa protein and 72 kDa protein were identified as the subunit of TM (α -TM) and supercoil of TM (α_2 -TM) by peptide mass fingerprint, and α_2 -TM consisted of two α -TM.

IgG/IgE-Binding Capacity Analysis of α/α_2 -TM. As shown in Figure 2A, α/α_2 -TM underwent positive reactions with the HTM polyclonal antibody and sera from abalone-sensitive patients (No. 1–10) by dot blot analysis. The degrees of IgG/IgE-binding capacity were as follows: α_2 -TM > α -TM (Figure 2B). Moreover, to analyze the IgG/IgE-binding capacity of α/α_2 -TM, a specific IgG/IgE-binding capacity was performed by an iELISA. On the analysis of IgG-binding capacity, IC_{50} of α/α_2 -TM inhibition to α -TM was 0.93 and 0.84 pmol, respectively, whereas IC_{50} of α/α_2 -TM inhibition to α_2 -TM was 3.75 and 1.94 pmol, respectively (Figure 2C,D). For further comparing the IgE-binding capacity of α/α_2 -TM, IC_{50} of α/α_2 -TM inhibition to α -TM was 900 and 449 pmol, respectively. Similarly, IC_{50} of α/α_2 -TM inhibition to α_2 -TM was 788 and 510 pmol, respectively (Figure 2E,F).

Prediction Analysis of Conformational Epitopes in α_2 -TM. A homology model of α/α_2 -TM was developed based on

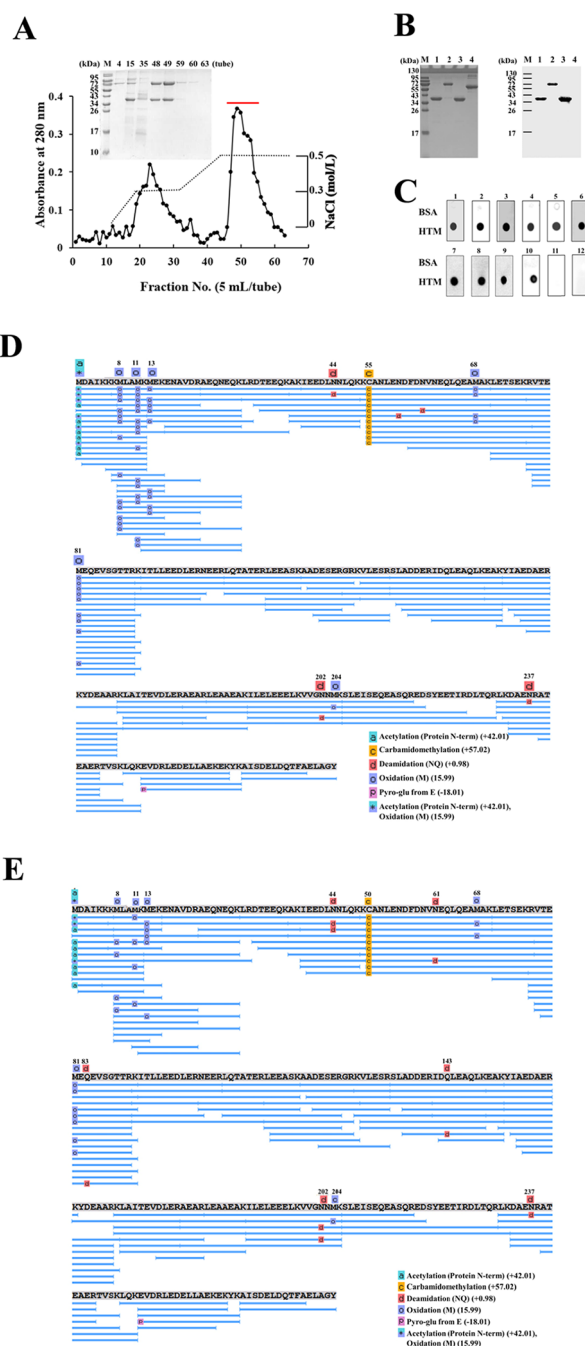


Figure 1. Purification and identification of α/α_2 -TM from *H. discus hannai*. (A) Purification of the target proteins by Q-Sepharose chromatography. A flowsheet represented the purification process of TM, and SDS-PAGE represented the fraction number in the flowsheet. Lane M, protein marker. The numbers 4, 15, 35, 48, 49, 55, 60, and 63 on the top of the lanes correspond to the fraction number. (B) SDS-PAGE and Western blot analysis of the purified proteins. Primary antibody: *Chlamys nobilis* TM polyclonal antibody. Lane M: protein marker; lane 1, the purified 36 kDa protein; lane 2, the purified 72 kDa protein; lane 3, *Chlamys nobilis* TM; lane 4, BSA. (C) IgE-binding capacity analysis of TM by dot blot. No. 1–10 were the sera of abalone-sensitive individuals, and No. 11–12 were the sera of nonallergic individuals; 1:3 dilution. (D,E) 36 kDa protein and 72 kDa protein confirmed by LC–MS/MS, respectively. Sequence alignment results of the mass spectrometry peptides were matched with TM (Genbank accession number: BAH10148.1) from *Haliotis discus discus*.

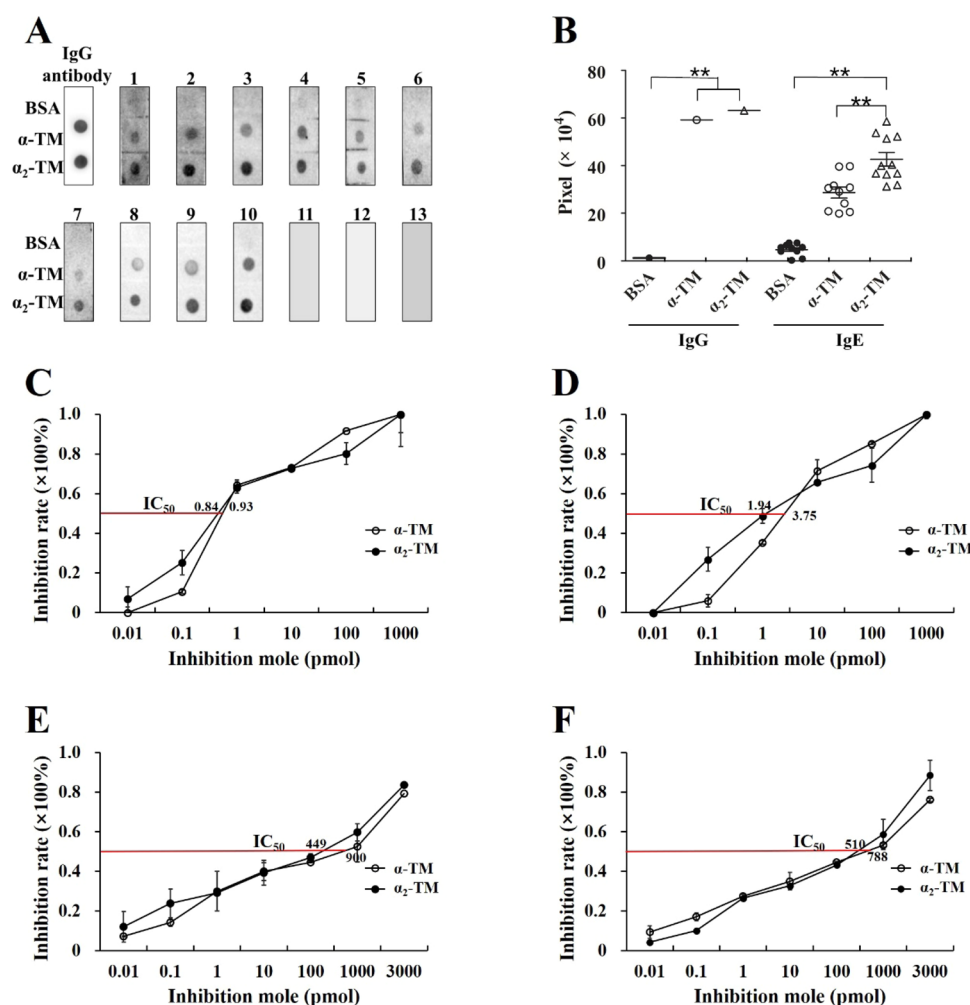


Figure 2. Analysis of IgG/IgE-binding capacity of α/α_2 -TM. (A) IgG/IgE-binding capacity analysis of α/α_2 -TM by dot blot. No. 1–10 were the sera of abalone-sensitive individuals, and No. 11–13 were the sera of nonallergic individuals; 1:3 dilution. (B) Average intensity of the dots shown on the nitrocellulose members in Figure 2A by image J software. (C,D) IgG-binding capacity analysis of α/α_2 -TM by iELISA using the HTM polyclonal antibody. α/α_2 -TM on the solid phase respectively. (E,F) IgE-binding capacity analysis of α/α_2 -TM by iELISA using abalone-sensitive patients' serum pool (serum pool including serum No. 1–10 from Table 1); α/α_2 -TM on the solid phase. All data are presented as the mean $\pm$ SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$.

Sus scrofa TM (PDB: 1C1G) via the online website, and the amino acid sequence of α/α_2 -TM was from *Haliotis discus discus* TM, which was matched with α/α_2 -TM. Based on the primary sequence and 3D structure, two conformational epitopes (C-1: AA_{6, 8, 9, 12, 13, 16, 285–298, 300, 301, 304} and C-2: AA_{268, 269, 271–284, 565–568}) of α_2 -TM were predicted (Table S1). Additionally, the two predicted conformational epitopes mapped to α_2 -TM in Figure 3A.

Prediction and Verification Analysis of Linear Epitopes in α/α_2 -TM. Bioinformatics tools were employed to analyze allergenic linear epitopes of α/α_2 -TM. Seven mimotopes (P1–P7) were synthesized for verification (Table 2). As shown in Figure 3B, the peptides and positive control HTM showed a specific IgE-binding capacity with 10 sera from abalone-sensitive patients by dot blot and negatively with sera from nonallergic individuals. The negative control BSA did not bind with sera from abalone-sensitive patients or nonallergic individuals. Meanwhile, P1–P4 displayed lower IgE-binding capacity than HTM and P5–P7.

Moreover, to investigate the relevance of IgE-binding capacity of individual synthesized peptides and exclude the difference of sera samples, an iELISA analysis was executed by the IgE

competitive interaction between P1–P7 and α/α_2 -TM using the serum pool. In Figure 3C, P1–P7 and α -TM showed different inhibition rates to α -TM. P6 displayed the highest inhibition rate (86.01%), whereas P1 displayed the least inhibition rate (44.16%) at 50 nmol. P6 displayed the highest (83.33%), whereas P1 displayed the lowest rate (41.67%) at 25 nmol. P6 displayed the highest rate (82.38%), whereas P1 displayed the lowest inhibition rate (31.99%) at 5 nmol. P5 displayed the highest rate (38.01%), whereas P1 displayed the lowest inhibition rate (10.53%) at 0.5 nmol.

However, P1–P7 also revealed different inhibition capacities to α_2 -TM (Figure 3D). P6 displayed the highest inhibition rate (96.10%), whereas P2 displayed the least rate (50.56%) at 50 nmol. P6 displayed the highest rate (78.36%), whereas P2 displayed the lowest rate (41.90%) at 25 nmol. P5 displayed the highest rate (74.65%), whereas P1 displayed the lowest inhibition rate (24.90%) at 5 nmol. P5 displayed the highest rate (71.29%), whereas P1 displayed the lowest inhibition rate (25.88%) at 0.5 nmol. As shown in Figure S2, when P4 and P6 were used as inhibitors to α -TM, the inhibition rate was over 80% at the highest inhibitor concentration. When P4–P6 were used as inhibitors to α_2 -TM, the inhibition rate was over 80% at

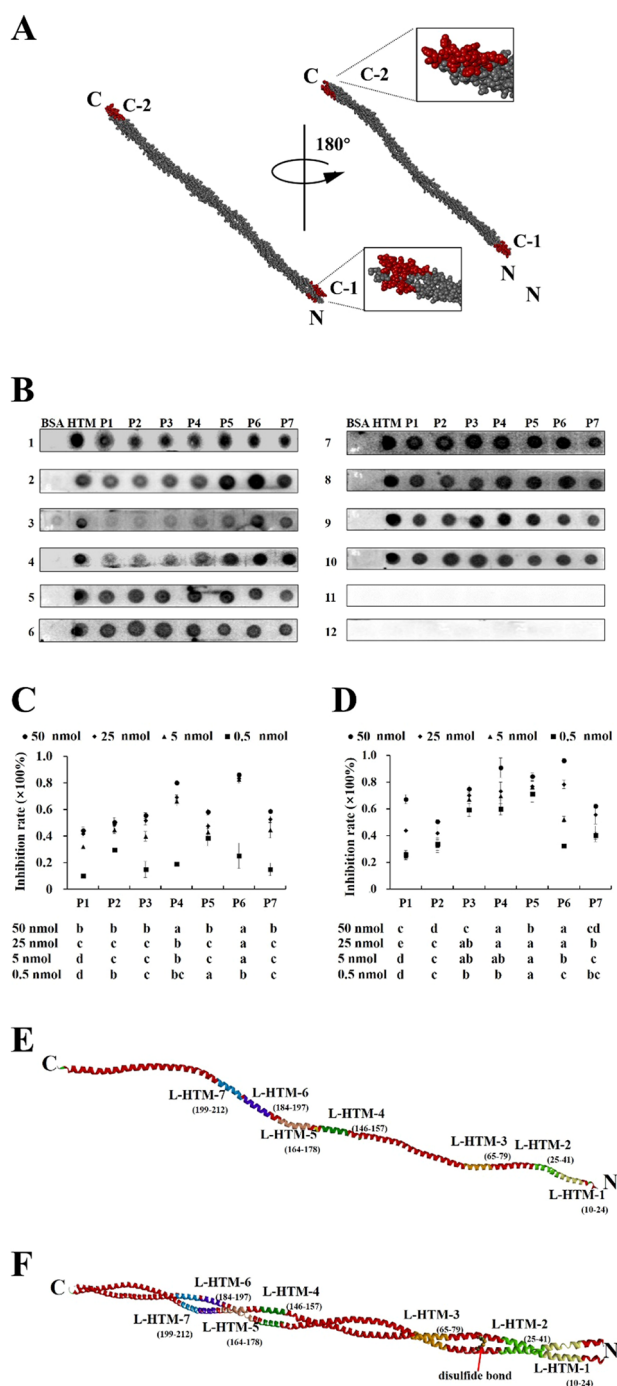


Figure 3. Prediction and identification of HTM IgE epitopes. (A) Molecular graphics of the conformational epitopes in α_2 -TM. The gray chains indicate α_2 -TM, and the red regions indicate the conformational epitopes. (B) Dot blot analysis of seven potential IgE linear epitopes. No. 1–10 were the sera of abalone-sensitive individuals, and No. 11 and 12 were the sera of nonallergic individuals; 1:3 dilution. BSA was used as the negative control; HTM was used as the positive control. (C,D) iELISA analysis of seven potential IgE linear epitopes; α/α_2 -TM on the solid phase. The IgE binding of the serum pool was determined with different peptides (P1–P7). All the data are presented as the mean $\pm$ SD ($n = 3$). $*p < 0.05$, $**p < 0.01$. (E,F) Distribution of IgE linear epitopes in the 3D structure of α/α_2 -TM. IgE linear epitopes: L-HTM-1 (AA_{10–24}), L-HTM-2 (AA_{25–41}), L-HTM-3 (AA_{65–79}), L-HTM-4 (AA_{146–157}), L-HTM-5 (AA_{164–178}), L-HTM-6 (AA_{184–197}), L-HTM-7 (AA_{199–212}); and disulfide bond: bronze ball.

the highest inhibitor concentration. In addition, the inhibition rates of α_2 -TM were higher than those of α -TM, when P3–P5 were used as inhibitors at different inhibited moles. The seven identified linear epitopes of HTM are listed in Table 1 and named L-HTM-1 (P1), L-HTM-2 (P2), L-HTM-3 (P3), L-HTM-4 (P4), L-HTM-5 (P5), L-HTM-6 (P6), and L-HTM-7 (P7). Seven IgE linear epitopes were mapped to the structures of α/α_2 -TM (Figure 3E,F).

Thermal Stability, pH Stability, and Digestibility Analysis of α/α_2 -TM. In the thermal stability analysis, the bands of α -TM at different temperatures were retained as shown in Figure 4A. The molecular weight of α_2 -TM was gradually reduced to near 36 kDa (Figure 4B) when the temperature was up to 60 °C, while the degradation gradually increased with the increase of temperature. After thermal treatment, the IgG-binding capacity of α/α_2 -TM was decreased slightly at 100 °C (Figure 4C,D). However, there was no obvious change in the IgE-binding capacity of α/α_2 -TM by dot blot analysis (Figure 4E,F).

In the experiments for pH stability, α -TM stabilized in the alkaline condition (pH 7.0–11.0) and aggregated in the acid condition (pH 2.0–6.0), as seen in Figure 4G. During the strongly acidic condition (pH 1.0), α/α_2 -TM diffused clearly. However, α_2 -TM remained stable when exposed to acidic and alkaline conditions (Figure 4H) and degraded slightly in high alkaline conditions (pH 11.0). As shown in Figure 4I,J, the bands of α/α_2 -TM reacted positively with the HTM polyclonal antibody. All the samples revealed high IgE-binding capacity by the dot blot assay in Figure 4K,L.

In vitro vitrodigestion analysis was performed to assess the digestibility of α/α_2 -TM. As shown in Figure 5A,B, α -TM was digested slightly within 30 min after digesting with pepsin, and α_2 -TM was degraded to α -TM absolutely within 1 min. All the samples revealed the positive IgG/IgE-binding capacity, as shown in Figure 5C–F. As seen in Figure 5G, α -TM was degraded rapidly into small fragments by trypsin within 1 min; with increasing time, the digested fragments became smaller, even unclear. In the mass, the molecular weights of degradation bands of α_2 -TM were lower than α -TM (Figure 5H). The trypsin digested fragments of α_2 -TM were mainly in 20 and 10 kDa, and the majority of trypsin digestive fragments in α -TM were in 20 and 15 kDa. The digestive samples were recognized by HTM polyclonal antibody and abalone-sensitive patients' serum pool (Figure 5I–L). Different from digestion with trypsin in SIF, the degraded bands were similar to the pepsin digestive pattern, after digestion with chymotrypsin (Figure 5M and N). All the digested fragments of α/α_2 -TM were recognized clearly by HTM polyclonal antibody and patients' serum pool (Figure 5O–R). Moreover, a total of 8 pepsin cleavage sites, 49 trypsin cleavage sites, and 7 chymotrypsin cleavage sites were predicted and mapped (Figure S3).

Secondary Structure, Chemical Bonds, and Surface Hydrophobicity Analysis of α/α_2 -TM. The secondary structures of α/α_2 -TM were similar in components, which were composed of α -helix (Figure 6A–C). Figure 6A,B show that the secondary structure of α -TM was reversible, whereas the secondary structure of α_2 -TM displayed nonreversibility when returning to room temperature after heating processes. Moreover, the proportion of α -helix in α -TM at the groups of control, heated to 100 °C, and renaturation was $98.6 \pm 0.2\%$, $66.9 \pm 3.0\%$, and $98.4 \pm 0.1\%$, respectively. The α -helix content in α_2 -TM was $88.9 \pm 3.9\%$, $48.9 \pm 0.7\%$, and $54.6 \pm 1.2\%$ at the groups of control, heated to 100 °C, and renaturation,

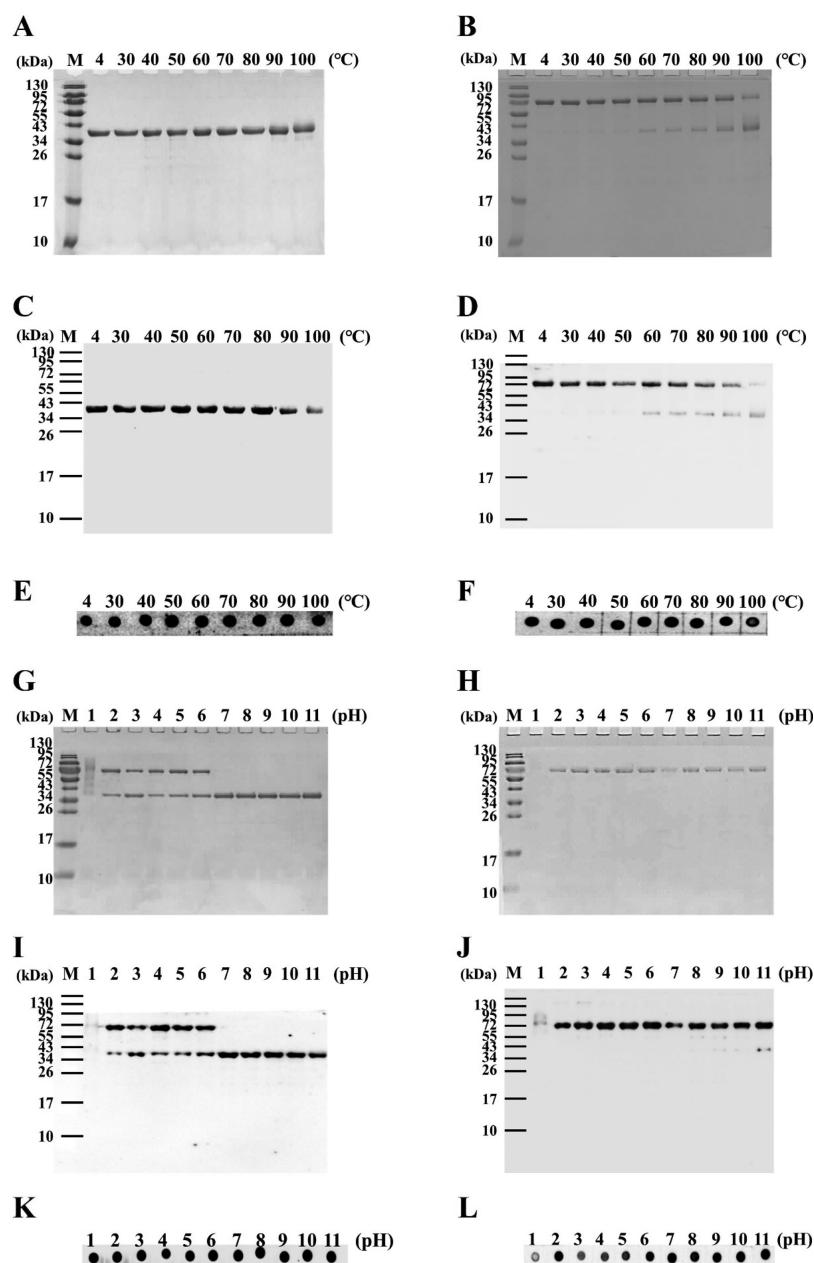


Figure 4. Analysis of the thermal stability and pH stability of α/α_2 -TM. (A,B) SDS-PAGE analysis of thermal stability for α/α_2 -TM, respectively. Lane M, protein marker. (C,D) Western blot analysis of thermal stability of α/α_2 -TM using HTM polyclonal antibody, respectively. Lane M, protein marker. (E,F) Dot blot analysis of thermal stability of α/α_2 -TM using abalone-sensitive patients' serum pool, respectively. (G,H) SDS-PAGE analysis of pH stability for α/α_2 -TM, respectively. Lane M, protein marker. (I,J) Western blot analysis of pH stability of α/α_2 -TM using HTM polyclonal antibody, respectively. Lane M, protein marker. (K,L) Dot blot analysis of pH stability of α/α_2 -TM using abalone-sensitive patients' serum pool, respectively.

respectively, as shown in Figure 6C. The thermal denaturation temperature (T_m) values of α/α_2 -TM were 42.0 ± 0.2 °C and 46.7 ± 0.2 °C, respectively (Figure 6D,E). The residues of AA₁, AA₆, AA₂₁, and AA₁₃₈ and region of AA_{281–284} were the other secondary structures rather than α -helix in α -TM, which could be shortened to non- α -helix. The residues of AA₁, AA₆, AA₃₁, AA₁₃₈, AA₂₈₅, and AA₅₃₀ and regions of AA_{281–284}, AA_{298–301}, AA_{311–313}, AA_{450–454}, and AA_{565–568} were the non- α -helix in α_2 -TM (Figure S3). According to Figure 6F, the proportion of the hydrogen bond, hydrophobic force, and disulfide bond of α -TM was $15.59 \pm 0.1\%$, $0.58 \pm 0.2\%$, $78.53 \pm 0.1\%$, and $0 \pm 0\%$, respectively. The content of hydrogen bond, hydrophobic force, and disulfide bond of α_2 -TM was $7.91 \pm 0\%$, $7.88 \pm 0.1\%$, $29.56 \pm 0.1\%$, and $54.63 \pm 0.3\%$, respectively. The main chemical

bond of α -TM showed hydrophobic interaction, whereas the main chemical bond of α_2 -TM was the disulfide bond. As shown in Figure 6G,H, the surface hydrophobicity of α/α_2 -TM exhibited a redshift (10 nm). The surface hydrophobicity of both α/α_2 -TM increased and so did the fluorescence intensity peaks (10 and 20 nm, respectively) after heating at 100 °C. In the meantime, the fluorescence intensity of α_2 -TM was higher than α -TM both in the groups of control and heated to 100 °C.

DISCUSSION

IgE-mediated shellfish allergies play an important role in human health.³¹ Abalone is a traditional functional food that is nutritious and delicious.³² Although shellfish allergens have

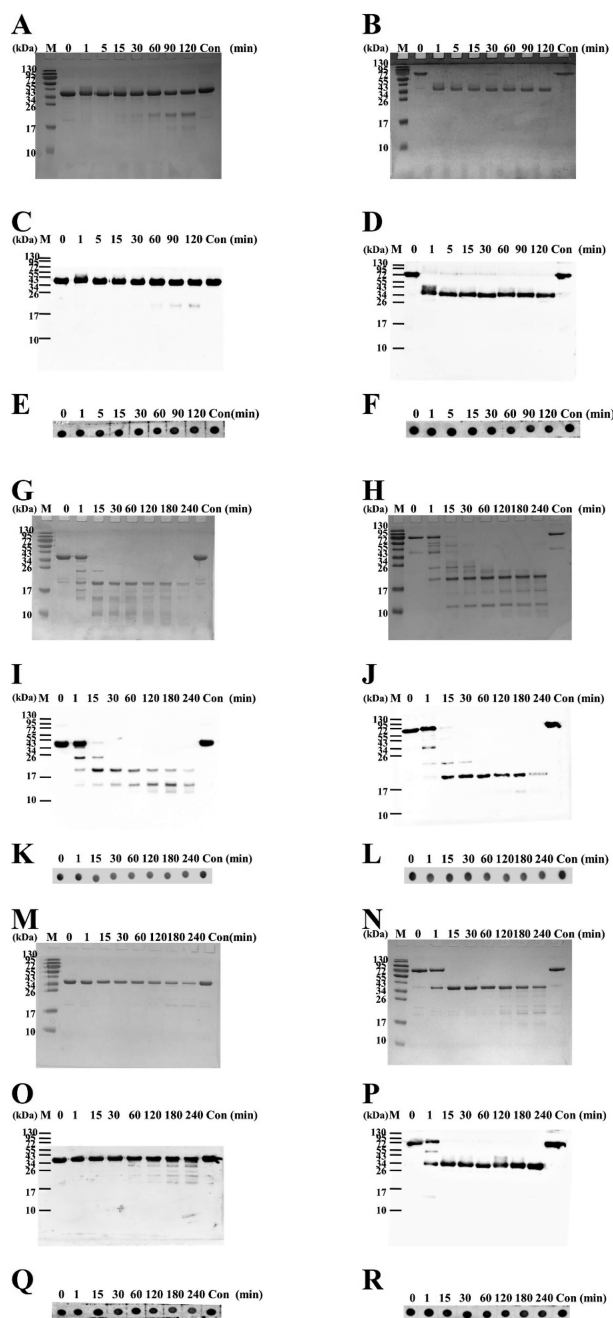


Figure 5. Analysis of the digestion stability of α/α_2 -TM. (A,B) SDS-PAGE analysis of pepsin digestion stability for α/α_2 -TM, respectively. Lane M, protein marker. (C,D) Western blot analysis of pepsin digestion stability of α/α_2 -TM using HTM polyclonal antibody, respectively. Lane M, protein marker. (E,F) Dot blot analysis of pepsin digestion stability of α/α_2 -TM using abalone-sensitive patients' serum pool, respectively. (G,H) SDS-PAGE analysis of trypsin digestion stability for α/α_2 -TM, respectively. Lane M, protein marker. (I,J) Western blot analysis of trypsin digestion stability of α/α_2 -TM using HTM polyclonal antibody, respectively. Lane M, protein marker. (K,L) Dot blot analysis of trypsin digestion stability of α/α_2 -TM using abalone-sensitive patients' serum pool, respectively. (M,N) SDS-PAGE analysis of chymotrypsin digestion stability for α/α_2 -TM, respectively. Lane M, protein marker. (O,P) Western blot analysis of chymotrypsin digestion stability of α/α_2 -TM using HTM polyclonal antibody, respectively. Lane M, protein marker. (Q,R) Dot blot analysis of chymotrypsin digestion stability of α/α_2 -TM using abalone-sensitive patients' serum pool, respectively.

been widely reported, there is little information on abalone.³³ Hence, it is necessary to identify the allergens in abalone to estimate allergenicity for further diagnosis.

TM has largely been identified in invertebrates and vertebrates, and its sensitization was widely found in invertebrates, especially in shellfish.¹² As a pan-allergen in most species,^{2,5} the identified TM in abalone could improve the understanding of allergens in shellfish. In this study, TM was purified and identified from *H. discus hannai*. Moreover, HTM identified in this paper existed as a subunit in the supercoil form and had IgG/IgE-binding capacity with sera of abalone-sensitive patients, indicating that HTM is an allergen.

Different from TM in most shellfish (*Scylla paramamosain*, *Macrobrachium rosenbergii*, *Patinopecten yessoensis*, and *Penaeus monodon*), abalone TM was found to contain a Cys in its primary sequence using LC-MS/MS. As a special TM in shellfish, the immunoreactivity difference of abalone α/α_2 -TM was still unknown. In this study, α_2 -TM displayed a higher IgG/IgE-binding capacity than α -TM, as determined by dot blot and iELISA assays; the possible reason would be that α_2 -TM contains two Cys, while α -TM contains only one. The formation of disulfide bonds can strengthen the allergenicity of allergens,²¹ such as Ara h 2 and Ara h 6.³⁴ A disulfide bond existed in α_2 -TM, which could generate different epitopes between α -TM and α_2 -TM. Recent studies showed that the epitope is related to the allergenicity of allergens.¹⁵ Thus, seven epitopes of α/α_2 -TM including L-HTM-1 (AA_{10–24}), L-HTM-2 (AA_{25–41}), L-HTM-3 (AA_{65–79}), L-HTM-4 (AA_{146–157}), L-HTM-5 (AA_{164–178}), L-HTM-6 (AA_{184–197}), and L-HTM-7 (AA_{199–212}) were verified. Three epitopes of α/α_2 -TM, including L-HTM-4, L-HTM-5, and L-HTM-6, displayed higher IgE-binding capacity, which might be the key regions of α/α_2 -TM. Moreover, the inhibition rate of L-HTM-5 to α_2 -TM was higher than that of α -TM. Meanwhile, the unique non- α -helix region of α_2 -TM (AA_{164–170}) was overlapped with L-HTM-5 (AA_{164–178}). It was easier to mask L-HTM-5 in α_2 -TM and thus create a highly significant difference in the rate of inhibition of L-HTM-5 to α/α_2 -TM. The conformational epitopes, including C-1 (AA_{1–16}, 285–304) and C-2 (AA_{268–284}, 565–568), could be generated due to the partially overlapped non- α -helix regions of the first chain and the second chain in α_2 -TM. Pekar et al.²¹ concluded that disulfide bonds could contribute to the formation of conformational IgE epitopes. It is necessary to illustrate the generation conformational epitopes of α_2 -TM. These findings indicated that conformational epitopes in α_2 -TM might lead to the higher immunoreactivity of α_2 -TM. Meanwhile, the knowledge of epitopes holds huge potential for allergy diagnosis and food processing.

Physicochemical properties, including thermal stability, pH stability, and in vitro digestibility, could influence the sensitization of allergens.²⁰ Here, α -TM showed high thermal stability, which is consistent with scallop TM that remained stable even at 100 °C.²⁶ However, α_2 -TM degraded to α -TM when the temperature was over 60 °C. It is indicated that α -TM is more heat-stable than α_2 -TM because α -TM contains one Cys, whereas α_2 -TM contains two Cys and has an interchain disulfide bond that is disrupted during heating. Creamer et al.²² found that the disulfide bond broke and showed interchange between β -lactoglobulin non-native monomers or polymers during the heating process. Visschers et al.³⁵ revealed that the disulfide bond could be broken during the thermal treatment. In addition, the contents of α -helix decreased and the surface hydrophobicity increased in α_2 -TM during the thermal process.

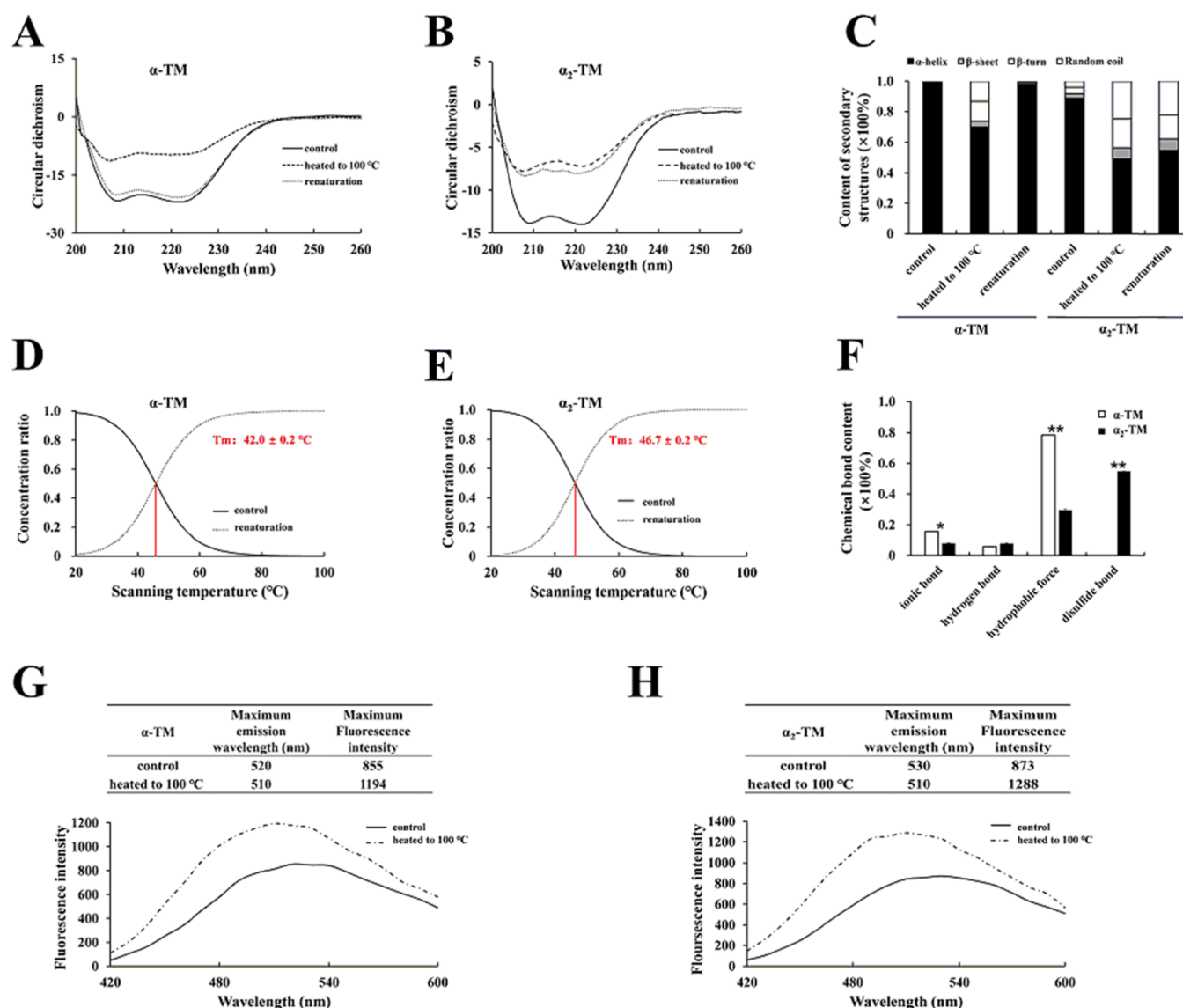


Figure 6. Structure analysis of α/α_2 -TM. (A,B) Secondary structural analysis of α/α_2 -TM. The control group indicated that α/α_2 -TM were at room temperature, the heated to 100 °C group indicated that α/α_2 -TM were heated to 100 °C, and the renaturation group indicated that α/α_2 -TM returned to room temperature after heating to 100 °C. (C) Contents of different secondary structures of α/α_2 -TM. (D,E) Determination of the T_m values of α/α_2 -TM. (F) Chemical bonds analysis of α/α_2 -TM. (G,H) Surface hydrophobicity analysis of α/α_2 -TM. The control group indicated that α/α_2 -TM were at room temperature, and the heated to 100 °C group indicated that α/α_2 -TM were heated to 100 °C. All data are presented as the mean $\pm$ SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$.

However, α -TM could aggregate as α_2 -TM at the acid solution (pH 1.0–6.0). The formation of intermolecular disulfide bonds in acid-induced gels of heat-denatured whey protein or globular protein was observed fully.^{36–38} The spatial structure is considered to verify the interchange of the disulfide bond during thermal or acid–alkali treatment. Moreover, α/α_2 -TM showed different digestion patterns in SGF and SIF. α -TM displayed high tolerance of pepsin, while α_2 -TM was digested to α -TM within 1 min, which showed the easy digestibility of disulfide bonds with pepsin.³⁹ After trypsin digestion, the different fragments between α -TM and α_2 -TM were exhibited in both SDS-PAGE and LS-MS/MS, especially in AA_{29–49}, AA_{92–100}, AA_{124–161}, and AA_{170–183}. The immunobinding activity of the digested samples was still contained because antigen epitopes were not fully digested. Meanwhile, the digestive degree of TM by the three digestive enzymes is consistent with the number of cleavage sites of the corresponding enzymes. In

pepsin and chymotrypsin digestion, there was only one pepsin/chymotrypsin cleavage site (Y₁₅₃) in epitopes (L-HTM-4). Therefore, the pepsin digestive samples were performed with strong IgG/IgE-binding capacity. In trypsin digestion, there were 17 trypsin cleavage sites in epitopes, and the epitopes still existed in digestive fragments. It is indicated that TM still has IgG/IgE-binding capacity after trypsin digestion. This phenomenon was consistent with the fact that the number of pepsin or chymotrypsin cleavage sites was fewer than that of trypsin. Like TM from many species,^{5,26,40} α/α_2 -TM presented a strong IgG/IgE-binding capacity after heating, acid–alkali, and digestive treatment. In other words, the thermal stability and pH stability of α/α_2 -TM were only related to the disulfide bond, whereas the digestion stability in vitro of α/α_2 -TM was mainly related to cleavage sites combined with the disulfide bond. These findings will improve the understanding of TM and are helpful to provide suitable processing of abalone products in the future.

The spatial structure is closely related to the allergenicity of allergens.³³ The secondary structure, chemical bonds, and surface hydrophobicity are common indexes for assessing structural changes of proteins. According to spectroscopy analysis, α/α_2 -TM mainly consisted of α -helix, which showed a similar secondary structure with TM as described previously.^{12,25} However, the α -helix content of α_2 -TM is lower than α -TM in the result of CD spectrum analysis and prediction. The main chemical bond of α -TM was hydrophobic force. The hydrophobic force is considered to be the key chemical bond for the stability of some thermophilic allergens.⁴¹ Therefore, α -TM showed higher thermal stability than α_2 -TM in this study. However, the main chemical bond of α_2 -TM was the disulfide bond, which could contribute to the formation of conformational epitopes.²¹ Furthermore, the fluorescence intensity peaks of α_2 -TM displayed a redshift in surface hydrophobicity, compared with α -TM. The increased and redshifted fluorescence intensity peaks could increase the allergenicity of allergens by exposing more hydrophobic regions on the surface of allergens.⁸ Thus, the conformational epitopes of α_2 -TM could be predicted, and α_2 -TM displayed stronger IgG/IgE-binding capacity than α -TM.

In conclusion, α/α_2 -TM were identified as allergens in *H. discus hannai*. The IgG/IgE-binding capacity of α_2 -TM was higher than that of α -TM, which may be responsible for the predicted conformational epitopes of α_2 -TM. The disulfide bond in α_2 -TM and hydrophobic force in α -TM were revealed by physicochemical property and chemical bond assays. Furthermore, the different components of the secondary structure and surface hydrophobicity were performed to explain the different epitopes of α/α_2 -TM. These findings would advance the knowledge on TM from abalone and contribute to the verification of these conformational epitopes. Meanwhile, it is worth establishing the cell models and animal models for further verification of linear epitopes.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.1c06401>.

Titer of HTM polyclonal antibody, iELISA analysis of IgE linear epitopes at different moles, analysis of the amino acid sequence in HTM, and amino acid position of the predicted conformational epitopes in α_2 -TM (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

α -TM, subunit of TM; α_2 -TM, supercoil of TM; α/α_2 -TM, α -TM and α_2 -TM; AA, amino acid; ANS, 8-anilino-1-naphthalenesulfonic acid; BSA, bovine sera albumin; CD, circular dichroism; Cys, Cysteine; HTM, *H. discus hannai* TM; iELISA, inhibition enzyme-linked immune sorbent assay; IgE, Immunoglobulin E; IgG, Immunoglobulin G; LC-MS/MS, liquid chromatography mass spectrometry–mass spectrometry; NCBI, National Center for Biotechnology Information; OD, optical density; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; TM, tropomyosin; T_m , thermal denaturation temperature; 3D, three-dimensional.

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