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Cite this: DOI: 10.1039/d1fo00270h

Effects of the Maillard reaction on the epitopes and immunoreactivity of tropomyosin, a major allergen in *Chlamys nobilis*†

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Scallop (*Chlamys nobilis*) causes an IgE-mediated food allergy; however, studies of the allergens in its musculus are not sufficiently comprehensive. In this context, the target protein was purified from scallops and confirmed to be the major allergen tropomyosin (TM) using proteomic technology and serological testing. Subsequently, seven potential IgE epitopes of TM were obtained using phage display technology with IgE enrichment from the serum of scallop-sensitized patients and identified via inhibition enzyme-linked immunosorbent assays. A method for the Maillard reaction of TM and xylose was established, and Maillard-reacted TM (MR-TM) showed significantly decreased immunobinding activity and CD63 and CD203c expression in basophils compared with TM. Furthermore, shotgun proteomics analysis showed that eleven specific amino acids (K12, R15, K28, K76, R125, R127, K128, R133, R140, K146, and K189) of the six IgE epitopes of TM were modified after the Maillard reaction. Overall, the immunoactivity of MR-TM was reduced, which provides a theoretical reference for the development of hypoallergenic foods.

Received 27th January 2021

Accepted 13th April 2021

DOI: 10.1039/d1fo00270h

rsc.li/food-function

Introduction

Shellfish allergy is a common cause of immunoglobulin (Ig) E mediated food allergies, which affect up to 2% of the world population and last for life in most patients.^{1,2} The prevalence of shellfish allergy varies in different geographical regions, and it is more common in the Asia-Pacific region than in Europe and North America.³

Chlamys nobilis (*C. nobilis*) is a commercially important shellfish bivalve that has been cultured in the South China Sea for decades.⁴ However, scallop allergy is one of the most common, severe, and long-lasting food allergies among children and adults.⁵ At present, shellfish tropomyosin (TM), arginine kinase, myosin light chain, sarcoplasmic calcium-binding protein, and hemocyanin have been identified as the cause of

sensitive reactions, of which TM is the only well recognized allergen in *C. nobilis*.⁵ In a previous study, recombinant TM of *Haliotis diversicolor*, *C. nobilis*, and *Perna viridis* was cloned and expressed using genetic engineering. Additionally, immunoblot analysis showed that cross-sensitization toward the recombinant proteins of abalone, scallop, and mussel TM is observed.⁵ As *E. coli* does not have intracellular mechanisms for post-translational modifications such as glycosylation or disulfide bonds, the recombinant food allergen may be structurally different from the natural allergen.⁶ Therefore, the natural protein can better reflect the real sensitized state in the serum response and effector cell activation of patients. Based on the research status described above, the characterization and physicochemical properties of *C. nobilis* TM are worthy of further investigation.

The allergenic potential of an allergen is determined by its IgE epitopes, which trigger the effector cells to release various sensitive mediators that induce the consequences of the allergy.⁷ The analysis of IgE epitopes is important for providing good indicators of IgE-allergen complex formation and the activation of effector cells.⁸ The protein region that causes a sensitive reaction may be a simple stretch of a few amino acids along the primary structure, or it may be a unique three-dimensional (3D) motif of the protein structure; these are referred to as linear or conformational epitopes, respectively.⁹ In the past decades, antigenic epitopes have been mapped by

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†Electronic supplementary information (ESI) available: For additional experimental details. See DOI: 10.1039/d1fo00270h

overlapping synthetic peptide approaches, immunoinformatics tools, phage peptide display technology, and X-ray crystallography.¹⁰ At present, biopanning allergen IgE epitopes using a phage display random peptide library is becoming more popular and easier.^{11–13} Yang *et al.* used phage display to comprehensively analyze the IgE epitopes of crab myosinogen allergens.¹¹ Eight linear epitopes and seven conformational epitopes of TM from *S. serrata* were identified using phage display.¹² Fatemeh *et al.* first reported the identification of a linear mimotope of *Naja oxiana* venom with antibodies using phage display.¹³ Identification using the peptides displayed on the phage that bind to antibodies is more accurate and more reliable than prediction using immunoinformatics tools, because the antibody selects specific peptides mimicking the epitope of the antigen.¹⁴ TM is the main allergen in *C. nobilis*, and its epitope information has not been reported. Therefore, the identification of TM epitopes would lay a foundation for the study of the mechanism of processing subtraction in reducing the immunoreactivity of *C. nobilis* TM.

The structure of TM is a dimer consisting of two alpha helical chains associated into a parallel coiled-coil, which is highly stable to heat treatment and can maintain its ability to induce hypersensitivity even after cooking and high pressure treatment.^{15,16} The Maillard reaction is a non-enzymatic reaction between the carbonyl groups of reducing sugars and the free amino groups of a protein (typically arginine and lysine).¹⁷ Several factors, such as the reaction temperature, time, ratio of the amino groups and reducing sugars, water activity, and pH, as well as the intrinsic characteristics of the protein, influence the Maillard reaction.¹⁸ The allergenicity of some thermally stable food allergens is affected by the Maillard reaction. Zhang *et al.* found that *Exopalaemon modestus* TM was modified by fructooligosaccharides *via* the Maillard reaction to produce a large amount of advanced glycation end products (AGEs), which enhanced the sensitization towards TM.¹⁹ The Maillard reaction increases the allergenicity of *Patinopecten yes-soensis* TM by structural changes that lead to protein cross-linking, which introduced novel IgE epitopes.²⁰ In contrast, the Maillard reaction with ribose, galacto-oligosaccharide or chitosan-oligosaccharide reduced the allergenicity of *Penaeus chinensis* TM by inducing conformational changes.²¹ The Maillard reaction may have an impact on allergenicity, but there is no defined rule describing how these modifications alter allergenic properties. Therefore, studying the mechanism of the Maillard reaction in *C. nobilis* TM sensitization has practical significance.

In this study, we aimed to elucidate effects of the Maillard reaction on the epitopes and immunoreactivity of TM from *C. nobilis*. The native allergen in *C. nobilis* was purified and identified, and its immunoactivity was validated by serological testing. The epitopes were biopanned using phage display. Additionally, TM was characterized before and after the Maillard reaction, and glycation sites of TM were identified by shotgun proteomics after the Maillard reaction. The results of the present study might provide new insight into the desensitization of *C. nobilis*-induced allergy.

Materials and methods

Materials

Live *C. nobilis* were purchased at Jimei Market (Xiamen, Fujian, China) and frozen at -20°C until use. Q-Sepharose was purchased from GE Healthcare (Waukesha, Wisconsin, USA). Various reducing sugars (arabinose, Arab; xylose, Xyl; glucose, Glu; mannose, Man; and galactose, Gal) were obtained from Macklin (Shanghai, China). The Ph.D.-12 Phage Display Random Peptide Library and *Escherichia coli* ER2738 were purchased from New England Bio-Laboratories (Beverly, MA, USA). Horseradish-peroxidase-labeled goat anti-human IgE antibody and horseradish-peroxidase-labeled anti-rabbit IgG antibody were purchased from Southern Biotech (Birmingham, AL, USA). The peptides were synthesized by Genscript Biotech (Nanjing, Jiangsu, China).

Human whole blood and sera

Whole blood and sera from 11 scallop-sensitized patients (no. 5–15) and 4 nonallergic individuals (no. 1–4) were collected from the Second Affiliated Hospital of Xiamen Medical College (Xiamen, Fujian, China). All serological procedures were performed in accordance with the guidelines of the Second Affiliated Hospital of Xiamen Medical College, and the experiments were approved by the Human Ethics Committee (no. 2020018). All the samples were tested *in vitro*. Signed informed consent was obtained from all individuals. Levels of scallop-specific IgE $\geq 0.35 \text{ kUA L}^{-1}$ by ImmunoCAP (Phadia AB, Uppsala, Sweden) were defined as positive, and food-allergy-free sera was used as the negative control. The samples were stored at -80°C until further use. Table S1 of the ESI† shows the individual sensitivity profiles and clinical symptoms, which were used in the present study. The serum pool was prepared by mixing the sera of patients no. 5–9 in Table S1 of the ESI† equal to 300 μL per tube without dilution.

Purification of native protein

The preliminary treatment was carried out as described previously, with slight modifications.²² The sliced striated adductor muscles of *C. nobilis* were washed and homogenized using a homogenizer in 10 volumes of 50 mM PBS (pH 7.0), and then the homogenate was extracted in cold storage at 4°C for 1 h. The homogenate was centrifuged at 10 000g for 15 min at 4°C , and the precipitate was re-suspended in the same washing medium and further centrifuged four times. The last precipitate, which contained the myofibrillar proteins, was washed four times with cold absolute acetone and dried completely. The dried powder was extracted overnight with 10 volumes (v/w) of 100 mM PBS (pH 7.0) containing 1 M NaCl. The mixture was then centrifuged at 12 000g for 20 min at 4°C . The supernatant was collected, and the pH was adjusted to 4.5 with 0.2 M HCl. After further centrifugation at 12 000g for 10 min at 4°C , the precipitate was dissolved in 20 mM Tris-HCl (pH 7.5). The supernatant was eluted (flowrate of 1 mL min^{-1}) with two linear gradients (0–0.3 M, 0.3–0.5 M KCl) on a Q-Sepharose column. The samples were loaded and

subjected to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Identification of native protein

The obtained single protein band was entrusted to Shenzhen Wininnovate BIO (Shenzhen, Guangdong, China) for liquid chromatography mass spectrometry–mass spectrometry (LC/MS–MS) by Thermo Scientific (Billerica, MA, USA).⁶ The resulting peptide spectrum was compared with those in the National Center for Biotechnology Information (NCBI) database.

Immunoactivity analysis

The specific IgG/IgE-binding activity of TM was detected using dot blotting and western blotting, which were performed as described previously.²³

The basophil activation test (BAT) is a flow-cytometry-based assay that reproduces IgE-mediated sensitive reactions *in vitro*.²⁴ The sera of the patients was verified by dot blotting, and the whole blood was used to further detect the effects of the allergen on the effector cells of the patients *via* BAT. The allergen concentration (50 µg mL⁻¹) was chosen as described in the paper of Yang *et al.*¹¹ Up-regulation of CD63 and CD203c expression on basophils was measured using flowcytometry as reported. The stimulation index (SI) was calculated as the ratio of the mean fluorescence intensity of the allergen (MFistim) basophils to the mean fluorescence intensity of unstimulated (MFicon) basophils, defined as MFistim/MFicon.²⁵

Thermal, pH, and digestion stability analysis of TM

The investigation of thermal and pH stability of TM was performed as described previously.²² Briefly, processing temperatures from 30–100 °C were used in the thermal stability test. Additionally, protein stored at 4 °C was used as the negative control. To test its pH stability, TM was incubated for 1 h at room temperature in buffers with different pH values (1.0–11.0), and the samples incubated at pH 7.0 were used as controls. The stability of the target protein *in vitro* in simulated gastric fluid or simulated intestinal fluid was determined in accordance with previously described methods,²⁶ with minor modifications. Pepsin and trypsin (0.25 U µg⁻¹) were selected, and the tests of the stability of TM (1:50 pepsin/trypsin to protein, w/w) were carried out at 37 °C with continuous rocking.

Phage display random peptide library and peptide synthesis

The Ph.D.-12 Phage Display Random Peptide Library was used to analyze antigenic epitopes according to the method of Liu *et al.*¹² Briefly, for first round of biopanning, overnight coating of 100 µL goat anti-human IgE (ε-chain specific) in enzyme-linked immune sorbent assay (ELISA) plates was used to enrich IgE from the serum of patients no. 5–9. The samples were then blocked with bovine sera albumin for 2 h and incubated with human IgE overnight at 4 °C. Overnight coating 250 µL (~2 × 10⁹) of the phage peptide library at 4 °C was then

carried out. After the specific binding phages were eluted and neutralized, *E. coli* ER2738 was infected with the bound phages for amplification. After the third-round mapping, the bound phages were titered, and the blue monoclonality was randomly selected from the LB-Tet plate. Then, the positive phage clones detected by ELISA were collected and sent to the company for sequencing.²⁷ The software ClustalW was used to align and identify the potential epitopes of TM. Finally, seven epitope peptides (Table 1) were synthesized and stored at –20 °C until use.

Inhibition ELISA

Inhibition ELISA was carried out to evaluate the IgE-binding activity of the epitope peptides according to the method of Fu *et al.*,²⁸ with some modifications. The peptide concentration (3 mg mL⁻¹ with 3 mg mL⁻¹ BSA) that had been determined to be the most appropriate was prepared, and (100 µL) of the serum pool (1:5 dilution) was used for the test, which was carried out for 18 h at 4 °C. A positive control (TM and serum pool), negative control (BSA and serum pool), and blank control (serum only) were also performed. The absorbance was measured at 450 nm using a Versa max Microplate reader (Molecular Devices, Sunnyvale, USA), and the inhibition rates were calculated as follows: $[1 - OD_{\text{inhibitor}}/OD_{\text{blank}}] \times 100\%$.

Alignment of TM amino acid sequences of different species

The amino acid sequence of TM in *C. nobilis* was obtained from the protein database of the NCBI (<https://www.ncbi.nlm.nih.gov/>) with Genbank accession number Q9GZ69. Multiple sequence alignment of the TMs from shellfish was carried out using ClustalW, and their sequences were also obtained from the NCBI.

Preparation of MR-TM

TM dissolved in 20 mM PBS was mixed individually with the reducing sugars Arab, Xyl, Glu, Man and Gal. The concentrations of the protein and the five reducing sugars were adjusted to 5 × 10⁻³ mM and 4 mM with heating at 100 °C for 60 min (pH 8.5), to explore the reducing sugar type, with some modifications.²⁹ The optimal conditions for the Maillard reaction were determined by the changes in the IgG-binding activity and cell viability analysis. The effect of MR-TM on RBL-2H3 cell viability was analyzed using Cell Counting Kit 8 (CCK-8).³⁰ RBL-2H3 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in

Table 1 Synthetic peptide information

Peptide no.	Sequence	Position	Length of sequence
P1	KMQAMKVDRENAQDL	7–21	15
P2	QDLAEQMEQKLKDE	19–33	15
P3	NNYDTVNEQLQE	55–66	12
P4	KQITQLESVDVGG	76–87	12
P5	ADESERNRKVLEGRS	120–134	15
P6	SNSYEERIDELEKQL	134–148	15
P7	KVLELEELTVVGA	189–202	14

an incubator with 5% CO₂ at 37 °C. All the samples were treated by dialysis in 20 mM PBS (pH 7.4) after reaction.

Identification of glycation sites

Glycation site testing was conducted according to the method of Zhao *et al.*,³¹ with some modifications. First, MR-TM was digested by trypsin to form peptides, which were separated according to hydrophobicity by high performance liquid chromatography (Eksigent Nano-LC 415 System) and then loaded onto a Q-Exactive mass spectrometer. The Q-Exactive mass spectrometer was used to collect and amplify the ion signal *via* their entering the ion detector according to the mass-charge ratio of ions of different masses at the same time, and then draw the mass spectrogram after computer processing. Finally, analysis of the original RAW file was performed using the software PEAKS.

Circular dichroism spectra and surface hydrophobicity analysis

According to the methods previously described by Zhang *et al.*,²² the secondary structures of TM and MR-TM were measured using a circular dichroism (CD) spectrophotometer (Applied Photophysics, Ltd, Surrey, UK). All samples were homogeneously mixed and adjusted to a concentration of 0.1 mg mL⁻¹. CD spectra were collected from 190 to 260 nm at a scanning rate of 100 nm min⁻¹ with a bandwidth of 1.0 nm.

ANS is a fluorescent hydrophobic probe that has been widely used to measure protein surface hydrophobicity.⁶ The surface hydrophobicities of TM and MR-TM were determined using ANS as the fluorescence probe. Briefly, 4 µL of 5 mM ANS was mixed with 200 µL of the samples and measured using an excitation wavelength of 375 nm; the emission wavelength was 400–600 nm.

Statistical analysis

Data from the studies are presented as the mean ± SD. Data were analyzed using the general linear model and Tukey test. Differences between groups were considered significant when $p < 0.05$.

Results

Purification and identification of the target protein from *C. nobilis*

The target protein was purified from *C. nobilis* striated adductor muscles using isoelectric precipitation and column chromatography on a Q-Sepharose column. The target protein was eluted at 0.3–0.5 M KCl and separated as a single band in SDS-PAGE analysis (Fig. 1A and B). The purified target protein band was excised from the SDS-PAGE gel, destained, and digested in-gel with trypsin. The resulting peptides were confirmed by LC/MS-MS, and the effective peaks were compared with the entire NCBI database using the Mascot search engine. The search for homologous proteins in the NCBI protein database found that the target protein covered 98% of the TM

sequence in *C. nobilis*. Hence, the target protein was determined to be TM in *C. nobilis* (Genbank accession number: Q9GZ69, Fig. 1C). Moreover, TM showed a positive reaction to the serum from scallop-sensitized patients and a negative reaction to that of nonallergic individuals by dot blotting (Fig. 1D). However, whether this sensitization is clinically relevant remains to be further explored. BAT was used to analyze the apparent correlation *via* the reactivity of the basophils from voluntary donors. Fig. 1E shows that in comparison to the NC, TM stimulation induced a significantly higher SI level of CD63 ($p < 0.01$) and CD203c basophils ($p < 0.05$) in the blood of the five allergic patients. These results indicated that TM was able to induce basophil activation *in vitro*, and that the serum can be used in future follow-up experiments.

Thermal, pH, and digestion stability analysis of TM

To further validate the purified proteins, a rabbit anti-*C. nobilis* TM IgG polyclonal antibody (1:10⁴) was used for immuno-blotting analysis. The protein presented obvious IgG-binding activity with the rabbit anti-*C. nobilis* TM polyclonal antibody (Fig. S1C†).

The thermal, pH and digestion stability of food allergens are also important factors affecting their allergenicity. In thermal stability experiments, TM was stable after exposure to various temperatures, even up to 100 °C (Fig. 2A), and the IgG-binding activities of TM were barely affected (Fig. 2C). As illustrated in Fig. 2B, the TM band did not change significantly in the pH range of 2.0–7.0 (acid condition), whereas under strong acid (pH 1.0) and strong alkali conditions (pH 10.0, 11.0), the band became diffuse and widened with a vague fringe, accompanied by the appearance of small fragments of degradation. Furthermore, the main TM band still retained strong IgG-binding activity (Fig. 2D).

In vitro digestion assays were performed to evaluate the digestibility of TM. The digestion patterns of TM by pepsin and trypsin were quite different. After digestion with pepsin, the molecular weight of TM determined by SDS-PAGE decreased slightly over time (Fig. 2E). Fig. 2G indicates that pepsin digestion had little effect on the IgG-binding activity of TM. After 1 min of digestion with trypsin, the main band of TM degraded rapidly into small fragments. As the digestion time was increased from 15 min to 120 min, TM main band completely disappeared, accompanied by the formation of lower-molecular-weight fragments below 18.4 kDa (Fig. 2F). When the time was extended from 180 to 240 min, the abundance of the degradation bands became increasingly lower. IgG-binding activity results revealed that compared with the control, TM digested with trypsin for 1–15 min was still highly reactive with rabbit-*C. nobilis* TM polyclonal antibody. However, with increasing digestion time (30–240 min), the IgG-binding activity of the digestion products became increasingly weak (Fig. 2H).

Biopanning and identification of TM IgE epitopes

Eighteen individual mimotopes were identified using enriched IgE from the serum of the five scallop-sensitized patients (Fig. S2†). The sequence alignment results showed that six of

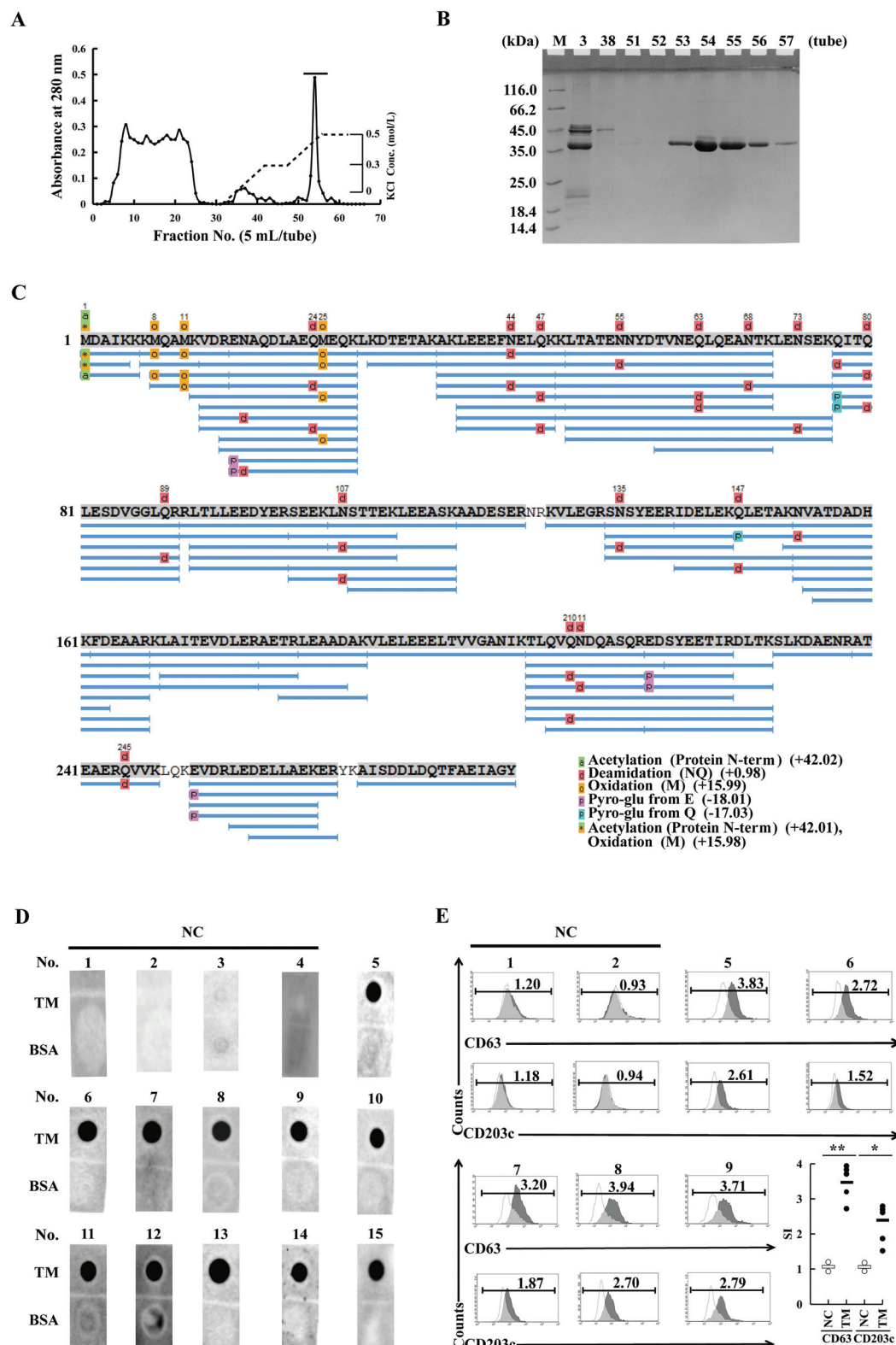


Fig. 1 Purification and identification of the target protein from *C. nobilis*. (A) Purification of the target protein via Q-Sepharose chromatography. (B) SDS-PAGE of Q-Sepharose chromatography; lane M, protein marker; the numbers 3, 38, 51, 52, 53, 54, 55, 56, and 57 at the tops of the lanes correspond to the fraction number. (C) Confirmation of the target protein using LC-MS/MS. Sequence alignment results of the mass spectrometry peptides matching TM (Genbank accession number: Q9GZ69) from *C. nobilis*. (D) IgE binding activity analysis of TM via dot blotting using serum from scallop-sensitized patients (1:5 dilution). (E) Basophil activation analysis of TM using whole blood from scallop-sensitized patients (no. 5–9). White and black represent cell populations stimulated by PBS and TM, respectively. NC represents two nonallergic individuals, and TM indicates scallop-sensitized patients. The horizontal lines represent the average SI values obtained by PBS or TM stimulation. **: $p < 0.01$ compared to NC.

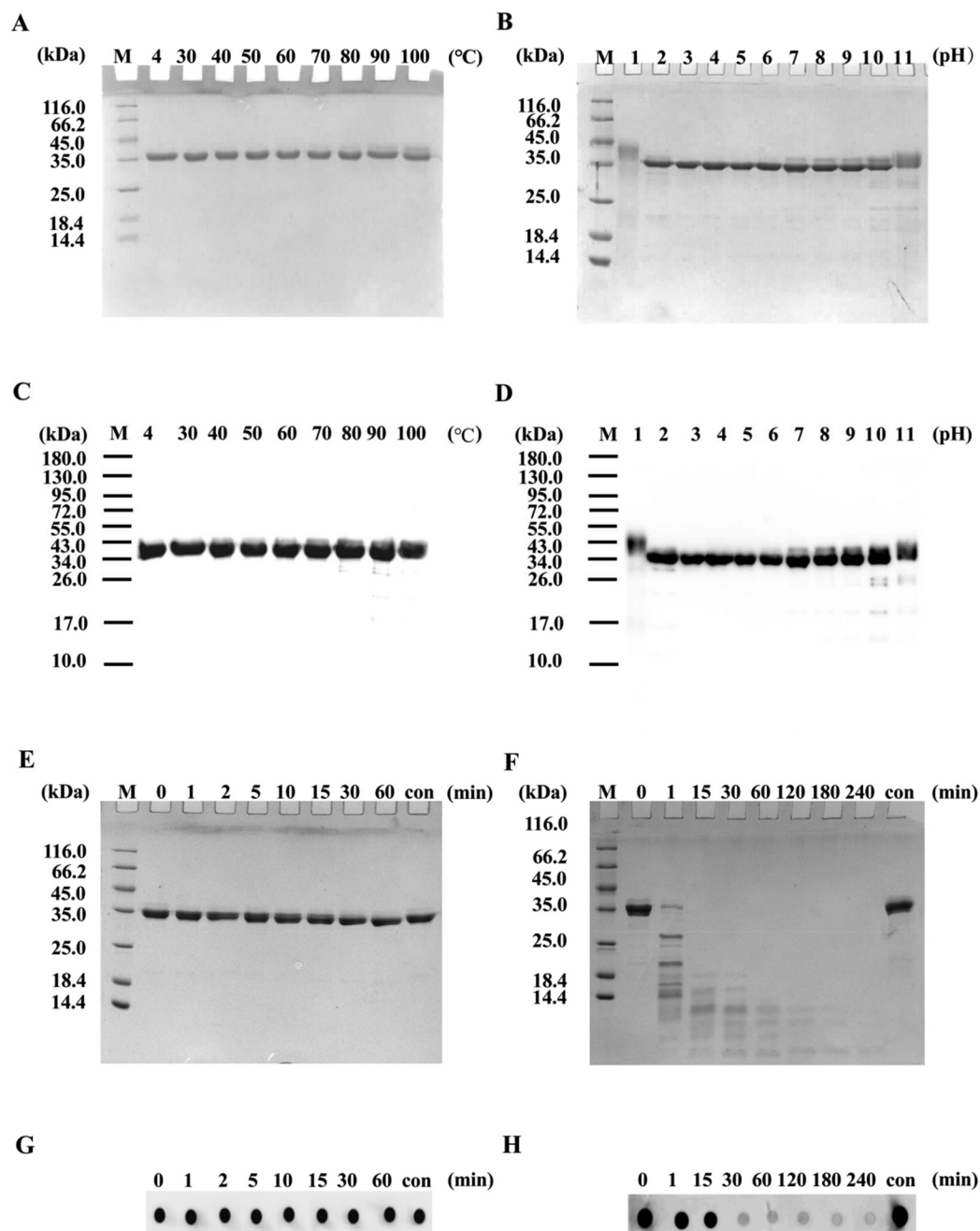


Fig. 2 Thermal, pH, and digestion stability analysis of TM (A and B) SDS-PAGE analysis of the thermal stability and the pH stability of TM, respectively; lane M, protein marker. (C and D) Western blot analysis of the thermal stability and the pH stability of TM, respectively; lane M, protein marker. (E and F) SDS-PAGE analysis of the pepsin digestion stability and the trypsin digestion stability of TM, respectively; lane M, protein marker. (G and H) Dot blotting analysis of the pepsin digestion stability and the trypsin digestion stability of TM, respectively.

the mimotopes matched a segment of more than three amino acids in the TM sequence (Fig. 3A). Among the six mimotopes, there were four repeated peptide sequences, including AMKVSSDPLQK (4 repeated), SENRKVPLKEL (3 repeated), GDASERGPFLHG (3 repeated), and SYLGLSGLVTGA (2 repeated). Other mimotopes were not mapped to the sequence of TM. Based on the alignment results, seven potential IgE epitope peptides were designed and synthesized (Table 1).

A homology model of *C. nobilis* TM was developed. TM from *Sus scrofa*, which is deposited in the Protein Data Bank as 1C1G, was selected as the template structure. The locations of the seven potential IgE epitopes, which were named P1–P7, were mapped on the TM structure (Fig. 3B). In an effort to determine which of the seven epitope peptides were immunodominant, inhibition ELISA was carried out based on the IgE competitive interaction between the epitope peptides and TM

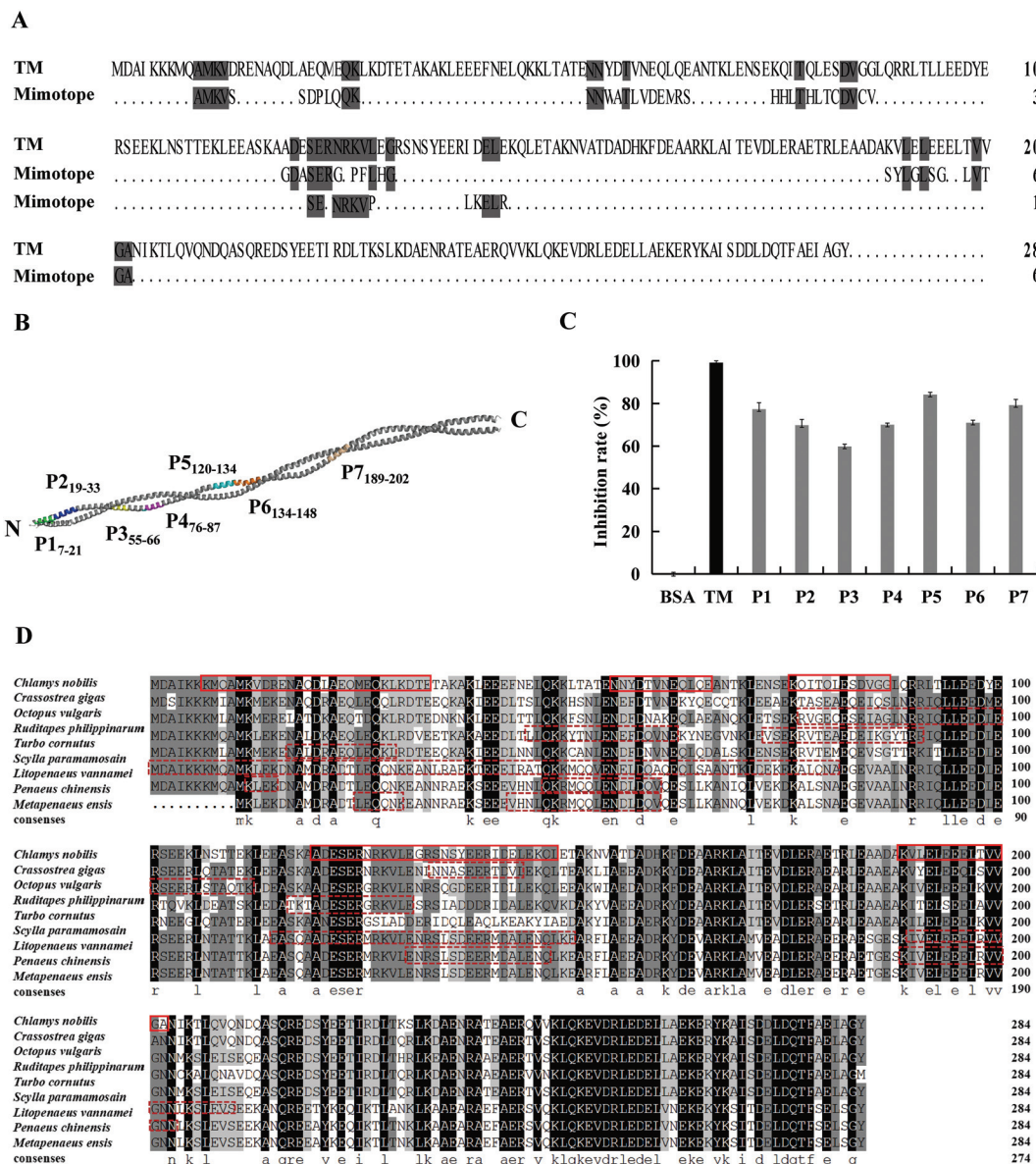


Fig. 3 Biopanning and identification of TM IgE epitopes. (A) Alignment of the TM amino acid sequence and six mimotopes derived from biopanning. Amino acid sequences common to TM and the mimotopes are marked in gray. (B) Position of the potential IgE linear epitopes on the 3D structure of TM. P1–P7 represent the seven potential IgE linear epitopes. (C) Validation of the seven potential IgE linear epitopes of TM based on inhibition ELISA. The IgE binding activity was determined with different epitope peptides using the serum pool (1:5 dilution); BSA, negative control. All data are presented as the mean $\pm$ SD ($n = 3$), while the different letters indicate significant differences ($p < 0.05$). (D) Sequence alignment of TM from different species. TM sequences are from nine species (Genbank accession numbers): *C. nobilis* (Q9GZ69), *C. gigas* (ARX70262.1), *O. vulgaris* (BAE54433), *R. philippinarum* (BAH10157), *T. cornutus* (BAH10149), *S. paramamosain* (MN218640.1), *L. vannamei* (XP_027229138), *P. chinensis* (ADA70137), and *M. ensis* (AAA60330). The solid red boxes represent the IgE-binding epitopes of *C. nobilis* TM, and the dotted red boxes represent the IgE epitopes of TM of other species.

using the serum pool (no. 5–9). As shown in Fig. 3C, compared with the positive control (BSA), the seven epitope peptides revealed significant inhibition effects ($p < 0.05$), in which the inhibition rates varied from 59.8 to 84.2%. Among them, P5 displayed the highest inhibition efficiency (84.2%), followed by P7 (79.3%). Although P3 had the lowest inhibition rate compared to the other six peptides, it could still inhibit the binding ability of TM to the sera of the patients with an inhi-

bition rate of 59.8%. Therefore, the seven epitope peptides demonstrated the potential to cause a sensitive reaction, suggesting that they were the primary epitopes.

C. nobilis TM is a highly conserved protein and contains 284 amino acid residues. In Fig. 3D, the solid red boxes represent IgE epitopes of *C. nobilis* TM. In recent years, there have been some new reports of the study of IgE epitopes of other shellfish TM (dotted red boxes), such as *Crassostrea gigas*,

Octopus vulgaris, *Ruditapes philippinarum*, *Turbo cornutus*, *Scylla paramamosain*, *Litopenaeus vannamei*, *Penaeus chinensis* and *Metapenaeus ensis*.

IgG-binding activity and cell viability analysis of TM after the Maillard reaction

To evaluate the effect of the Maillard reaction on the immunobinding activity of TM, the optimal Maillard reaction conditions were explored. TM and the reducing sugars Arab, Xyl, Glu, Man, and Gal were incubated at 100 °C for 1 h (pH 8.5), respectively. The SDS-PAGE profiles of TM and the various glycosylated monosaccharides are represented in Fig. 4A. The molecular weights of all the TM glycoconjugates (TM-Arab, TM-Xyl, TM-Glu, TM-Man and TM-Gal) were higher than that of TM. Fig. 4B shows that the IgG-binding activity of all the TM glycoconjugates (TM-Arab, TM-Xyl, TM-Glu, TM-Man and TM-Gal) was weaker compared to that of TM, and that TM-Xyl had the lowest binding activity. Therefore, in this study, Xyl (3 mM) was chosen to react with TM. 3.5×10^{-3} mM was selected as the most appropriate the concentration and could be used in future follow-up experiments. As shown in Fig. 4C, the optimized Maillard reaction conditions were heating at 100 °C for 60 min (pH 8.5) with Xyl. With further increasing the heating time, the IgG-binding activity of TM gradually decreases (Fig. 4D).

The cytotoxic effects of MR-TM on RBL-2H3 cells were assessed using a CCK-8 assay. In Fig. 4E, when the reaction time of MR-TM was 50 or 60 min, the cell viability decreased (approximately 75%) with increasing MR-TM concentration. However, when the reaction time of MR-TM was 30 or 40 min, the cell viability remained approximately 100%, even when the MR-TM concentration reached $10 \mu\text{g mL}^{-1}$. Thus, MR-TM with a reaction time of 30 min was chosen for further study.

Immunoreactivity, secondary structure, and surface hydrophobicity analysis of MR-TM

IgG/IgE-binding activity and BAT analyses were conducted to analyze the immunoreactivity of MR-TM. In Fig. 5A and B, the results revealed lower IgG/IgE-binding activity for MR-TM than TM using the rabbit-*C. nobilis* TM polyclonal antibody and scallop-sensitized patient serum pool, respectively. The BAT results demonstrated that MR-TM stimulation induced a significantly lower SI level of CD63 (average SI = 1.9, $p < 0.01$) and CD203c basophils (average SI = 1.4, $p < 0.05$) compared to TM (average SI of CD63 = 2.9, average SI of CD203c = 1.8) in six patients. In nonallergic individuals, no upregulation was seen after stimulation with TM. These results indicated that MR-TM could significantly reduce the activation of basophils compared with TM.

The secondary structures of MR-TM were analyzed using far-UV CD. It can be seen in Fig. 5D that TM exhibits a positive peak at about 190 nm and two negative peaks at 210 nm and 220 nm, showing typical features of α -helical proteins. However, the content of α -helices was significantly lower in MR-TM with transformations in the peak values, accompanied by flattening of the spectrum.

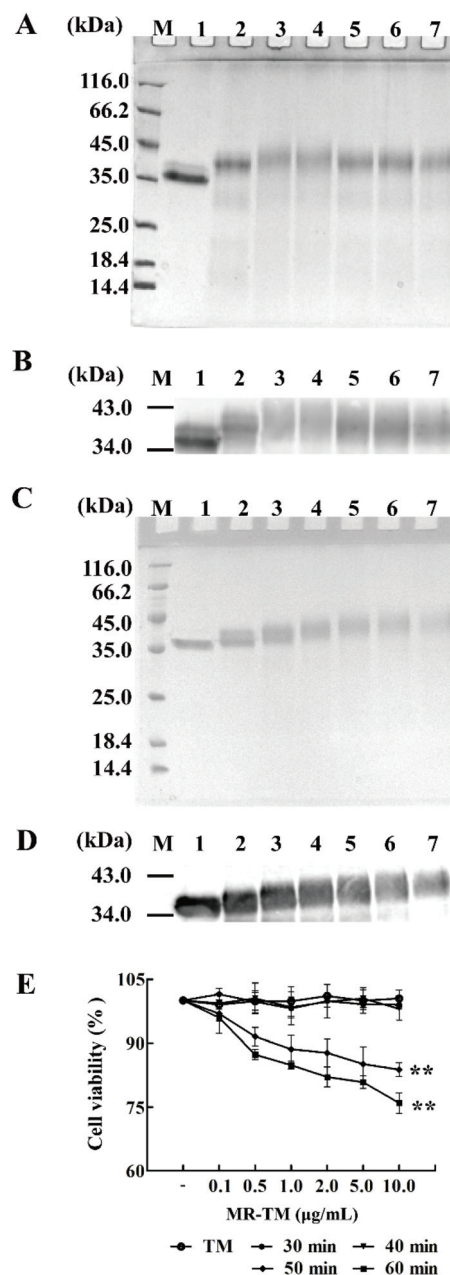


Fig. 4 IgG-binding activity and cell viability analysis of TM after Maillard reaction. (A) SDS-PAGE analysis of TM incubated with different reducing sugars in the Maillard reaction. Lanes 1–7: TM, TM (H): TM heated under the same conditions, TM-Arab, TM-Xyl, TM-Glu, TM-Man, and TM-Gal; lane M, protein marker. (B) Western blot analysis of TM incubated with different reducing sugars in the Maillard reaction. Lanes 1–7: TM, TM (H): TM heated under the same conditions, TM-Arab, TM-Xyl, TM-Glu, TM-Man, and TM-Gal; lane M, protein marker. (C) SDS-PAGE analysis of TM incubated with Xyl for different Maillard reaction times. Lanes 1–7: TM, 10 min, 20 min, 30 min, 40 min, 50 min, and 60 min; lane M, protein marker. (D) Western blot analysis of TM incubated with Xyl for different Maillard reaction times. Lanes 1–7: TM, 10 min, 20 min, 30 min, 40 min, 50 min, and 60 min; lane M, protein marker. (E) Cell viability analysis of MR-TM produced with different reaction times. The data represents the mean $\pm$ SD of three independent experiments.

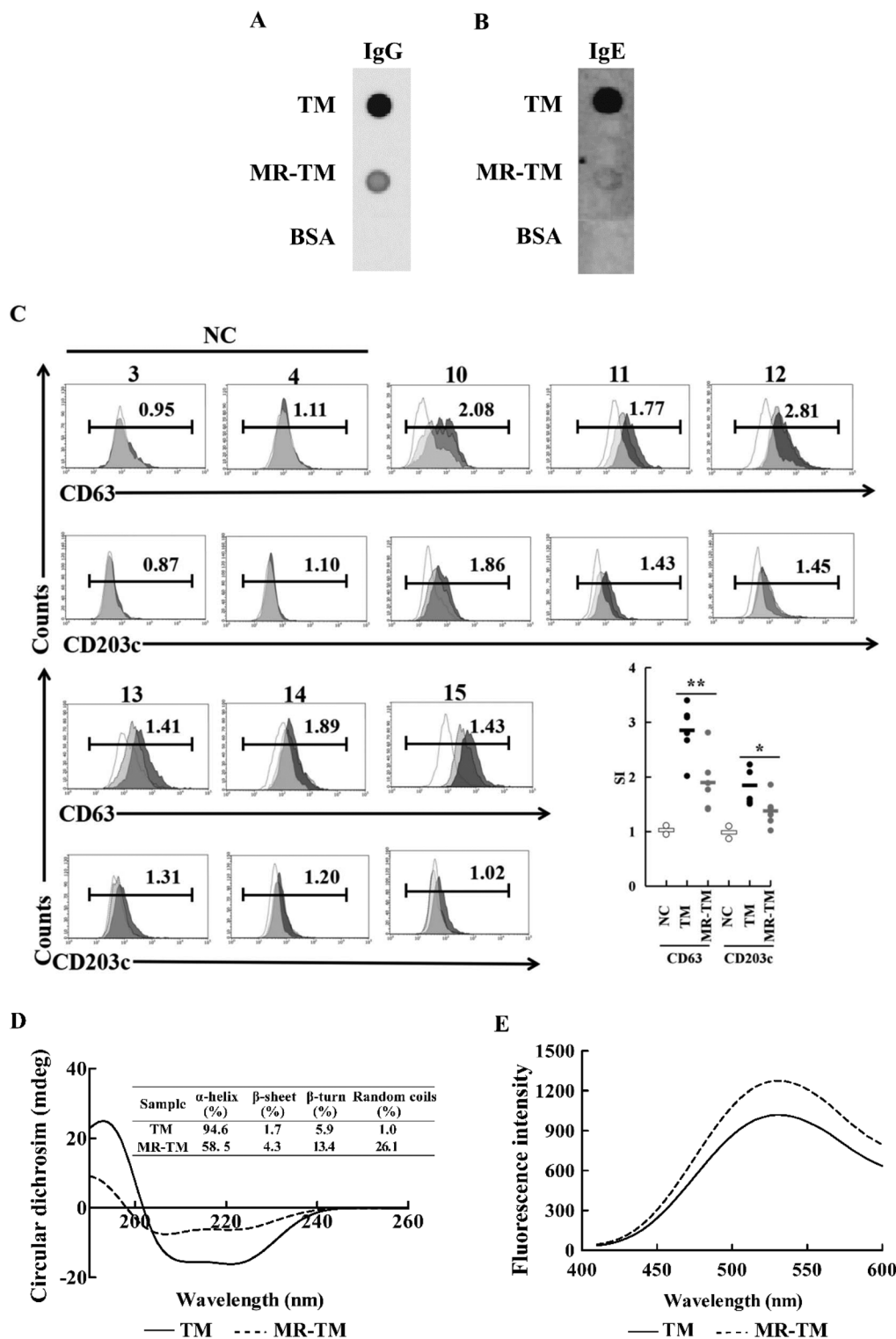


Fig. 5 Immunoactivity, secondary structure, and surface hydrophobicity analysis of MR-TM. (A) IgG-binding activity analysis of MR-TM. MR-TM was detected with rabbit anti-*C. nobilis* TM polyclonal antibody ($1:10^4$ dilution). (B) IgE-binding activity analysis of MR-TM. The serum pool of scallop-sensitized patients ($1:5$ dilution) was the primary antibody. (C) Basophil activation analysis of MR-TM using whole blood from scallop-sensitized patients (no. 10–15). White, black, and grey represent cell populations stimulated by PBS, TM, and MR-TM, respectively. NC represents two nonallergic individuals, and TM indicates scallop-sensitized patients. The horizontal lines represent the average values of SI by PBS, TM, and MR-TM stimulation. *: $p < 0.05$, **: $p < 0.01$ compared with NC. Numbers in the figure represent the SI of MR-TM. (D) Secondary structure analysis of MR-TM. (E) Surface hydrophobicity of MR-TM. The data represent the mean $\pm$ SD from determinations in triplicate.

Table 2 Identification of glycation sites on the seven IgE epitopes

Modified amino acids on IgE epitopes	Peptide sequence	MVobs (M + H)	Δ mass (Da)	Modification
K12, R15	MQAMKVDR	1071.48	94	Furfural
K28	ENAQDLAEQMEQK	1546.68	14	Methyl
K76	LENSEKQITQLESDVGGGLQR	2295.14	52	Acetaldehyde
R125, R127, K128	AADESENRK	1284.57	110	Pyrroline
R133	VLEGRSNSYEER	1505.68	68	Furan
R140	SNSYEERIDELEK	1668.74	72	Carboxyethyl
K146	IDELEKQLETAK	1467.75	52	Acetaldehyde
K189	LEAADAKVLEEEELTVVGANIK	2525.33	58	Carboxymethyl

Bold type represents the specific amino acids that were modified.

As shown in Fig. 5E, the fluorescence absorption peak of MR-TM was significantly different from that of TM. MR-TM exhibited enhanced absorption, indicating its higher surface hydrophobicity compared to TM. Therefore, the structure of MR-TM is significantly changed.

Modification of specific TM amino acids *via* the Maillard reaction

The modification of specific TM amino acids was analyzed after the Maillard reaction using shotgun proteomics. Shotgun proteomics analysis showed that lysine and arginine were glycosylated by one or more compounds (furfural, acetaldehyde, carboxymethyl, carboxyethyl, methyl, methoxy, furan, pyrroline). The glycation site identification results for the six IgE epitopes are shown in Table 2, and suggest the possible direct destruction or blockage of the IgE epitopes by the glycation. K189 was modified by carboxymethyl, and the molecular weight increased by 70 Da (Fig. 6A). The glycosylated amino acids (red spheres) were mapped to the corresponding IgE epitopes (Fig. 6B); however, P3 was not modified by glycation.

Discussion

The annual production of scallops has been increasing, and reached 180 000 tons in 2019.³² This food is widely loved for its rich nutrition and delicious taste; however, it is increasingly causing allergic phenomena.⁵

TM, a major allergen protein consisting of two α -subunits with a molecular weight range of 33–38 kDa, is a glycoprotein that is relatively stable to heat, acid and alkali. It may retain its immunoactivity following pepsin digestion, but is intolerant to trypsin digestion.³³ After digestion by trypsin, the main band of TM did not disappear even after 240 min of digestion, although it was accompanied by the appearance of three steady and small-molecular-weight fragments.²⁶ In this study, 36 kDa native TM was obtained from *C. nobilis*, and physico-chemical property analysis found that this TM was also resistant to heat, acid, alkali, and pepsin digestion, and had a strong response with the rabbit-*C. nobilis* TM polyclonal antibody. In contrast, trypsin digestion was very rapid and the main band was almost gone after 1 min; the small molecule fragments gradually disappeared as the digestion time was

increased. The trypsin digestion rate of TM from *C. nobilis* was significantly different from that of TM from other species (such as *Penaeus monodon* and *Penaeus vannamei*).²⁶ It is speculated that *C. nobilis* TM has more trypsin cleavage sites and these were exposed to the protein surface, thus accelerating the trypsin digestion.

The specific chemical groups in an antigen molecule that determine the specificity of the antigen are called an epitope. In this study, seven IgE epitopes were obtained, and all of them inhibited the binding of TM to patient sera to varying degrees. It was found that P1–P7 could overlap with the IgE epitopes of TM in eight shellfish species with high sequence homology, including *C. gigas* (73.94%), *O. vulgaris* (71.83%), *R. philippinarum* (68.66%), *T. cornutus* (68.31%), *S. paramamosain* (58.45%), *L. vannamei* (57.75%), *P. chinensis* (57.75%) and *M. ensis* (54.23%).^{34–40} Vidal *et al.*⁴¹ discovered that the sensitization pattern of crustacean-allergic individuals can indicate allergy to molluscs. This is the primary reason for clinical and immunological cross-reactivity due to the presence of shared IgE epitopes and high sequence homology between *C. nobilis* TM and TM from other species.⁴² Therefore, the IgE epitopes of *C. nobilis* TM could play an important role in creating intact data for the high frequency of cross-reactivity during routine allergy diagnosis.

The Maillard reaction, which takes place between the carbonyl groups of a reducing sugar and the free amine groups of amino acids, could influence the immunoreactivity of food proteins by changing the protein structure and modifying the free amino groups in allergens.²⁹ In this study, the MR-TM formed by the reaction of TM and Xyl showed changes in secondary structure and surface hydrophobicity. The changes in the TM protein structure may have resulted in decreased immunoreactivity due to the exposure of more hydrophobic regions, which made TM less likely to bind to the IgE antibody. Moreover, the formation of high-molecular-weight aggregates during the Maillard reaction help to mask the epitopes. TM was reacted with various reducing sugars under the same conditions, resulting in different reductions in its IgG-binding activity. The reactivity of the five-carbon sugars (Arab, Xyl) in the Maillard reactions was faster than that of six-carbon sugars (Glu, Man, Gal), which might be because the saccharides with a smaller molecular size (Arab, Xyl) had less steric hindrance and greater accessibility to react with TM. The Maillard reac-

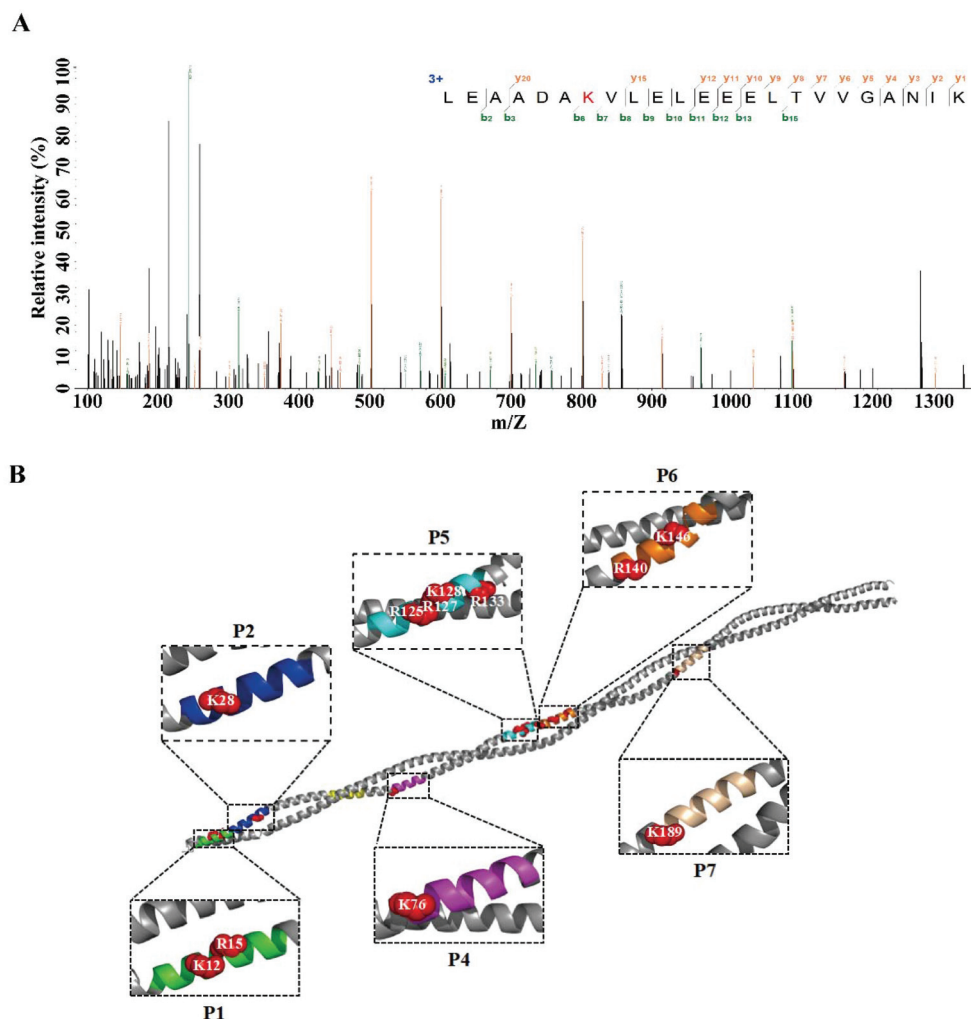


Fig. 6 Analysis of glycation sites. (A) Identification of the specific amino acids of TM modified by the Maillard reaction using shotgun proteomics. (B) Localization of the specific modified amino acids on the TM IgE epitopes; green: P1, blue: P2, yellow: P3, magenta: P4, cyan: P5, orange: P6, and wheat: P7. The specific amino acids are represented by red spheres.

tion dramatically consumes free amino acids (such as lysine, arginine, and cysteine), minerals and other nutrients in some food.²⁹ In the present study, to avoid unnecessary massive loss of nutrients, 30 min was chosen as the optimal reaction time for the Maillard reaction. The Maillard reaction masks lysine and arginine on the IgE-binding epitope of TM in *S. paramamosain*, which reduces the sensitization of TM.²⁹ Some of the lysines located on IgE epitopes of silver carp recombinant parvalbumin are modified by glycation, which decreases its immunoreactivity.³² Shotgun proteomics analysis showed eleven glycation sites (K12, R15, K28, K76, R125, R127, K128, R133, R140, K146, K189) located on the six IgE epitopes of TM, which is the main reason for the reduced TM immunoreactivity.

In summary, TM was identified to be a major allergen in *C. nobilis* with strong immunoactivity. Furthermore, seven IgE epitopes were obtained using a phage display random peptide library, and their sequences were highly conserved in different

shellfish. The glycation sites after the reaction of TM with Xyl for 30 min were identified using shotgun proteomics, and the results indicated that specific amino acids on the IgE epitopes were modified *via* glycation. As a consequence, TM epitopes with lysine and arginine could be destroyed and masked by saccharide residues on the TM surface to reduce their affinity for IgE binding, resulting in lower immunoreactivity. This work might be useful for understanding the effects of the Maillard reaction on the epitopes and immunoreactivity of shellfish allergens for the control of shellfish allergy.

Author contributions

Tian-Liang Bai: Conceptualization, methodology, validation, investigation, formal analysis, writing – original draft, visualization. Xin-Yu Han: Investigation, data curation. Meng-si Li: Conceptualization, methodology. Yang Yang: Methodology. Meng Liu: Methodology. Nai-Ru Ji: Investigation. Chen-Chen Yu: Investigation. Dong Lai: Resources. Min-jie Cao:

Supervision, writing – review & editing. Guangming Liu: Writing – review & editing, supervision, project administration, funding acquisition.

Abbreviations

Arab	Arabinose
BAT	Basophil activate test
BSA	Bovine sera albumin
CD	Circular dichroism
ELISA	Enzyme-linked immune sorbent assay
Gal	Galactose
Glu	Glucose
IgE	Immunoglobulin E
IgG	Immunoglobulin G
MFI	Mean fluorescence intensity
Man	Mannose
MR-TM	Glycation of tropomyosin with xylose
NC	Negative control
PBS	Phosphate buffered saline
SI	Stimulation index
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TM	Tropomyosin
Xyl	Xylose

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by grants from the National Key R & D Program of China (2019YFD0901703).

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Online Supplementary Material

Supplementary Methods

Preparation of rabbit anti-*C. nobilis* TM polyclonal antibodies

The specific polyclonal antibody was prepared by subcutaneously injecting of the *C. nobilis* TM in an adult female New Zealand white rabbit by immunizing with purified protein at Xiamen University (Xiamen, Fujian, China), the specific polyclonal antibody was similar with the previous study.¹ Determination of the rabbit anti-*C. nobilis* TM polyclonal antibody titer was shown in Figure S1A.

1. T. J. Han, M. Liu, F. Huan, M. S. Li, F. Xia, Y. Y. Chen, G. X. Chen, M. J. Cao and G. M. Liu, Identification and cross-reactivity analysis of sarcoplasmic-calcium-binding protein: a novel allergen in *Crassostrea angulata*, *J. Agr. Food Chem.*, 2020, **68**, 5221-5231.

Table S1. Specific IgE levels and symptoms of the scallop-sensitized individuals

Serum No.	Age (years)	Sex ^a	Scallop specific IgE (kUA/L) ^b	Symptoms
1 ^c	10	M	0.1	— ^d
2 ^c	28	F	0.3	— ^d
3 ^c	53	M	0.1	— ^d
4 ^c	5	F	0.2	— ^d
5	15	M	13.6	atopic dermatitis
6	40	F	15.8	allergic purpura
7	58	M	11.4	urticaria
8	25	F	14.6	cough
9	6	M	18.4	anaphylactic rhinitis
10	11	F	11.8	cough
11	25	F	8.4	edema
12	43	M	7.4	edema
13	32	F	5.4	bronchitis
14	12	F	8.7	cough
15	22	F	6.2	urticaria

^aM, male; F, female. ^bA sera with specific IgE ≥ 0.35 kUA/L is defined as positive. ^cThe person was nonallergic individual. ^dMeans no symptoms when the experiment.

Table S2. Maillard reaction modification of eleven specific amino acids on seven IgE epitopes.

Modified amino acids on IgE epitopes	Modification
K12	Furfural
R15	Acetaldehyde, Carboxymethyl, Carboxyethyl
K28	Methyl, Methoxy, Furfural
K76	Acetaldehyde
R125	Acetaldehyde, Furfural, Furan, Carboxyethyl, Pyrroline
R127	Acetaldehyde, Furfural, Furan, Carboxyethyl, Pyrroline
K128	Acetaldehyde, Furan, Carboxyethyl, Pyrroline
R133	Furan, Carboxymethyl, Carboxyethyl
R140	Methyl, Carboxymethyl, Carboxyethyl
K146	Acetaldehyde
K189	Acetaldehyde, Carboxymethyl, Carboxyethyl, Pyrroline

Supplementary Figure Legends

Figure S1. Preparation and verification of rabbit anti-*C. nobilis* TM polyclonal antibodies

(A) Determination of the rabbit anti-*C. nobilis* TM polyclonal antibody titer.

Dot blot analysis of the rabbit anti-*C. nobilis* TM polyclonal antibody titers, 1: 100-1: 2×10^5 is the polyclonal antibody dilution.

(B) SDS-PAGE analysis verification of TM. Lane M: protein marker; Lane 1-2, TM, BSA.

(C) Western blot verification of TM. (primary antibody: specific TM-polyclonal antibodies, dilution 1: 1×10^4). Lane M: protein marker; Lane 1-2, TM, BSA.

Figure S1

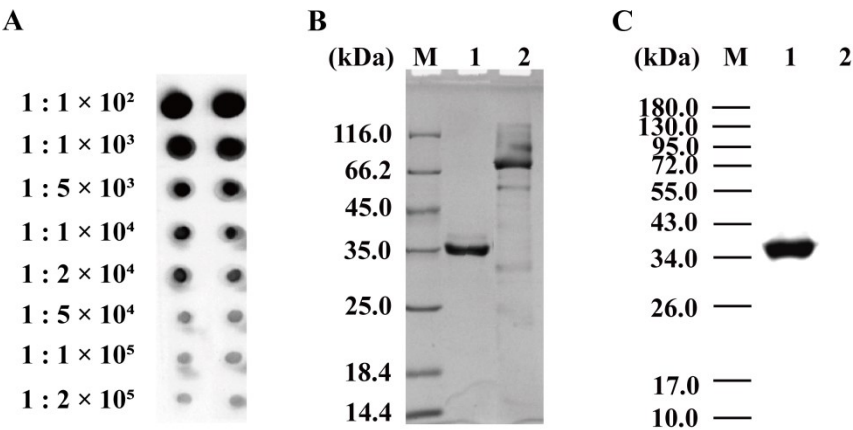


Figure S2. Eighteen individual mimotopes were identified by Phage display random peptide library.

Figure S2

---LQWVPAPNSFNI
---LRWVPAINSENI
SIPLTVVPTANA---
--KLLRVVNQNPIQ-
MPQVNLFPFKRT---
-SEN RKVPLKELR-
---NNWATLVDEMRS
-GLINSAPT KIMR-
-HTTASLATKTTI-
-LATNYHKS KIR-
-SYIGISGLVTGA-
-GQVYAGSSPFAK-
AMKVSSDPIQQK---
---TFRQPGFTKVST
-PFHDFQLISTHE-
ASHTGTAQISRQ---
GDASERGPFLHG---
-HHLTHLTCDVCV-