

Crystal Structure Analysis and Conformational Epitope Mutation of Triosephosphate Isomerase, a Mud Crab Allergen

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Supporting Information

ABSTRACT: The triosephosphate isomerase (TIM), Scy p 8, is a crab allergen and shows cross-reactivity in the shellfish. Here, recombinant Scy p 8 was expressed, and its crystal structure was determined at a resolution of 1.8 Å. The three-dimensional structure of Scy p 8 is primarily composed of a $(\beta/\alpha)_8$ -barrel motif prototype. Additionally, Scy p 8 showed cross-reactivity with high sequential and secondary structural identity among TIMs from shellfish species. The site-directed mutagenesis of critical amino acids of conformational epitopes was carried out, and the mutants of Trp 168 and Lys 237 to Ala reduced immunoglobulin E (IgE)-binding activity by approximately 30%, compared with wild-type TIM in an inhibition ELISA; however, it still induced basophil activation despite the interpatient variability between patients. These results can help to provide an accurate template for the analysis of the IgE binding and establish meaningful relationships between structure and allergenicity.

KEYWORDS: crab allergen, cross-reactivity, crystal structure, site directed mutagenesis, triosephosphate isomerase

INTRODUCTION

Food allergy is a major issue related to food safety and has aroused concern throughout the world. It has been estimated that the prevalence of food allergy has increased over the past 2 to 3 decades, and primary food allergies include peanut, tree nuts, fish, shellfish, egg, milk, wheat, soy, and seed allergies.^{1,2} Moreover, the worldwide prevalence of shellfish allergy is increasing in countries with high seafood consumption (e.g., Australia, Asia, and parts of Europe), and the incidence rate in the Asia Pacific region is higher.^{3,4}

Several shellfish allergens have been identified and characterized, among which tropomyosin and arginine kinase are considered to be pan-allergens and have high immunoglobulin E (IgE)-mediated cross-reactivity with other invertebrates.⁵ Several other allergenic components, including the myosin light chain, sarcoplasmic calcium-binding protein, filamin C, and triosephosphate isomerase (TIM), have also been identified. TIM is a key enzyme involved in glycolysis, which is essential for efficient energy production.⁶ TIM in North Sea shrimp (*Crangon crangon*, Cra c 8) was the first identified allergenic crustacean TIM, having approximately 20% sensitization frequency.⁷ We also isolated and characterized the TIM allergens from Octopus (*Octopus fangsiao*),⁸ red swamp crayfish (*Procambarus clarkia*, Pro c 8),⁹ and mud crab (*Scylla paramamosain*)¹⁰ in previous studies. Meanwhile, TIM in *S. paramamosain* was named Scy p 8 by the official database of allergen nomenclature that is approved by the World Health Organization and International Union of

Immunological Societies (WHO/IUIS) Allergen Nomenclature Subcommittee.¹⁰ In addition, TIM has been identified to be an allergen present in several other food and nonfood sources, including prawns, cockroach, dust mites, and wheat flour.^{11–14}

The structural features of allergens can provide important insight into the biological functions of allergens that may affect their ability to induce an IgE response and increase our understanding of protein allergenicity.¹⁵ Thus, the structural biology of allergens provides the foundation for IgE epitope mapping and the rational design of allergens with reduced IgE-binding activity.¹⁶ The coiled-coil structure imparts structural stability to tropomyosin, which can withstand both heat and high-pressure processing and retain its allergenicity.¹⁴ The structure of arginine kinase has also been documented.¹⁷ The first structural characterized TIM in crustacean was the TIM from *Litopenaeus vannamei* (PDB entry SEYW).¹⁸ Compared to the in-depth investigation of the structure–allergenicity relationship of tropomyosin and arginine kinase, the physicochemical properties and epitopes of TIM have been investigated, but the structure and allergenic activity of TIM remain unknown. Thus, to gain a better understanding of the

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biochemical and structural basis of TIM allergenicity, the three-dimensional structure should be elucidated.

There is a wide-range of crab farming and consumption in China, which has both economical and nutritional importance; however, the crab allergy cannot be ignored. *S. paramamosain* TIM exhibited cross-reactivity with different crustacean species,⁸ amplifying the concern and risk. When the sequence comparison of allergenic proteins displays a higher sequence identity or a similar three-dimensional fold of the protein, immunological cross-reactivity likely exists. Moreover, the cross-reactivity between the 2S albumin, Ara h 2, and the cupins, Ara h 1 and 3, indicates that it is based on the presence of short similar structural motifs.¹⁹ Maleki et al.²⁰ examined the IgE cross-reactivity between walnut and peanut allergens from known and predicted peanut allergen epitopes. However, little is known about the relationship between the structural features and epitopes that cause cross-reactivity in TIM from different species. Therefore, investigating the mechanism of TIM cross-reactivity based on the relationship between the structure and epitopes is of critical importance.

Conformational epitopes are composed of amino acids that are not in a linear sequence but are brought together by the folding of the amino acid chain, arranged by the tertiary structure of the allergen.²¹ Comparing the structure and epitopes of allergens will provide basic insight into the creation of modified allergens and improve the safety of immunotherapy. At present, immunotherapy for food allergy takes the form of allergens based on epitope mutations by destroying the conformational IgE epitopes; in this manner, the recombinant allergen leads to a reduction of IgE-binding and decreased allergenic potential.²² Reese et al.²³ performed the first site-directed mutations on the major shrimp allergen Pen a 1 (tropomyosin) with regard to reduced allergenic potency. In our previous studies, both the conformational and linear epitopes of TIM in *S. paramamosain* have been well studied;¹⁰ however, the use of site-directed mutagenesis to generate hypoallergenic mutations based on TIM conformational epitopes requires further investigation.

In this context, we aimed to obtain the crystal structure of TIM and explored the structure-based cross-reactivity between different species. Moreover, mutations on conformational epitopes were performed to obtain low-immunobinding activity mutants. To this end, wild-type TIM (wtTIM) from *S. paramamosain* was expressed, purified, and crystallized. TIM structures of different species were compared, and the allergenic epitopes of TIM from *S. paramamosain* were mapped to discuss the cross-reactivity between the structure and epitopes. Furthermore, TIM mutations based on the key amino acids of the conformational IgE epitopes were generated, and the IgE-binding activity was measured using serum from crab-sensitive individuals.

MATERIALS AND METHODS

Chemicals. The Ni²⁺-NTA chromatographic column was purchased from Transgen Biotech (Beijing, China). The rabbit anticrayfish TIM polyclonal antibody was prepared in our laboratory as previously described,⁹ and a goat antirabbit IgG-HRP antibody was purchased from Abmart (Berkeley Heights, NJ, USA). All other reagents were of analytical grade.

Patient Sera. Sera from 12 crab-sensitive patients were provided by Xiamen Maternity and Child Health Care Hospital (human ethical approval no. KY-2018-018 and no. KY-2019-014). The diagnosis of crab allergy was based on a combination of clinical history of anaphylactic reactions, positive sIgE levels to crab extracts. Crab-

specific sIgE (Table 1) was quantified in vitro using an ImmunoCAP (Phadia AB, Uppsala, Sweden). Levels lower than 0.35 kU_A/L were

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

serum ID	age (years)	sex ^a	crab specific IgE (kU/L) ^b	symptoms
P1	27	F	>100.0	^c
P2	41	M	32.5	chronic urticaria
P3	32	F	56.4	asthma and chronic urticaria
P4	52	F	77.9	^c
P5	11	M	44.3	nausea, dyspnea and diarrhea
P6	7	F	>100.0	nausea and emesis
P7	38	M	>100.0	allergic eczema and itchy skin
P8	27	M	56.0	acute urticaria
P9	34	F	33.4	acute urticaria
P10	36	F	86.5	allergic eczema
P11	35	F	83.4	asthma and dyspnea
P12	31	F	53.0	^c
C1	28	F	0.1	^c
C2	25	M	0.2	^c

^aM, male; F, female. ^bA serum with specific IgE < 0.35 kU_A/L is defined as negative. ^cMeans no symptoms when the experiment. C1/C2 represents that the person was nonallergic individual.

considered negative. Adult patients and the parents of minors provided signed informed consent. All sera were stored at −80 °C until analysis.

Expression and Purification of Allergens. The mature protein-coding sequence of wild-type TIM (wtTIM) (GenBank accession number no. APP94292.1) encodes 248 amino acids and was inserted in the pET-28a expression vector between the NdeI and SalI sites to generate a recombinant plasmid and transformed into *E. coli* BL21 (DE3) competent cells. The wtTIM clones were grown in Luria–Bertani liquid medium until the OD₆₀₀ reached 0.6–0.8 and induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM at 37 °C for 4 h. The cells were harvested by centrifugation at 4 °C at 10 000g for 10 min. The pellet was resuspended in buffer A (20 mM Tris-HCl, pH 8.0, 200 mM NaCl) and subjected to obtain supernatant by centrifugation (12 000g, for 30 min). The filtered supernatant was bound to the Ni²⁺-NTA chromatographic column, washed with binding buffer containing 10 mM imidazole, and eluted with a buffer containing 100 mM imidazole to obtain wtTIM. Mutant constructs were generated using a two-step PCR procedure to replace the codons by the overlapping extension PCR method with specific primers (Table S1) containing W168A, T172A, Q180A, and K237A. The variants were expressed and purified as described above for wtTIM with slight modifications. The mutant TIM clones were grown at 37 °C in LB medium until the OD₆₀₀ reached 0.6–0.8 and induced by adding IPTG at a final concentration of 1 mM at 16 °C for 15 h and then centrifuged. The purified proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250. Molecular mass analysis was performed with AlphaView 3.0 software (AlphaInnotech, San Leandro, CA, USA). Western blot analysis was performed as described by Chen et al.,²⁴ with some modifications. The protein samples separated with SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane followed by blocking with 5% skim milk in TBST (20 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.05% Tween-20) at room temperature for 1.5 h. After it was washed with TBST, they were then incubated with the anticrayfish TIM polyclonal antibody as the primary antibody and washed with TBST. The membrane was then incubated for 1.5 h with

goat antirabbit IgG-HRP antibody (diluted 1:20 000) as the secondary antibody. After being extensively washed with TBST, the proteins were visualized by enhanced chemiluminescence.

Crystallization and Structural Determination of wtTIM. The wtTIM was concentrated in 20 mM Tris-HCl to a concentration of 30 mg/mL, and crystallization experiments were performed at room temperature using the hanging-drop vapor diffusion method. The selected conditions were further optimized, and the proteins were obtained at room temperature by mixing 1 μ L of the protein solution with 1 μ L of the reservoir consisting of 22% (w/v) PEG 6000, 0.1 M citric acid (pH 4.6). Glycerol was used as a cryoprotectant during rapid cooling in liquid nitrogen to protect the single crystal. X-ray diffraction data were collected at Shanghai Synchrotron Radiation Facility beamline BL17U or BL19U1 and were scaled and processed with HKL2000²⁵ and XDS.²⁶ The structure was solved by molecular replacement using the TIM of *L. vannamei* (PDB entry 5EYW) as a model in PHENIX.²⁷ Structural refinement was performed with CCP4i²⁸ and Coot.²⁹ Data collection statistics and refinement statistics are summarized in Table 2. Molecular graphic images were created using PyMOL software (<http://www.pymol.org/>).

Table 2. X-ray Data Collection and Refinement Parameters for wtTIM

data collection	
space group	P3 ₁ 21
unit cell (<i>a</i> , <i>b</i> , <i>c</i>) (Å)	154.712, 154.712, 52.713
(α , β , γ) (deg)	90, 90, 120
resolution (Å) ^a	50–1.80 (1.83–1.80)
no. of reflections (total/unique)	660 169/66 802
redundancy ^a	9.9 (8.8)
completeness (%) ^a	100.0 (100.0)
<i>I</i> / σ (<i>I</i>) ^a	20.1 (2.2)
<i>R</i> _{meas} ^{a,b}	0.060 (1.017)
CC(1/2) ^a	N/A (0.827)
refinement	
resolution (Å)	50–1.80
no. of protein atoms	3738
B-factor of protein atoms	24.26
no. of solvent/hetero-atoms	328
B-factor of solvent/hetero-atoms	38.58
rmsd bond lengths (Å)	0.006
rmsd bond angles (deg)	0.773
<i>R</i> _{work} (%) ^c	15.62
<i>R</i> _{free} (%) ^d	17.91
Wilson B-factor	23.05
Ramachandran plot (favored/disallowed) ^e	96.71/0
PDB code	6JOX

^aNumbers with asterisks correspond to the last resolution shell. ^b $R_{\text{meas}} = \sum_i (n_i/n - 1)^{1/2} \sum_j |I_i(h) - \langle I(h) \rangle| / \sum_j I_i(h)$, where $I_i(h)$ and $\langle I(h) \rangle$ are the *i*th and the mean measurement, respectively, of the intensity of reflection *h*. ^c $R_{\text{work}} = \sum_h |F_{\text{obs}}(h) - |F_{\text{calc}}(h)|| / \sum_h |F_{\text{obs}}(h)|$, where $F_{\text{obs}}(h)$ and $F_{\text{calc}}(h)$ are the observed and calculated structure factors, respectively. No *I*/ σ cutoff was applied. ^d*R*_{free} is the *R* value obtained for a test set of reflections consisting of a randomly selected 10% subset of the data set excluded from refinement. ^eValues from the Molprobity server at <http://molprobity.biochem.duke.edu/>.

Structural Comparison among Shellfish TIMs. The surface conservation was calculated using the ConSurf server.³⁰ The protein surface color-coded according to sequence conservation was drawn in PyMol. The amino acid sequences of the TIMs were aligned via CLUSTALW,³¹ and the structure-based sequence alignment was completed by ESPrnt 3.0.³² To confirm immunologic cross-reactivity, different crude extracts of *S. paramamosain*, *L. vannamei*, *Penaeus monodon*, *C. crangon*, and *P. clarkia* were prepared. The crude extracts

were purified according to the method of Chen et al.²⁴ with some modifications. The fresh muscle was minced, homogenized, and centrifuged. The supernatant was precipitated with 40% ammonium sulfate fractionation and then centrifuged. The supernatant was termed myosinogen protein. The IgG-binding activity of the crude extracts was analyzed by Western blot using rabbit anticrab TIM pAb. A detailed description of the rabbit anticrab TIM pAb preparation has been presented in the Supporting Information methods.

The tertiary structure superposition and the *Scylla paramamosain* TIM epitope mapping was displayed with PyMOL. The electrostatic potential of the protein surface was calculated by APBS.

Immunoreactivity Analysis of Mutant TIMs. The specific IgE-binding activity of wtTIM and mutant TIMs was identified by dot blot and an inhibition ELISA. The wtTIM and mutant TIMs (1 mg/mL) were spotted on nitrocellulose membranes. Crab-sensitive patient serum (1:5 dilution) was spotted on the membranes as the primary antibody, and horseradish peroxidase-labeled goat antihuman IgE antibody (1:2000 dilution) was used as the secondary antibody. Quantification of the dots was performed using public-domain ImageJ software (<http://rsb.info.nih.gov/ij/>) and presented as a scatter diagram.

The inhibition ELISA was performed with the crab-sensitive patient serum pool (Table 1). The wtTIM (200 ng/well) was coated onto a 96-well ELISA plate, mutant TIMs (200 ng/well) were used as inhibitors, preincubated with serum pool (1:5 dilution) which mixed serum of numbers P1–P12 in Table 1, equal to 500 μ L/tube for 2 h at 37 °C, and were added to the coated plate incubated for 2 h at 37 °C. IgE-binding activity of different proteins was shown by the percentage of inhibition based on the following formula: $[1 - (I_e - I_n)/(I_p - I_n)] \times 100\%$ of which *I*_e, *I*_n, and *I*_p represent the experimental, negative control, and positive control groups, respectively.

CD Spectropolarimetry Analysis. The secondary structure and heating curve of wtTIM and mutant TIMs were examined by circular dichroism (CD) (Applied Photophysics Ltd., Surrey, UK) at 25 °C with the protein concentration adjusted to 0.29 mg/mL in 20 mM phosphate-buffered saline (PBS) as previously described.⁹

Basophil Activation Test. Basophil activation test (BAT) was performed with blood from crab-sensitive patients (patients P2, P4, P7, P8, P10, P11) and two nonallergic patient controls, as described previously.³³ The blood samples were stimulated with 50 μ g/mL wtTIM, mutant TIMs, or PBS for 30 min at 37 °C and washed with stain buffer. Leucocytes were stained with an antibody cocktail of HLA-DR-APC, CD63-FITC, and CD203c-PE (BioLegend, San Diego, CA, USA). After staining, red cells were lysed by erythrocyte lysis solution (BioLegend) for 30 min in the dark at 4 °C before being centrifuged. Subsequently, samples were washed twice with 3 mL of stain buffer (PBS containing 1% fetal bovine serum) and resuspended with 300 μ L of the same solution. Samples were analyzed using flow cytometry on a Guava instrument (Millipore, Massachusetts). Allergen-induced CD203c and CD63 up-regulation was calculated using the mean fluorescence intensities (MFIs) obtained with stimulated (MFIstim) and unstimulated (MFIcon) cells and expressed as the stimulation index (SI), defined as MFIstim/MFIcon.

Statistical Analysis. Data from the experiments were presented as the mean $\pm$ SD. Data were analyzed using a general linear model and a Duncan ANOVA test. Differences between the groups were considered significant when *p* values were <0.05.

RESULTS

Purification and Immunoreactivity Analysis of wtTIM. wtTIM was purified using Ni²⁺-NTA chromatography, and the purification results were demonstrated by SDS-PAGE (Figure 1A). After eluting with 100 mM imidazole, a single protein band which contained 6 His-tag after number 29 fraction of approximately 28 kDa was detected and concentrated in a 3 kDa ultrafiltration centrifuge tube for subsequent analyses. To further validate the purified proteins, a

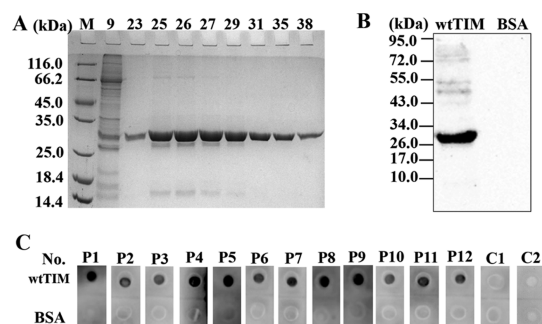


Figure 1. Purification and identification of *S. paramamosain* wtTIM. (A) SDS-PAGE analysis of wtTIM. Lane M, protein marker. Lane 9, 23, 25, 26, 27, 29, 31, 35, 38, the elution fraction number by the Ni^{2+} -NTA chromatographic column. (B) Western blot verification of wtTIM by rabbit anticrayfish TIM IgG pAb. (C) IgE-binding activity of wtTIM. Nitrocellulose membrane-dotted with wtTIM and BSA were tested with the sera from crab-sensitive patients (sera P1–P12). For control purposes, the sera from nonsensitive individuals (sera C1, C2) were included.

rabbit anticrayfish TIM IgG polyclonal antibody ($1:10^5$) and crab patient's serum (Figure 1B,C) were used in the immunoblotting analysis. Strong reactivity with the anti-TIM pAb and serum was observed, which revealed that the purified 28 kDa protein was *S. paramamosain* TIM.

High-Resolution Crystal Structure of wtTIM. To determine the crystal structure of the wtTIM allergen, the crystallization conditions were screened and optimized. The best crystals diffracted to 1.8 Å. The space group was determined to be $P3_121$ with unit cell parameters of $a = 154.712$ Å, $b = 154.712$ Å, and $c = 52.713$ Å. The final refined structural model yielded R_{work} and R_{free} values of 15.62 and 17.91%, respectively (Table 2). The stereochemical quality of the final model was good, and the clear electron density maps enabled a reliable interpretation of the structure. The root-mean-square deviation (rmsd) from the ideal empirical values was 0.006 Å for bond lengths and 0.773 Å for bond angles.

In the final refined structure, the asymmetric unit consists of a pair of dimeric molecules, which includes 248 amino acids in each chain. The three-dimensional structure of TIM is similar with that of other known TIMs, which is primarily composed of a $(\beta/\alpha)_8$ -barrel motif prototype formed by eight central β -strands surrounded by eight α -helices joined by loops (Figure 2A). A monomeric model is presented in Figure 2B, in which the α -helices and β -strands are numbered sequentially from the N-terminus as α_1 – α_8 and β_1 – β_8 ; these are connected by eight $\beta\alpha$ loops between the β -strands and the subsequent α -helices, known as loop-1 to loop-8. The active site is always located in β -strands, which is at the C-terminal end of the barrel with $\beta\alpha$ loops. The loops at the C-terminal end of the β -strands are much more extensive than the loops at the N-terminal end of the β -strands.

Structural Similarity of *S. paramamosain* TIM with Shellfish TIMs. A comparison of homologous proteins can provide a foundation for cross-reactivity by sequence transformation. The evolutionary conservation of amino acid positions was estimated based on the homologous sequence of phylogeny by Consurf. The result of conserved features reveals a high degree of homology (Figure 3A). Although the β -strands inside the barrel were found to be relatively well-conserved, the α -helices were more variable.

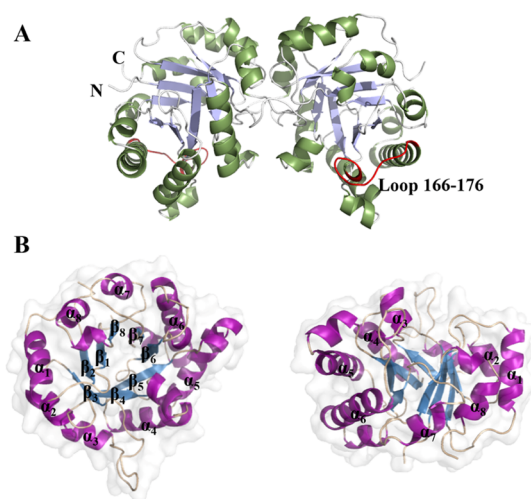


Figure 2. X-ray crystal structure of wtTIM. (A) A TIM dimer is shown with the α -helix-colored smudge, β -sheet-colored light blue, and random coil-colored white. The loop 6 (166–176) is colored in red and is in a typical “open” conformation. (B) TIM monomers are presented as an illustration and surface representation, in which the α -helix, β -strand, and loop are colored in purple, sky blue, and wheat, respectively.

The sequence alignment of TIM from *S. paramamosain*, *L. vannamei*, *P. monodon*, *C. crangon*, and *Procambarus clarkii* is presented in Figure 3B. *S. paramamosain* TIM shared an 87% amino acid sequence identity with other shellfish TIMs. Variable regions found in the α -helices and the β -strands were mainly conserved in shellfish TIMs, which are consistent with the primary structure analysis. To analyze the cross-reactivity of TIMs from different species, an immunologic assay was performed using a rabbit anticrab TIM IgG pAb prepared in our laboratory (Figure 3C). The proteins in 28 kDa in the crude extracts could be detected via SDS-PAGE, and all shellfish TIMs exhibited strong reactivity to rabbit anticrab TIM IgG pAb by Western blot.

The structural alignment between the TIM of *S. paramamosain* and *L. vannamei* (TIM-2PG, PDB: SEYW) is presented in Figure 4A. The structures of the two proteins are largely superimposable, with differences in three regions: residues 29–35, 130–141, and 168–182. The most significant structural difference was found to be a position at loop 6 (amino acids [AA] 166–176).

Mapping of the IgE Epitopes on the Structure of *S. paramamosain* TIM. We mapped the previously defined six linear epitopes and two conformational epitopes on the monomeric structure of TIM (Figure 4). The reported linear epitopes, including AA 45–60, 79–93, 96–110, 174–188, 208–223, and 227–241 (Figure 4B), and the conformational epitopes with W168, T172, Q180, and K237 are the key amino acid/regions for IgE recognition. They are further divided into two regions: epitope 1 ($\text{L}_{131}\text{E}_{132}\text{R}_{134}\text{E}_{135}\text{T}_{139}\text{P}_{166}\text{I}_{167}\text{W}_{168}\text{A}_{169}\text{I}_{170}\text{G}_{171}\text{T}_{172}\text{G}_{173}\text{K}_{174}\text{T}_{175}\text{A}_{176}\text{T}_{177}\text{P}_{178}\text{E}_{179}\text{Q}_{180}\text{S}_{183}\text{V}_{184}$) and epitope 2 ($\text{I}_{24}\text{S}_{211}\text{S}_{213}\text{G}_{233}\text{L}_{236}\text{K}_{237}\text{P}_{238}\text{D}_{239}$) (Figure 4C). While most of these epitopes were distributed on the protein surface, six linear epitopes were distributed in α -helices and conserved between shellfish species (Figure 3B). In addition, the conformational epitope of the key amino acids was conserved and primarily distributed in loop 6.

Mutant TIMs Reduce IgE-Binding Activity. To determine the specific epitopes on the TIM surface, the

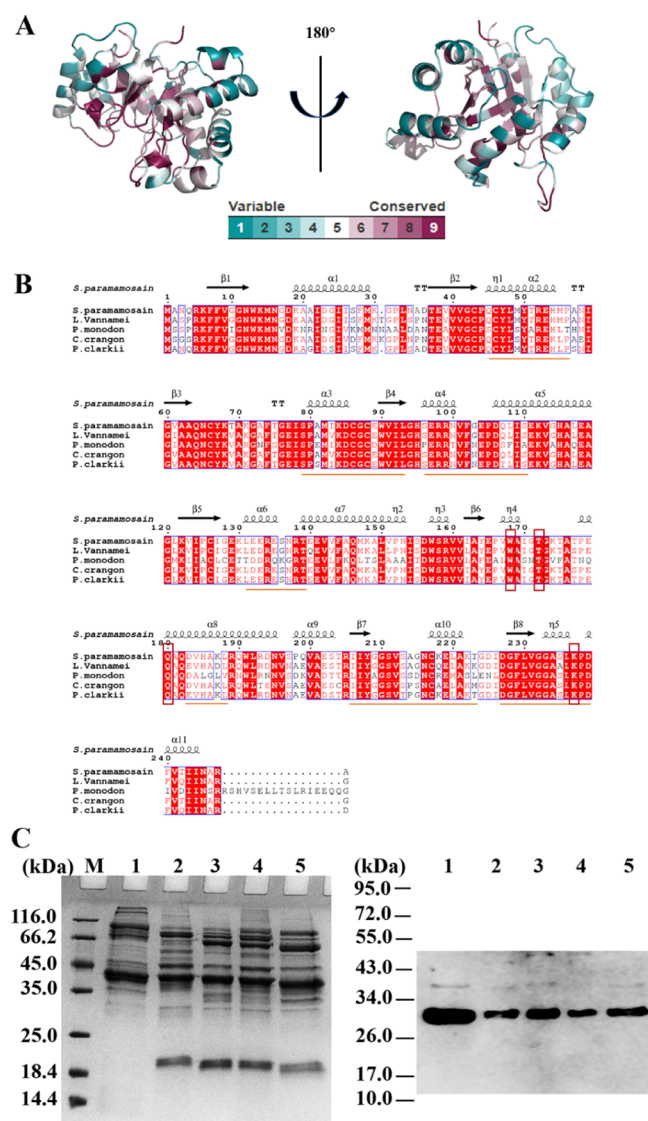


Figure 3. Structural analysis of TIM in *S. paramamosain* and other species. (A) Sequence conservation mapped on the surface of *S. paramamosain* TIM using the ConSurf web server. A score of one to four represents variable, a score of five to six is intermediate, and a score of seven to nine indicates conserved. The most well-conserved residues are presented in dark magenta, and the most variable residues are shown in dark cyan. (B) Comparison of the sequences, secondary structures, and epitopes of the TIMs from different species. The secondary structure of *S. paramamosain* TIM was considered the standard. The GenBank accession numbers of the sequence as follows: *S. paramamosain* (mud crab, APP94292.1), *L. vannamei* (white shrimp, AFT92034.1), *P. monodon* (black tiger shrimp ADG86240.1), *Crangon crangon* (North Sea shrimp, ACR43476.1), and *P. clarkia* (red swamp crayfish, AEB54655.1). The color represents residue similarity: red background, identical; red text, strongly similar. The orange lines represent the linear epitopes of *S. paramamosain* TIM. The red box represents the key amino acids for the conformational epitopes. (C) SDS-PAGE and Western blot of the cross-reactivity in TIM between different species. Lanes 1–5, *S. paramamosain*, *L. vannamei*, *P. monodon*, *C. crangon*, and *P. clarkia*.

mutants of key amino acids in conformational epitopes, including W168A, T172A, Q180A, and K237A were generated and subsequently verified by SDS-PAGE and Western blot analysis (Figure 5A). The wtTIM and mutants can be detected by rabbit anticrayfish TIM pAb. The results of the dot blot

using the crab-sensitive patient sera are presented in Figure 5B, in which the IgE-binding activity of K237A was significantly lower than that of wtTIM ($p < 0.05$). Except for patient P6, W168A also showed a mean reduction of the patient IgE reactivity, whereas T172A and Q180A displayed subtle increased on IgE-binding in the allergic patients. Mutant TIMs were individually tested for any changes in the IgE-binding capacity by inhibition ELISA compared with wtTIM (Figure 5C). The inhibition rate of W168A and K237A was approximately 70%, compared to that of T172A and Q180A at approximately 90%. These results suggest that the IgE-binding activity of W168A and K237A was decreased, which was in agree with the results of the dot blot experiment.

The CD spectra of wtTIM and mutant TIMs were equivalent, with a minimum between 208 and 222 nm (Figure 5D), indicating a similar overall folding of the wtTIM and mutant TIMs. Moreover, the effects of temperature on the secondary structure of wtTIM and mutant TIMs were evaluated (Figure S1), and the thermal denaturation temperature of the proteins was measured using Global 3.0 software (Figure S1). The denaturation temperatures of wtTIM, W168A, T172A, Q180A, and K237A were 56.0 ± 0.1 , 50.8 ± 0.3 , 53.1 ± 0.3 , 55.7 ± 0.2 , and 52.1 ± 0.3 °C, respectively.

The BAT was performed by assessing the ability of wtTIM and mutant TIMs to induce upregulation in the level of CD63 and CD203c expression on basophils from six crab allergic patients (Figure S2). The basophil of each patient was effectively activated in the presence of the recombinant allergens. The mutant TIMs, W168A and K237A, displayed a decrease in the SI (Figure 6A), and the reduction of BAT was patient-dependent: patients P2, P4, and P10 with K237A exhibited reduced stimulation by approximately 30%; meanwhile, W168A also exhibited reduced stimulation for the patients P7 and P10. By contrast, the mutants T172A and Q180A appeared to have an increased capacity to bind IgE in patients P11.

Furthermore, the electrostatic potential of W168A, K237A, T172A, and Q180A was calculated and mapped onto the protein surface (Figures 6B and S4). Compared with the wtTIM, the surface charge distribution of K237, W168, T172, and Q180 changed after the mutation. In detail, the surface contact area of T172A and Q180A increased, while the surface positive charge of K237A decreased and that of W168A increased.

DISCUSSION

In the present study, we determined the first crystal structure of TIM, Scy p 8, from *S. paramamosain* and performed an in-depth study of its structure and compared it with that of other homologous shellfish TIMs. Furthermore, the relationship between the distribution of its epitopes and structure was investigated, and we mutated key amino acids of conformational epitopes to obtain low immunoreactive proteins.

Crustacean allergy, which causes IgE-mediated type I hypersensitivity, is a chronic disease that usually persists for life,³⁴ and a number of crustacean allergens have been identified in various products over the past few decades. As a minor allergen, the abundance of TIM is substantially lower than primary allergens in myosinogen. Certain allergenic proteins might be underrepresented in diagnostic or therapeutic allergen extracts because of low concentration in the source material or unfavorable conditions during extract preparation.⁷ In the present study, the wtTIM was expressed as

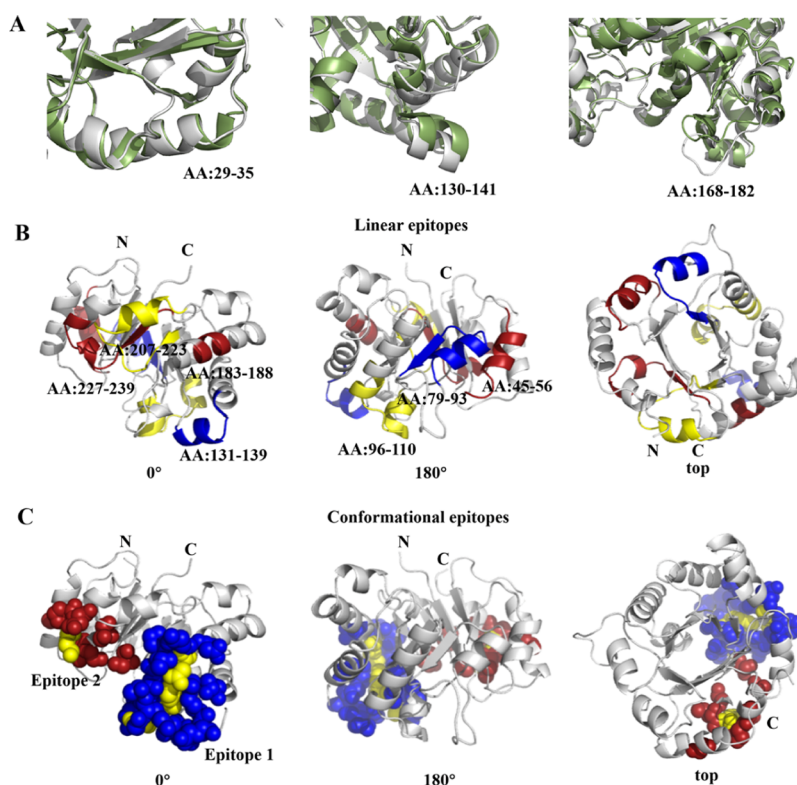


Figure 4. Structural superposition and IgE epitope mapping of *S. paramamosain* TIM. (A) Structural alignment of *S. paramamosain* TIM (white, 6J0X) with *L. vannamei* TIM (smudge, 5EYW), and the structural differences between the TIMs are squared with a solid line. (B) Reported linear epitopes were mapped on the TIM monomer shown on the diagram. AA: 45–56, 79–93, 96–110, 131–139, 183–188, 207–223, and 227–239. (C) Reported conformational epitopes were mapped on the TIM monomer shown on the diagram. Epitope 1 ($L_{131}E_{132}R_{134}E_{135}T_{139}P_{166}I_{167}W_{168}A_{169}I_{170}G_{171}T_{172}G_{173}K_{174}T_{175}A_{176}T_{177}P_{178}E_{179}Q_{180}S_{183}V_{184}$) and epitope 2 ($I_{24}S_{211}S_{213}G_{233}L_{236}K_{237}P_{238}D_{239}$). The key amino acids are colored yellow.

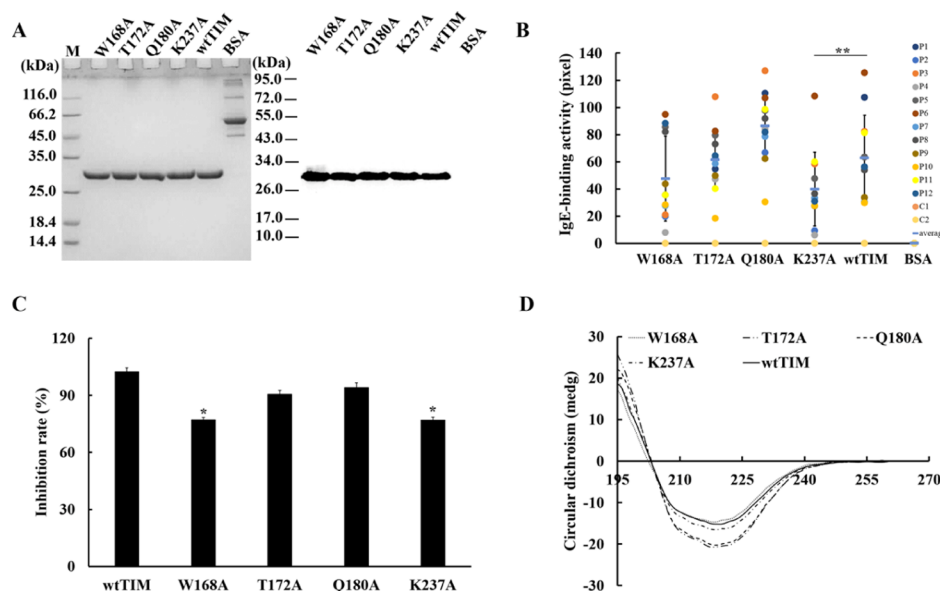


Figure 5. Analysis of the immunogenicity and the secondary structure of wtTIM and mutant TIMs. (A) SDS-PAGE and Western blot of the mutant TIMs (primary antibody: rabbit anticrayfish TIM IgG pAb, dilution 1:10⁵). Lane M, protein marker. Lane 1, W168A. Lane 2, T172A. Lane 3, Q180A. Lane 4, K237A. Lane 5, wtTIM. Lane 6, BSA. (B) IgE-binding activity analysis of wtTIM and mutant TIMs by dot blot using crab-sensitive patient serum. The intensity of the dots shown on the nitrocellulose membranes, and the quantification of grayscale dots was analyzed using ImageJ software. The horizontal line represents the average values of intensity. All data were presented as the mean $\pm$ SD ($n = 3$). ** $p < 0.01$, vs the wtTIM group. (C) Inhibition ELISA assay by the serum pool of crab-sensitive patients. All data were presented as the mean $\pm$ SD ($n = 3$). * $p < 0.05$, vs the wtTIM group. (D) The secondary structure of the wtTIM and mutant TIMs by CD spectra.

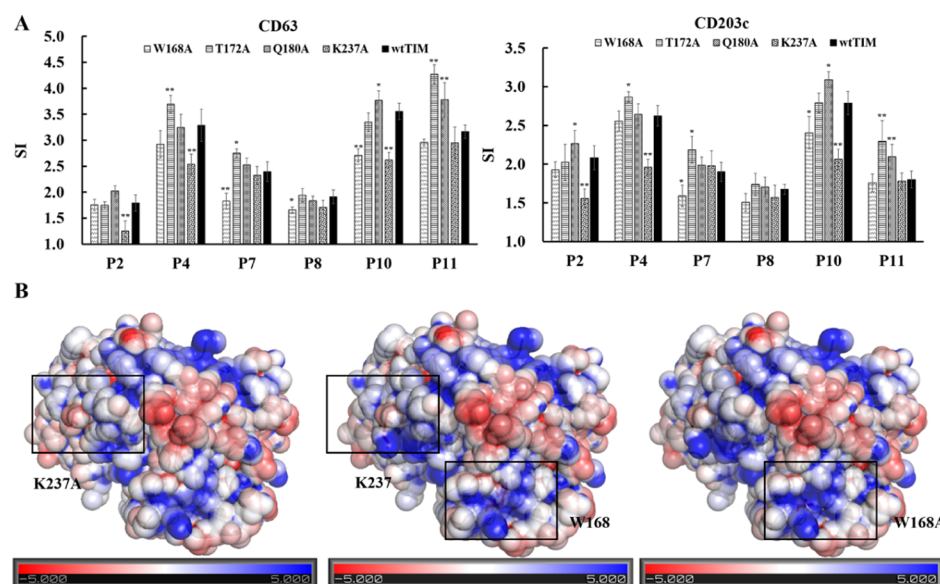


Figure 6. BAT and the charge distribution on the surface of wtTIM and mutant TIMs. A. BAT was performed to compare the immunoreactivity of TIM mutants from six crab-sensitive patients (P2, P4, P7, P8, P10, and P11). Data are present as mean $\pm$ SD ($n = 3$). * $p < 0.05$, vs the wtTIM group, ** $p < 0.01$, vs the wtTIM group. (B) Electrostatic surface potential of wtTIM, W168A, and K237A. Red and blue represent negative and positive charges, respectively.

a soluble protein in *E. coli* BL21 with His-tag. As for whether an additional tag influences the structure, the structure of wtTIM was superimposed with *L. vannamei* TIM and the structure basically overlapped. Lopez-Zavala et al.¹⁸ found the rmsd between *L. vannamei* TIM and chicken TIM (1TPH) was 0.5 Å, human (4POD) or rabbit (1R2R) 0.7 Å, proving that there is not much difference between their structures. It is reported that the His-tag is most commonly used for the expression of recombinant seafood allergens.³⁴ Li et al.³⁵ expressed recombinant myosin light chain 1 with His-tag, which retained the allergenicity. Bauermeister et al.⁷ expressed recombinant *C. crangon* proteins with His-tag. It means that the His-tag does not affect the structure and allergenicity of protein.

We have previously demonstrated that the *S. paramamosian* TIM consisted of a $(\beta/\alpha)_8$ -barrel, which is one of most common enzyme folds³⁶ and a common feature of allergen fold structures.³⁷ The internal β -barrel is protected by a bundle of α -helices, and the barrel fold is stable. Loop 6 is highly flexible and creates the TIM active site for efficient catalysis. The classical view is that loop 6 is open in the absence of a ligand, which interacts with loop 5, and is closed in the presence of a ligand, when it interacts with loop 7. As observed in Figure 4A, after the *L. vannamei* TIM loop 6 binds to its ligand, it produces a corresponding displacement at both ends of loop 6. Unique conformations of loop 6 have been observed in different complexes.³⁸ By shaping the active site into an open loop without substrate binding, a closed state occurs in the presence of bound ligand; however, loop 6 appears closed in the unliganded rabbit TIM.³⁹ In a malaria TIM structure, loop 6 is in an open conformation, whereas the ligand remains.⁴⁰ In some allergens, such activity may have a potential relationship with the allergenicity.⁶ As found in the wheat allergen β -amylase, Tri a 17⁴¹ and other homologous food allergens exhibit the same $(\beta/\alpha)_8$ -barrel architecture, which may induce species cross-reactivity.

The precise position of the conformational epitopes can be determined by X-ray crystallography, which depicts the true

structure of the epitopes. We found that the conformational epitopes are primarily located on the loop near the C-terminus, located at the vicinity of the active sites primarily in the flexible region. Loop 6 connects the front-end β sheet and the rear-end α helix, which forms protruding areas with high solvent accessibility. In addition, the protruding surface areas have been shown to contain conformational epitopes in the hyaluronidase-Fab complex structure.⁴² Linear epitopes are mainly located on the surface of the helices, which increase the possibility of epitope binding to the antibody.

The primary structure of wtTIM is highly conserved, and the variable residue regions are mainly distributed in α -helices. In parallel, TIMs of different species exhibit relatively high identity in the α -helices and β -strands by the secondary structures. The allergenic epitopes labeled in Figure 3B revealed that the residues forming the linear epitopes are conserved but except the *P. monodon* TIM; the key amino acids of the conformational epitopes, including W168, T172, Q180, and K237, were identical in all the examined species; thus, supporting the possibility of cross-reactivity among shellfish. Differences in the IgG-binding activity between *S. paramamosian* TIM with other shellfish TIMs, as well as the content in other species may be attributed to correlations with the energy metabolism of the species because of its important role in glycolysis and energy production, suggesting that some of allergen epitopes might also vary. The differences in the superposition of the tertiary structure are partial because of the deletion or replacement of amino acid residues in loops or helices.

The dot blot experiments of the mutant TIMs showed that W168A and K237A displayed a reduced IgE-binding capacity, and the BATs indicated a reduced biological activity; however, these results demonstrated that the allergenicity has not been eliminated, which may partially explain the presence of natural and polyclonal IgE antibodies in human sera. Interestingly, the effects of mutations on IgE antibody binding varied between the patients. Furthermore, we compared the structure of wtTIM and mutant TIMs by CD spectroscopy, and observed

that the mutants remained folded and the far-UV spectrum continued to display the typical characteristics of an α -helical protein. Temperature scan experiments revealed that the mutant unfolded at a temperature that was 7 °C lower than the melting temperature of wtTIM, which indicates a partial heat-induced destabilization of the mutants. These observations implicate a crucial role of W168 and K237 in mediating the interaction between IgE antibodies with the corresponding epitopes. Differences in the effects of mutations on IgE binding may be explained by the fact that they are involved in many interactions through their side chains. In addition, such mutations impact a larger area of the allergen surface, causing changes to the surface electrostatic potential. T172 and Q180 are likely of limited value for reducing IgE binding. The mutants of T172A and Q180A can be used for molecular detection because of increased allergenicity.

Several studies have used homologous modeling to predict epitopes; however, it has been well-established that the initial templates mimic native structures to a greater extent than homologous models.⁴³ Furthermore, allergen structural homologues can be used as scaffolds for epitope transfer, transplanting a single surface area of an allergen into a low or nonallergen member of the same family.⁴⁴ These results can lay the foundation for regulating allergen allergenicity. Meanwhile, it also can help to provide an accurate template for the analysis of the IgE binding and establish meaningful relationships between structure and allergenicity.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b05279.

Supplementary methods; determination of the rabbit anti-crab TIM polyclonal antibody titer; thermal denaturation analysis; effect of wtTIM and mutant TIMs in BAT; and electrostatic surface potential of mutants T172A and Q180A (PDF)

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Author Contributions

T.J. and G.-M.L. designed the study, participated in data analysis and extensively reviewed the manuscript. F.X. performed the experiments, analyzed the data and drafted the manuscript. M.-S.L., Q.-M.L., M.L., Y.Y. and M.-J.C. analyzed the data and revised the manuscript. G.-X.C. provided serum samples.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BAT, basophil activation test; BSA, bovine serum albumin; CD, circular dichroism; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; MFI, mean fluorescence intensity; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TIM, triosephosphate isomerase; wtTIM, wide-type triosephosphate isomerase

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Crystal structure analysis and conformational epitope mutation of triosephosphate isomerase, a mud crab allergen

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Running Title: Structure analysis and conformational epitopes mutation of Scy p 8.

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Online Supplementary Material

Supplementary Methods

Preparation of rabbit anti-crab TIM polyclonal antibodies

The specific polyclonal antibody was prepared by subcutaneously injecting of the recombinant *Scylla paramamosain* wtTIM in an adult female New Zealand white rabbit. The rabbit anti-crab TIM polyclonal antibody titer was measured by a dot blot analysis, following the method of Liu et al¹. The specific polyclonal antibody (1:5000 dilution) was then recruited as the primary antibody for the cross-reactivity Western blot.

Supplementary Figure Legends

FIGURE S1. Determination of the rabbit anti-crab TIM polyclonal antibody titer.

Dot blot analysis of the rabbit anti-crab TIM polyclonal antibody titers, 1:100 – 1:2 × 10⁴ is the polyclonal antibody dilution.

FIGURE S2. Thermal denaturation analysis of mutant TIMs by CD spectroscopy.

A – E. Determination of the thermal denaturation temperature values of wtTIM, W168A, T172A, Q180A, and K237A, respectively.

FIGURE S3. Effect of wtTIM and mutant TIMs on the expression of CD63 and CD203c in basophils.

A. Patients with AC (n = 6) were treated with wtTIM; the blood (n = 2) of NA was used as the negative control.

B and C. Patients P2, P4, P7, P8, P10, P11 were treated with W168A, T172A, Q180A, and K237A, respectively. PBS is shown in gray, orange for wtTIM, and the lines indicate mutant TIMs.

FIGURE S4. The electrostatic surface potential of mutants T172A and Q180A.

A. The electrostatic surface potential of wtTIM, T172A and Q180A. Red and blue are negative and positive charge, respectively.

B. The electrostatic surface potential of T172A at another Angle.

Table S1. Oligonucleotide primers used in PCR.

Primers	Oligonucleotide sequence (5'-3')
wtTIM-F	5'- CATATG ATGGCCAACCAGAGGAAAT-3'
wtTIM-R	5'- GTCGAC TTAAGCACGGGCATTAATAATT-3'
W168A-F	5'-AGTA GCA GCTATTGGAGCGGAAAG-3'
W168A-R	5'-TAGC TGC TACTGGTTCATAAGCAAT-3'
T172A-F	5'-CAGTATGGGCTATTGGA GCC GGAAA-3'
T172A-R	5'-AGGAGTTGCAGTCTTTCC GGC TCCA-3'
Q180A-F	5'-GAAAGACTGCAACTCCTGAA GCA GC-3'
Q180A-R	5'-GGACATCTTGGGC TGC TTTCAGGAGT-3'
K237A-F	5'-TTGTGGGAGGTGCTTCCCTC GCA CC-3'
K237A-R	5'-ATTGCAACAAAGTCTGG TGC GAGGG-3'

The nucleotide changes are underlined; **CATATG** and **GCTGAC** restriction sites for *Nde I* and *Sal I* enzymes, respectively.

FIGURE S1

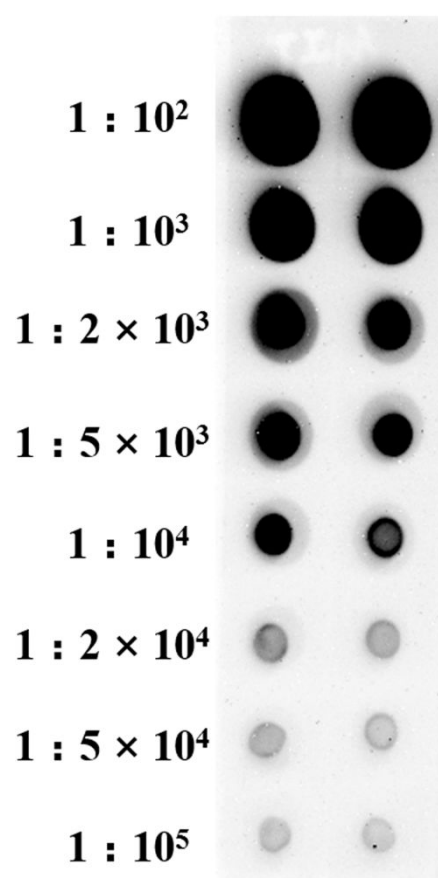


FIGURE S2

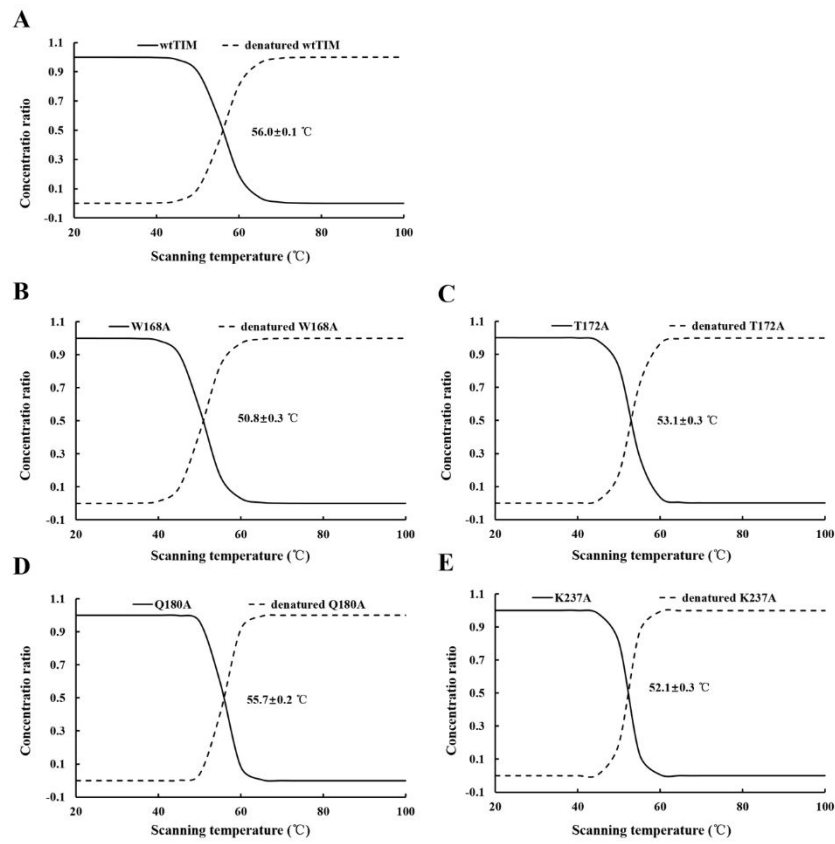


FIGURE S3

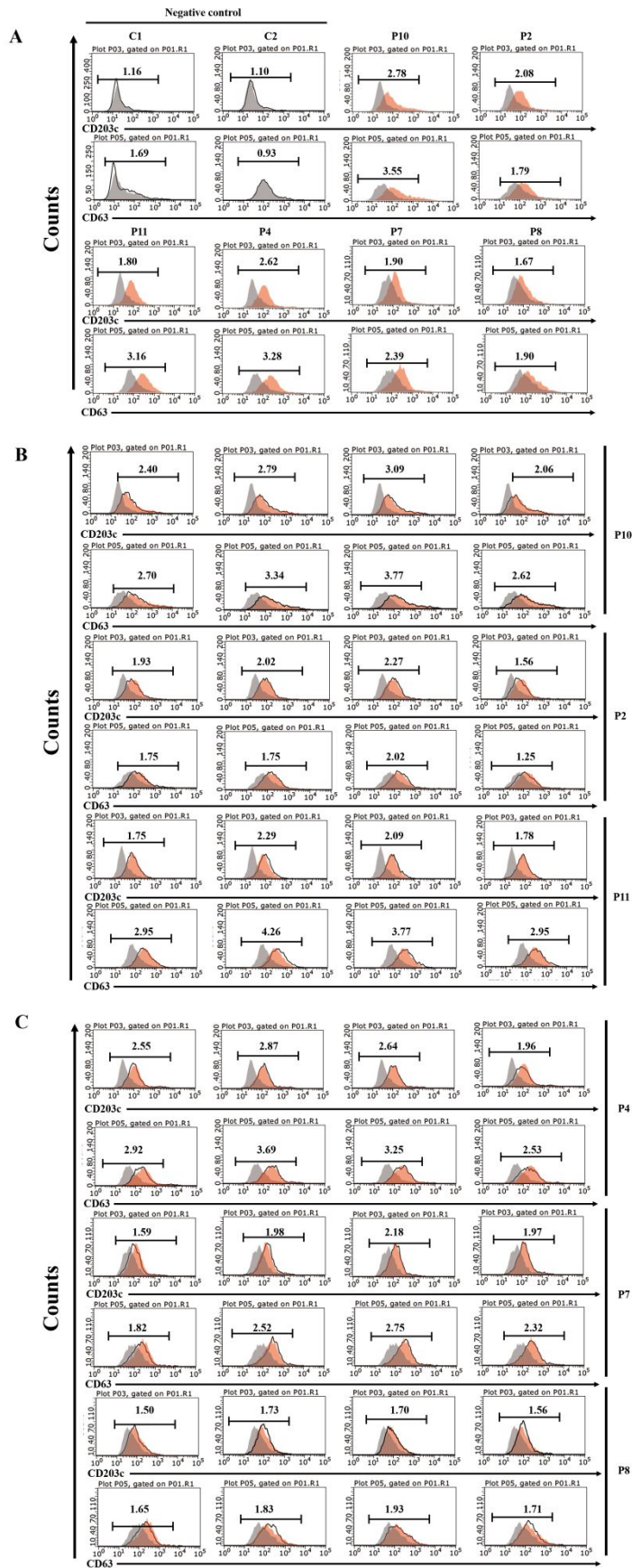
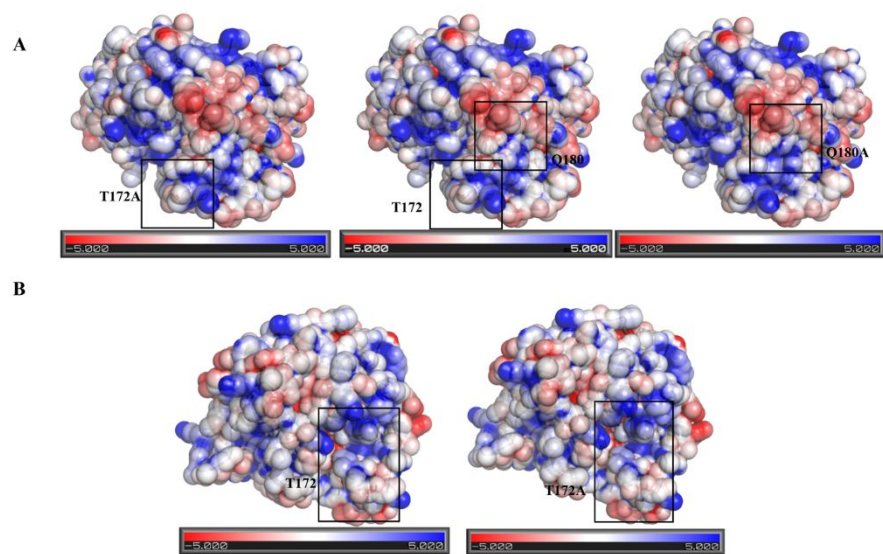


FIGURE S4



References

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