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Cross-linking of shrimp tropomyosin catalyzed by transglutaminase and tyrosinase produces hypoallergens for potential immunotherapy

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Tropomyosin (TM) is the major allergen in shellfish, which causes severe food allergy worldwide and shows wide cross-reactivity in different species. Transglutaminase (TG) and tyrosinase (Tyr) are protein modification enzymes that catalyze substrate cross-linking and change protein structures and properties. The aim of this work was to investigate the effect of TG and Tyr on TM. After TG/Tyr treatment, the cross-linking and protein structural change were observed by SDS-PAGE, scanning electron microscopy and circular dichroism. Furthermore, ELISA and mast cell degranulation assay with allergic sera demonstrated the allergenicity reduction after treatment. An *in vivo* TM-sensitization mouse model showed that both TG and Tyr treatments reduced the IgE-induction capacity of TM but maintained the IgG-induction ability, making the treated TM a potential hypoallergen. Moreover, TG-but-not-Tyr-treated TM promoted regulatory T cell proliferation and thus may facilitate the establishment of oral tolerance. This work provided an effective method to reduce TM allergenicity and produce hypoallergens, which has broad application prospects in low allergenic food production and allergen-specific immunotherapy.

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1. Introduction

Allergy is a worldwide immune disorder disease; about 30% to 40% of the world's population suffer from allergic diseases, and the incidence and mortality have been increasing year after year.^{1,2} In particular, food allergy has been a severe health problem in allergic diseases, affecting 1–3% of adults and 4–6% of children.³ Food allergy is mainly immunoglobulin E (IgE)-mediated type I hypersensitivity. Allergic patients will produce antigen-specific IgE antibodies when they first come in contact with the antigen, and when they come in contact with the same antigen again, the produced IgE interacts with the antigen, resulting in the release of inflammatory mediators (such as histamine and various cytokines) by mast cells or basophils, causing allergic symptoms including life-threatening anaphylactic shock.^{4,5} Therefore, it is of great practical significance to reduce the risk of food allergy, and eliminating food allergens by food processing methods is an effective way.

Enzymatic cross-linking refers to the intramolecular or intermolecular cross-linking reaction under the action of

enzymes, which can induce the polymerization of proteins.⁶ This modification method will alter protein properties such as digestion sensitivity and protein structures, and thereby may influence allergenicity.^{7–9} Transglutaminase (TG) is a commonly used cross-linking enzyme in the food industry. TG catalyzes the junction between the amidyl groups such as the epsilon amino group on the polypeptide lysine and the gamma hydroxyl amide group on glutamic acid.¹⁰ A study on peanut powder containing casein demonstrated that cross-linked casein by TG can result in a significant decrease in allergenicity.¹¹ Tyrosinase (Tyr) is one of the most common oxidases in natural resources. It can catalyze the oxidation of tyrosine residues, thus causing a cross-linking reaction between proteins and peptides.¹² Research has found that Tyr treatment effectively reduced the potential allergenicity and increased the bioavailability of peanut allergens.¹³

Tropomyosin (TM) is a highly conserved α -helix protein and has been identified as the major shellfish allergen, which is normally heat-stable and has a highly conserved amino acid sequence among different species.^{14,15} Our previous studies have revealed ten linear IgE epitopes in shrimp TM responsible for allergenicity, which contain glutamic acid, tyrosine and lysine abundantly; moreover, glutamic acid is the critical amino acid for allergenicity.¹⁶ Consequently, shrimp TM can be an ideal substrate for TG. In addition, enzymatic cross-linking with crab arginine kinase, another major shellfish allergen, by Tyr reduced protein allergenicity.¹⁷ Therefore, the

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cross-linking by enzymes such as TG or Tyr can be a promising way to modify shrimp TM properties and thus reduce allergenicity.

In addition to avoiding contact with allergens, active measures, especially allergen-specific immunotherapy, can be taken to treat food allergy; however, due to the potential risks of IgE-mediated side effects, too long therapeutic time period and relatively low effectiveness, this method has not been widely applied.¹⁸ In recent years, it has been reported that through particular methods such as physical treatment, chemical reaction, enzymatic modification or genetic engineering, some allergens may lose the IgE binding capacity but maintain the immune system activation ability (immunogenicity), which are termed hypoallergens and can be administered at higher doses and consequently increase the safety and efficacy of allergen-specific immunotherapy.^{19,20} Therefore, developing novel methods to produce hypoallergens may give a boost to the improvement of allergen-specific immunotherapy and the treatment of food allergy.

Overall, the major goal of the present work was to reduce the allergenicity of TM in *Penaeus chinensis* by TG/Tyr-catalyzed cross-linking. Both *in vitro* and *in vivo* methods were used to assess the efficiency of this method. Interestingly, we found that the treated TM showed potential hypoallergenic properties and might be used to develop novel immunotherapy methods.

2. Materials and methods

2.1. Ethics statement

The collection and application of human serum samples were approved by the Zhejiang Gongshang University Ethics Review Committee as it is part of a routine procedure where no additional consent is required by law. All animal procedures were carried out in strict accordance with the recommendations of the National Guidelines for the Care and Use of Laboratory Animals of China, and approved by the Institutional Animal Care and Use Committee of Zhejiang Provincial Experimental Animal Public Service System and the Zhejiang Gongshang University Ethics Review Committee.

2.2. Human sera

Serum samples were obtained from 20 subjects with a confirmed clinical history of allergic responses to *Penaeus chinensis* and five subjects without allergy. Shrimp-specific sera samples were obtained from the Chongqing MaNueAiKe Technology Company. These sera were frozen at -80°C until use.

2.3. Chemicals

Transglutaminase was purchased from Shanghai Yuan Ye Biotechnology Company (China). 1640 medium, fetal bovine serum, and penicillin–streptomycin liquid were purchased from Gibco (USA). Goat anti-mouse IgE and IgG2a antibodies were purchased from Abcam (USA). Tyrosinase and Freund's complete and incomplete adjuvants were purchased from

Sigma (USA). Human HIS ELISA Kit was purchased from Baoman Biology (China). Human IL-8 ELISA Kit, Human TNF- α ELISA Kit, and Mouse IL-4 ELISA Kit were purchased from eBioscience (USA).

2.4. Protein purification and enzymatic cross-linking reaction

Penaeus chinensis was purchased from a Yonghui supermarket in Xiasha district of Hangzhou. The extraction and purification of tropomyosin were performed according to the method of Fu *et al.*²¹ The optimal conditions for the cross-linking of TM are as follows: adding TG enzyme into the purified TM protein, the ratio of enzyme to substrate was 1 U mg^{-1} , reacting at 37°C for 24 hours, followed by inactivation at 90°C ; the product was named TM-TG. In another reaction, tyrosinase was added to the TM protein, and the volume ratio of the enzyme to the reaction system was 800 U mL^{-1} . The reaction time was 24 hours at 37°C . After the reaction, the enzyme was inactivated at 90°C , and the product was named TM-Tyr.

2.5. Electrophoretic and ELISA analysis

The target protein was collected and detected by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The IgE-binding activity of TM was measured by ELISA (enzyme linked immunosorbent assay): each sample was coated on 96-well plates in 0.05 M carbonate buffer overnight at 4°C , after washing and blocking with 3% bovine serum albumin/ 0.01 M pH 7.4 PBS for 2 h at 37°C , and the plates were washed again and incubated with serum samples (diluted 1:200) for 1.5 h at 37°C . Meanwhile, the wells supplemented with serum samples from non-allergic individuals served as the negative control. After washing, the plates were incubated with HRP goat anti-human IgE serum (diluted 1:2500, Novus Biologicals, USA) for 1.5 h at 37°C . After the final wash, the plates were incubated with TMB at 37°C for 15 min. The reaction was terminated using $2\text{ M H}_2\text{SO}_4$, and the samples were measured at 450 nm using a VersaMax microplate reader from Molecular Devices (USA).

2.6. Scanning electron microscopy (SEM) and circular dichroism (CD) analysis

For SEM observation, the samples were dropped onto the sample table with a conductive adhesive ($20\text{ }\mu\text{L}$), dried naturally overnight, coated with gold for 80 seconds (ACE 200, Leica, Germany), and then observed and photographed using a scanning electron microscope (Nova Nanosem 450, Thermo Fisher Scientific, USA).

CD spectroscopy was performed in the spectral range from 190 nm to 260 nm. Protein concentration was adjusted to 0.1 mg mL^{-1} (TM-TG) or 0.25 mg mL^{-1} (TM-Tyr) for analysis. A scan speed at 50 nm per min and a bandwidth of 1 nm were used. The contents of different secondary structures were calculated using JASCO secondary structure software based on the spectrum results.

2.7. LAD2 cell degranulation assay

LAD2 cells (ATCC, USA) were seeded in RPMI 1640 medium (Gibco, USA), supplemented with 9% (v/v) fetal bovine serum, and 1% (v/v) penicillin–streptomycin (1×10^6 cells per 10 cm dish) and incubated for 2 h with shrimp allergic sera at 37 °C/5% CO₂. After centrifugation at 900g for 5 min at room temperature, the cells were washed and resuspended in HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.38 mM Na₂HPO₄, 5.6 mM glucose, 1.8 mM CaCl₂, 1.3 mM MgSO₄, 0.4% BSA, at pH 7.4) and stimulated for 30 min with intact TM, cross-linked products or PBS at 37 °C/5% CO₂. Then the cells were centrifuged at 900g for 5 min at room temperature again, and the released β -hexosaminidase was quantified by the hydrolysis of *p*-nitrophenyl *N*-acetyl- β -D-glucosamide (Sigma, Canada) in 0.1 M sodium citrate buffer (pH 4.5) for 90 min at 37 °C/5% CO₂. In the cells, β -hexosaminidase was also measured after lysis with 0.01% Triton X-100. The reaction was terminated by the addition of Tris–HCl (1 M, pH 9.0) and the absorbance was measured at 405 nm. The percentage of β -hexosaminidase released was calculated as a percentage of the total content. Cell-free supernatants were also analyzed for the release of histamine and cytokine using an ELISA kit (eBioscience, USA).

2.8. Food allergy mouse model

Mice were randomly divided into 4 groups ($n = 5$) and sensitized by intraperitoneal (IP) injection according to the method of Fu *et al.*²² The mice were exposed to 0.6 mg protein sample (TM or cross-linked product) with isovolumetric Freund's complete adjuvant in the first week for one day. For the next two weeks, the mice were exposed once a week to 0.6 mg sample with isovolumetric Freund's incomplete adjuvant. In the last week, the mice were challenged with 1.2 mg sample with isovolumetric Freund's incomplete adjuvant. The negative control group received PBS instead of the protein sample. 30 minutes after the challenge, the symptoms of the mice were observed and the scores were recorded; the diarrhea and allergy symptom scoring table of the mice used is shown in Table 1. Then the blood samples were collected to measure TM-specific IgG2a, IgE and histamine. Splenocytes were prepared by aseptic removal, and cytokines in cell supernatants were measured using the 3-day culture. For regulatory T cell (Treg) quantification, the splenic cells were isolated and stained with

anti-CD4-PE and anti-CD25-FITC (eBioscience, USA) antibodies and then measured by flow cytometry (CytoFLEX, Beckman).²³

2.9. Statistical analysis

All the statistical results were analyzed with three replicates according to a completely randomized design. All data were analyzed by Student's *t*-test or analysis of variance (ANOVA). Data were analyzed statistically by repeated measures using SPSS 17.0 procedures and $p < 0.05$ was considered as statistically significant.

3. Results and discussion

3.1. TG and Tyr catalyze TM cross-linking

In order to eliminate the allergenicity of shrimp TM by changing the protein structure, two enzymes, TG and Tyr, were used to catalyze the cross-linking of TM. After treatment with the enzymes, the reaction products (TM–TG and TM–Tyr by TG and Tyr, respectively) were resolved by SDS-PAGE and detected by Coomassie Brilliant Blue staining. As shown in Fig. 1, compared with intact TM, both TG and Tyr treatments reduced the main TM band at 37 kDa and produced upper smears. In particular, Tyr treatment produced a high molecular weight product over 200 kDa; however, TG treatment did not result in

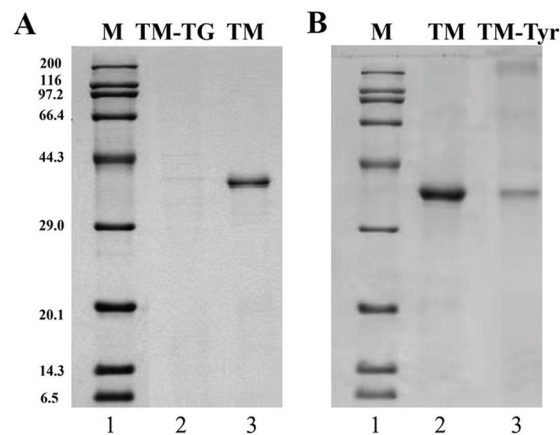


Fig. 1 TG and Tyr catalyze TM cross-linking. TM isolated from *Penaeus chinensis* was treated with TG or Tyr. The products were resolved by SDS-PAGE and detected by Coomassie Brilliant Blue staining.

Table 1 Diarrhea and allergic symptom scoring table

Score	Allergy symptom	Diarrhea symptom
0	No symptom	No symptom
1	Scratching ear and mouth repeatedly	Granular and viscous feces
2	Decreased activity, moving alone, red and swollen ears and eyes, flaring nares, anorexia	No granular feces
3	More than 2 minutes of rest time, significantly decreased activity, increased respiratory rate, erect hair	Shapeless feces with yellow mucus
4	Protrusive eyes, conjunctival congestion, no response after stimulation, tremble, convulsion	Shapeless feces with yellow mucus, fecal residues around the anus
5	Death	Liquid stool, blocked anus, fatal if not cleaned in time

a product with the exact molecular weight, and the total protein signal was much weaker than that of the untreated TM, which may be due to the generation of an extremely high molecular weight product that cannot enter the gel or even precipitate.

3.2. Structural change of TM after TG or Tyr treatment

To investigate the detailed structural change of TM after TG or Tyr treatment, the reaction products were analyzed by SEM and CD (Fig. 2). SEM results showed that compared with intact TM that formed a fiber-like structure, treated TM cross-linked with each other to form more compact and complex structures. Specifically, while TM-TG formed a tight network, TM-Tyr formed a film-like structure (Fig. 2A). Moreover, the CD results (Fig. 2B) showed that after TG and Tyr treatment, the molar residue ellipticity peaks near 192, 208 and 222 nm dramatically decreased, indicating the destructuring of the α -helix. Further analyses performed using JASCO software showed that the percentage of α -helix reduced by 20.1% and 15.2% in TM-TG and TM-Tyr, respectively; meanwhile, β -turn increased 5.8% in TM-TG and 6.2% in TM-Tyr. These results demonstrated that TG resulted in stronger TM structural change than Tyr.

3.3. TG or Tyr treatment reduced the IgE-binding capacity of TM

IgE-binding capacity is the primary feature of allergens, which is totally based on the protein structure. To investigate

whether the structural change of TM induced by TG and Tyr treatment influences the allergenicity, the IgE-binding capacity of reaction products was evaluated by ELISA with sera from shrimp allergy patients (Fig. 3). Compared with the negative control where TM was absent, TM showed a strong IgE-

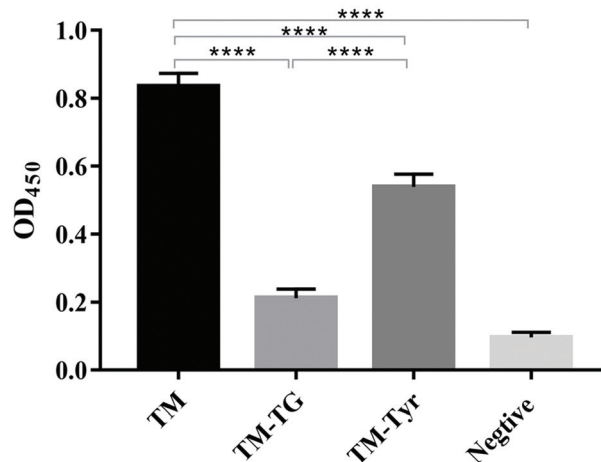


Fig. 3 TG or Tyr treatment reduced the IgE-binding capacity of TM. The IgE-binding capacities of TG or Tyr treated TM samples were evaluated by ELISA with sera from shrimp allergy patients. **** $p < 0.0001$.

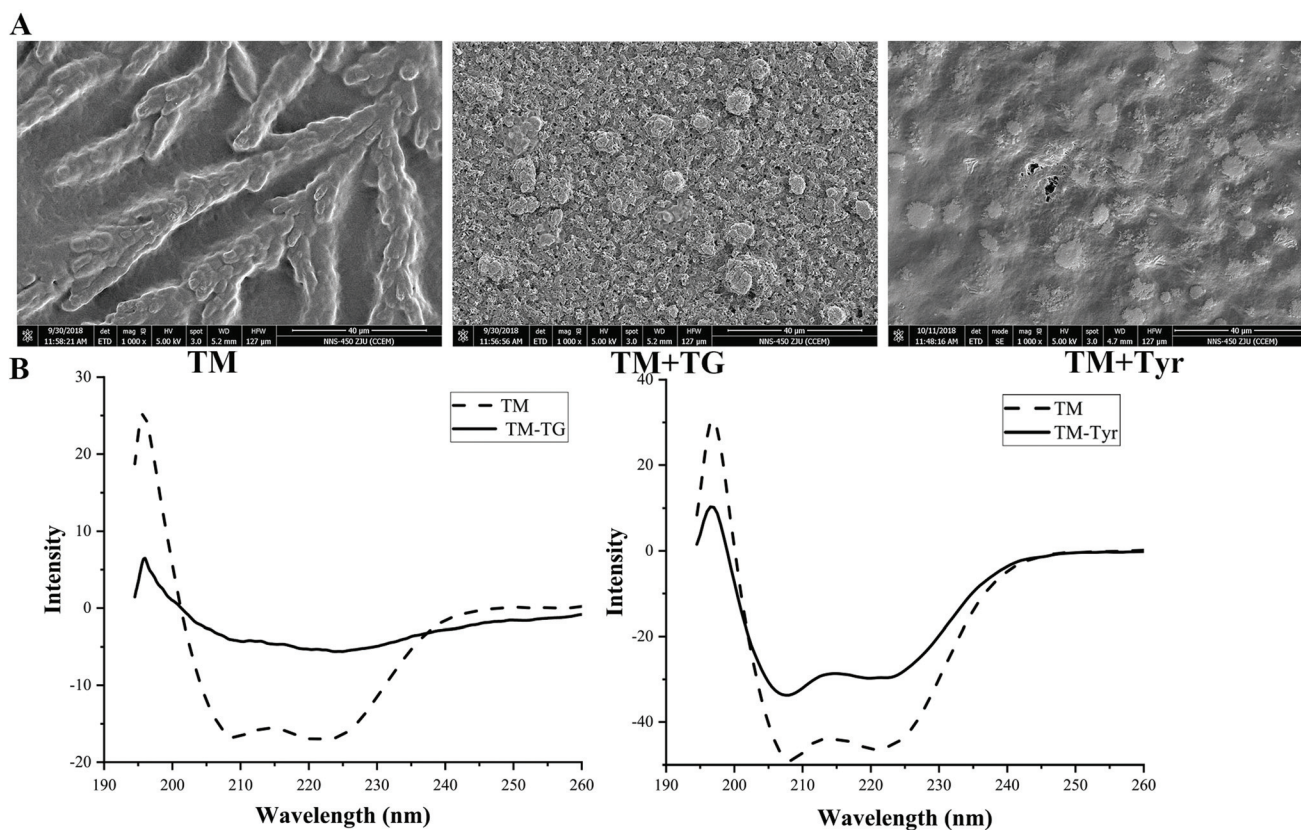


Fig. 2 Structural change of TM after TG or Tyr treatment. (A) TG or Tyr treated TM samples were observed by SEM. (B) TG or Tyr treated TM samples were analyzed by CD.

binding capacity with the OD₄₅₀ value increasing from 0.0965 to more than 0.8297 with statistical significance ($p < 0.0001$). Comparatively, Tyr treatment reduced about half of the IgE-binding capacity (OD₄₅₀ = 0.5397, $p < 0.0001$), and most IgE-binding capacity was eliminated in TM-TG (OD₄₅₀ = 0.2122, $p < 0.0001$). Consistent with the structural change level, the effect of TG on reducing the IgE-binding capacity was also stronger than that of Tyr, further confirming that the protein structure of TM is tightly correlated with allergenicity.

3.4. TG or Tyr treatment reduced TM-induced mast cell degranulation

Under pathological conditions, multivalent food allergens bind to a specific IgE on the surface of mast cells or basophils to trigger subsequent cell degranulation, leading to the release of allergic mediators such as β -hexosaminidase and histamine.²⁴ To assess the effect of TG and Tyr treatment on the process, treated TM was applied to an *in vitro* LAD2 human

mast cell line in the presence of allergic sera (Fig. 4). TM induced significant degranulation in LAD2 cells, whereas the induction by TM-TG and TM-Tyr was significantly weaker. In the TM-TG group, the releases of β -hexosaminidase, histamine, interleukin-8 (IL-8) and TNF- α reduced by 15.2437%, 22.1028%, 8.3733%, and 39.6458%, respectively, whereas the reduction in the TM-Tyr group was 14.1832%, 22.778%, 18.3555%, and 34.4309%, respectively. All these decrements were statistically significant, demonstrating the efficiency of TG and Tyr in eliminating TM allergenicity.

3.5. TG or Tyr treatment reduced TM-induced food allergy *in vivo*

To further evaluate the allergenicity change of TM after TG or Tyr treatment *in vivo*, a TM-induced food allergy mouse model was established. Compared with the negative control group that was not stimulated by TM, the TM-challenged mice showed slight (not significant) body weight gain delay, indicat-

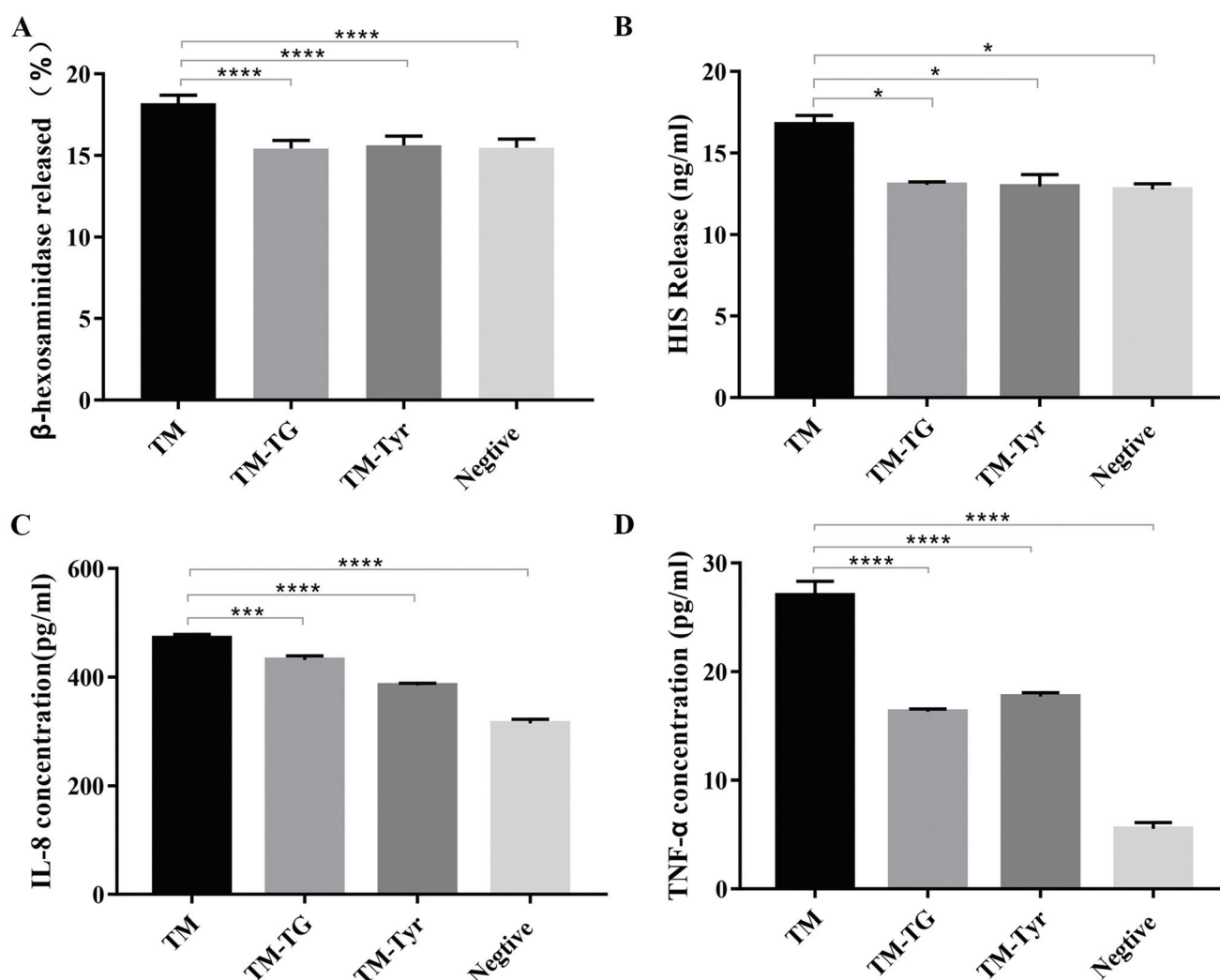


Fig. 4 TG or Tyr treatment reduced TM-induced mast cell degranulation. TG or Tyr treated TM samples were applied to LAD2 cells sensitized by allergic sera. The releases of β -hexosaminidase (A), histamine (B), IL-8 (C) and TNF- α (D) were quantified by ELISA. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

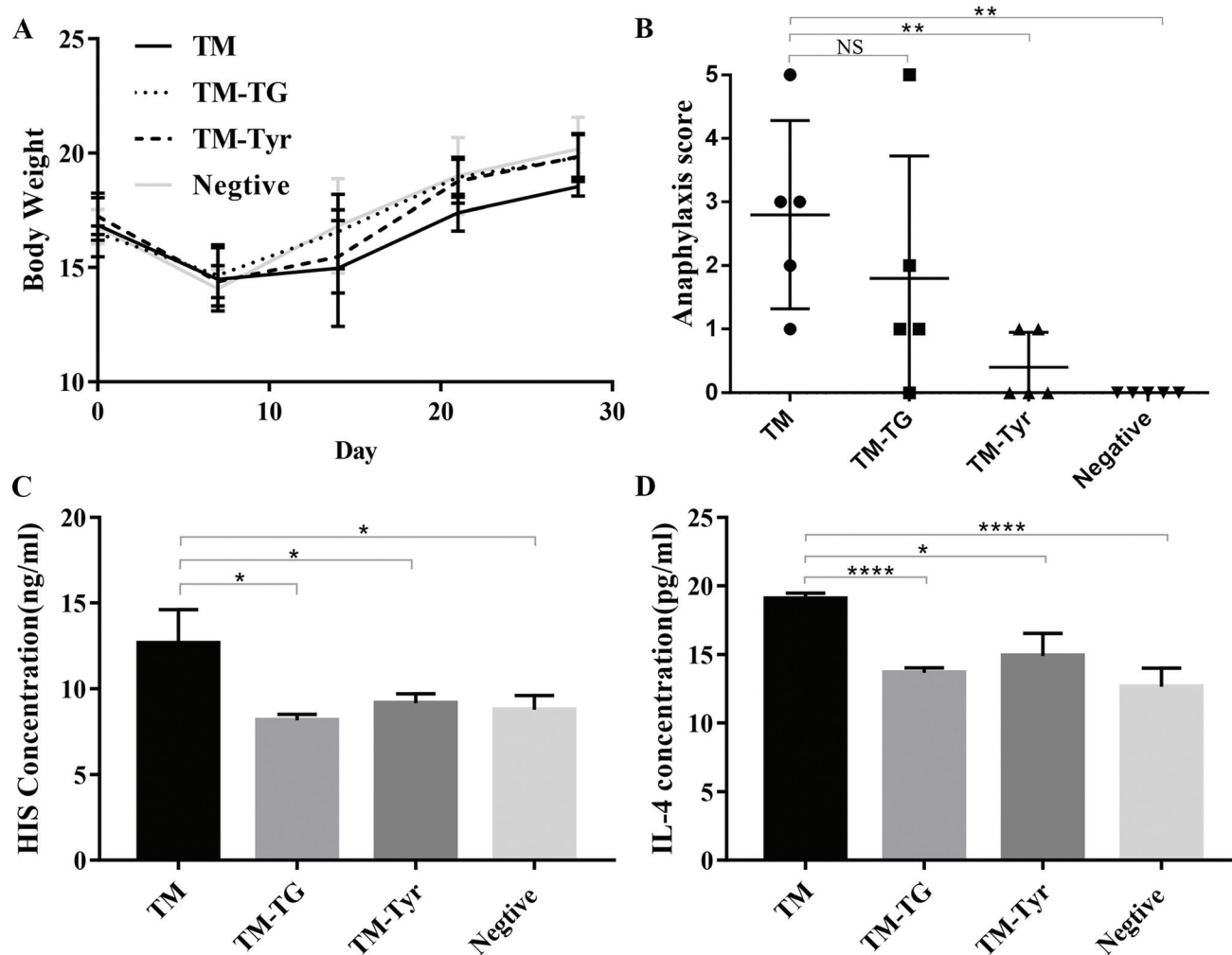


Fig. 5 TG or Tyr treatment reduced TM-induced food allergy *in vivo*. TG or Tyr treated TM samples were applied to a food allergy mouse model. Body weight (A), anaphylaxis responses (B), serum histamine concentration (C), splenic cell-produced IL-4 (D) and CD4⁺CD25⁺ Treg cell proportion (E) were determined. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. NS, not significant.

ing that the overall health of the mouse was impaired. In contrast, neither TM-TG nor TM-Tyr treatment decreased body weight gain (Fig. 5A). In addition, both the apparent anaphylaxis responses (Fig. 5B) and serum histamine increases (Fig. 5C) induced by TM were relieved when TM was treated with either TG or Tyr. Furthermore, compared with the splenic cells isolated from the TM group mice, those from the TM-TG or TM-Tyr group mice secreted a significantly lower level of IL-4, which is mainly produced by pro-allergic Th2 cells, implying that TG or Tyr treatment can eliminate the Th2-induction activity of TM (Fig. 5D).

3.6. TM-TG and TM-Tyr are potential hypoallergens

Based on the mouse model, we further measured the serum TM-specific antibody concentration. As shown in Fig. 6A and B, the TM-specific IgE level continued to rise throughout the sensitization process, but the level did not increase in the TM-TG and TM-Tyr groups, demonstrating that TG or Tyr treat-

ment eliminated the IgE-induction ability (allergenicity) of TM. Interestingly, after the last TM challenge, the serum TM-specific IgG2a concentrations in the TM, TM-TG and TG-Tyr groups were comparable to each other and all were significantly higher than that of the control group, indicating that the immunogenicity of TM was maintained after TG or Tyr treatment (Fig. 6C and D). To further investigate the immunological mechanisms of the phenomena, the percentage of CD4⁺CD25⁺ Tregs in the spleen was measured by flow cytometry (Fig. 6E). Surprisingly, this set of cells were dramatically expanded only in the TM-TG group, implying that TG treated TM resulted in the establishment of oral tolerance mediated by Tregs.

4. Discussion

The present study shows that the allergenicity of shrimp TM can be reduced by the cross-linking reaction with TG or Tyr.

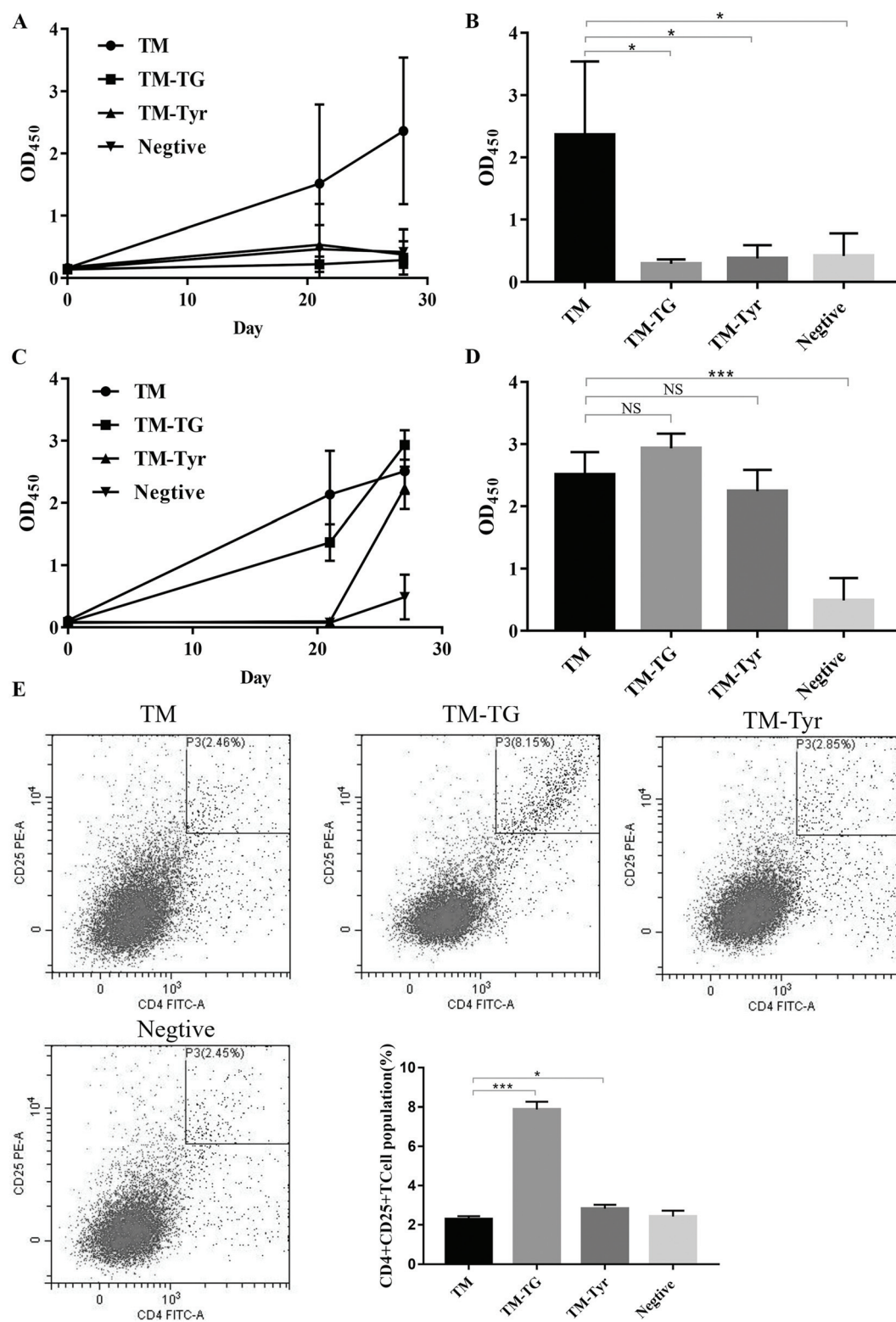


Fig. 6 TM-TG and TM-Tyr are potential hypoallergens. TG or Tyr treated TM samples were applied to a food allergy mouse model. Concentration of TM-specific IgE (A, B) and IgG2a (C, D) in the serum were measured by ELISA. Splenic CD4⁺CD25⁺ Treg cell proportion was determined by FACS (E). * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. NS, not significant.

Consistent with several previous studies,^{9,25,26} the cross-linking reaction resulted in the formation of a complicated protein complex, and thus changed the protein structure. SEM and CD results showed that TG or Tyr treatment greatly changed the protein structure of TM to different degrees, while TM-TG formed a tight network, TM-Tyr formed a film-like structure, and the structural change of TM-TG was larger than that of TM-Tyr. These discrepancies may explain the diverse effects of the products on the immune system and thus influence further applications of the method.

Because the protein structure is the basis of allergenicity, in principle the change of the protein structure may alter the allergenicity, either increase or decrease, which has been proved by plenty of research studies^{27–29} as well as the present work. After TG or Tyr treatment, the specific IgE-binding capacity, mast cell degranulation ability and *in vivo* allergenicity of TM were all reduced. The effects of TG and Tyr may be explained by the aggregation of cross-linked TM, which covered the surface epitopes and made them inaccessible to IgE and some specific immune cells. Interestingly, the results of these assays were not totally consistent: while TM-TG showed a lower IgE-binding capacity in ELISA, no significant difference was observed in the degranulation assay. This could be due to the different aggregation degrees of TM-TG and TM-Tyr, which produces multivalent allergens and is another factor affecting mast cell degranulation in addition to the IgE-binding capacity.³⁰

In the mouse model, we found that both TG and Tyr treatments abolished the IgE-induction activity of TM, demonstrating that the allergenicity of TM was eliminated. Interestingly, unlike IgE, TM-specific IgG2a could be induced in TM-TG and TM-Tyr groups after the last challenge; in other words, the immunogenicity of TM was maintained after TG or Tyr treatment, making TM-TG and TM-Tyr potential hypoallergens. This result demonstrated that the elimination of allergenicity was not due to the complete destruction of antibody epitopes, but it involved some more complex mechanisms. Similar results have been obtained by previous studies.⁹ Although enzymatic cross-linking has been already used in the processing of food allergens,^{31,32} the hypoallergenic property of the product has never been revealed. Consequently, TG and Tyr treatments are promising ways of producing hypoallergens that may be used for the immunotherapy of food allergy.

Despite more detailed immunological studies on the mouse model revealing similar effects on the reduction of apparent allergenicity, TM-TG and TM-Tyr used different mechanisms to communicate with the immune system. Although both TM-TG and TM-Tyr groups produced a comparable level of TM-specific IgG2a at last, they took different paths to yield similar results: TM-specific IgG2a gradually escalated throughout the whole sensitization process in the TM-TG group, but dramatically rose from the basal level at the last stage after allergen challenge in the TM-Tyr group, implying the discrepant immunogenicity between the two groups. Furthermore, flow cytometry results demonstrated that only TM-TG but not TM-Tyr promoted Treg cell proliferation. Interestingly, the pro-

portion of the CD4 + CD25 – T cell in the TM-TG group was reduced, implying that these cells might convert into Tregs. Because of the central role of Treg in establishing oral tolerance to food allergens,³³ these results suggested that TG treated TM could be a hypoallergen that gradually establishes tolerance by activating Tregs; however, Tyr treated TM regulates the immune system in a Treg-independent way. According to the anaphylaxis score, TM-Tyr showed weaker allergenicity than TM-TG; therefore, how TM-Tyr induces tolerance without the participation of Treg is worth exploring by further investigations. It has been reported that different epitopes may result in different immune responses; while IgE epitopes usually lead to sensitization, T cell epitopes may lead to desensitization and tolerance and thus can be used for allergen-specific immunotherapy.²⁰ According to this, it can be suggested that Tyr treatment may change the number and type of TM epitopes so as to trigger different responses.

It should be noted that to facilitate the mechanical study and improve the repeatability of the experiments as well as the reliability of the results, purified TM rather than whole shrimp protein was used in this study, which is different from the actual application situations. However, because TM is the predominant allergen in shrimp, establishing the tolerance to TM can be effective at treating most shrimp allergy cases to a certain extent. Nevertheless, the efficiency should be carefully verified before practical applications, and far more studies with whole shrimp protein are required for more universal applications.

Overall, in the present study, we investigated the effect of TG and Tyr on the shrimp allergen TM. Both TG and Tyr catalyzed the cross-linking of TM and changed the protein structure. Moreover, the allergenicity of TM was decreased but the immunogenicity remained after treatment, making the products potential hypoallergens. These results provided new insights into the production of low allergenic foods and the development of novel immunotherapy approaches.

Abbreviations

TM	Tropomyosin
TG	Transglutaminase
Tyr	Tyrosinase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ELISA	Enzyme linked immunosorbent assay
IL-10/4/8	Interleukin-10/4/8
CD	Circular dichroism
Treg cell	Regulatory T cell
IgE/IgG2a	Immunoglobulin E/G2a
SEM	Scanning electron microscopy

Conflicts of interest

The authors declare that they have no conflict of interest.

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References

- 1 L. Jing and M. Alcocer, Overview of the Commonly Used Methods for Food Allergens, *Methods Mol. Biol.*, 2017, **1592**, 1.
- 2 R. S. Geha, Allergy and hypersensitivity : Nature versus nurture in allergy and hypersensitivity, *Curr. Opin. Immunol.*, 2003, **15**, 603–608.
- 3 P. Tong, J. Gao, H. Chenab, Y. Zhang, S. Jian, H. Wichers, Z. Wu, A. Yang and F. Liu, Effect of heat treatment on the potential allergenicity and conformational structure of egg allergen ovomucoid, *Food Chem.*, 2012, **131**, 603–610.
- 4 A. M. Scurlock and S. M. Jones, An update on immunotherapy for food allergy, *Curr. Opin. Allergy Clin. Immunol.*, 2010, **10**, 587.
- 5 M. Pedrosa, T. Boyanomatinez, C. Garcíaara and S. Quirce, Shellfish Allergy: a Comprehensive Review, *Clin. Rev. Allergy Immunol.*, 2015, **49**, 203.
- 6 J. Wu, S. Tang, S. Huang and Y. Huo, Progress in the modification of food protein by enzymatic crosslinking, *Food Industry Sci. Technol.*, 2011, 511–513.
- 7 E. Monogioudi, G. Faccio, M. Lille, K. Poutanen, J. Buchert and M. L. Mattinen, Effect of enzymatic cross-linking of β -casein on proteolysis by pepsin, *Food Hydrocolloids*, 2011, **25**, 71–81.
- 8 G. Liu, Effects of processing methods on the digestive stability and anaphylaxis of crab myosin, *J. Chin. Inst. Food Sci. Technol.*, 2011, **11**, 14–22.
- 9 G. Liu, M. Hu, L. C. Sun, X. Han, Q. Liu, M. Alcocer, D. Fei, M. J. Cao and G. M. Liu, Allergenicity and Oral Tolerance of Enzymatic Cross-Linked Tropomyosin Evaluated Using Cell and Mouse Models, *J. Agric. Food Chem.*, 2017, **65**, 2205–2213.
- 10 B. Jiang, Properties of Glutamine Transaminase and Its Application in Food, *Chin. Food Addit.*, 2001, 34–36.
- 11 D. A. Clare, G. Gharst, S. J. Maleki and T. H. Sanders, Effects of Transglutaminase Catalysis on the Functional and Immunoglobulin Binding Properties of Peanut Flour Dispersions Containing Casein, *J. Agric. Food Chem.*, 2008, **56**, 10913.
- 12 E. Selinheimo, K. Autio, K. Kruus and J. Buchert, Elucidating the mechanism of laccase and tyrosinase in wheat bread making, *J. Agric. Food Chem.*, 2007, **55**, 6357–6365.
- 13 J. Radosavljevic, E. Nordlund, L. Mihajlovic, M. Krstic, T. Bohn, J. Buchert, T. C. Velickovic and J. Smit, Sensitizing potential of enzymatically cross-linked peanut proteins in a mouse model of peanut allergy, *Mol. Nutr. Food Res.*, 2014, **58**, 635–646.
- 14 L. Lv, H. Lin, Z. Li, J. Wang, I. Ahmed and H. Chen, Changes of structure and IgE binding capacity of shrimp (*Metapenaeus ensis*) tropomyosin followed by acrolein treatment, *Food Funct.*, 2017, **8**, 1028–1036.
- 15 D. R. Hoffman, E. D. Day and J. S. Miller, The major heat stable allergen of shrimp, *Ann. Allergy*, 1981, **47**, 17.
- 16 L. Fu, J. Wang, S. Ni, C. Wang and Y. Wang, Identification of allergenic epitopes and critical amino acids of major allergens in Chinese shrimp (*Penaeus chinensis*) by Immunoinformatics Coupled with Competitive-binding Strategy, *J. Agric. Food Chem.*, 2018, **66**, 2944–2953.
- 17 D. X. Fei, Q. M. Liu, F. Chen, Y. Yang, Z. W. Chen, M. J. Cao and G. M. Liu, Assessment of the sensitizing capacity and allergenicity of enzymatic cross-linked arginine kinase, the crab allergen, *Mol. Nutr. Food Res.*, 2016, **60**, 1707–1718.
- 18 L. Zuidmeerjongejan, M. Fernandezrivas, L. K. Poulsen, A. Neubauer, J. Asturias, L. Blom, J. Boye, C. Bindslevjensen, M. Clausen and R. Ferrara, FAST: towards safe and effective subcutaneous immunotherapy of persistent life-threatening food allergies, *Clin. Transl. Allergy*, 2012, **2**, 5–5.
- 19 H. P. M. van der Kleij, H. J. M. Warmenhoven, R. van Ree, S. A. Versteeg, R. H. H. Pieters, S. C. Dreskin, A. C. Knulst, E. V. Hoffen, D. J. E. Opstelten, S. J. Koppelman and J. J. Smit, Chemically modified peanut extract shows increased safety while maintaining immunogenicity, *Allergy*, 2018, DOI: 10.1111/all.13687.
- 20 M. Bublin, M. Kostadinova, C. Radauer, E. M. Varga, C. Hafner, K. Schmidthaler, A. Saidova, S. J. Maleki, Z. Szepefalusi, T. Eiwegger and H. Breiteneder, Engineering of structural variants of the major peanut allergens Ara h 2 and Ara h 6 for allergen-specific immunotherapy, *J. Allergy Clin. Immunol.*, 2018, DOI: 10.1016/j.jaci.2018.10.039.
- 21 L. Fu, S. Fu, Y. Wang, C. Wang, Y. Zhang, Q. Wu and A. MA, Optimization of separation and purification of myosin sulfate from *Penaeus vannamei*, *Food Sci.*, 2017, **38**, 187–192.
- 22 L. Fu, J. Peng, S. Zhao, Z. Yan, X. Su and Y. Wang, Lactic acid bacteria-specific induction of CD4 + Foxp3 + T cells ameliorates shrimp tropomyosin-induced allergic response in mice via suppression of mTOR signaling, *Sci. Rep.*, 2017, **7**, 1987.
- 23 L. Fu, J. Song, C. Wang, S. Fu and Y. Wang, Bifidobacterium infantis Potentially Alleviates Shrimp Tropomyosin-Induced Allergy by Tolerogenic Dendritic Cell-Dependent Induction of Regulatory T Cells and Alterations in Gut Microbiota, *Front. Immunol.*, 2017, **8**, 1536.
- 24 J. U. Rüggeberg, Y. Liu and S. Zuo, Allergy: Guidelines for the collection, analysis and description of case definition and vaccination safety data, *Chin. J. Vaccines Immun.*, 2017, 121–125.
- 25 K. Liu, S. Chen, H. Chen, P. Tong and J. Gao, Cross-linked ovalbumin catalyzed by polyphenol oxidase: Preparation,

- structure and potential allergenicity, *Int. J. Biol. Macromol.*, 2017, **107**, 1–12.
- 26 C. Li, Y. L. Xiong and J. Chen, Oxidation-induced unfolding facilitates Myosin cross-linking in myofibrillar protein by microbial transglutaminase, *J. Agric. Food Chem.*, 2012, **60**, 8020.
 - 27 G. Bu, Y. Luo, Z. Zheng and H. Zheng, Effect of heat treatment on the antigenicity of bovine α -lactalbumin and β -lactoglobulin in whey protein isolate, *Food Agric. Immunol.*, 2009, **20**, 195–206.
 - 28 Z. Hu, X. Wang and Q. Zhang, Relationship between structural changes of shrimp myosin induced by high pressure treatment and sensitization, *Food Sci.*, 2017, **38**, 33–39.
 - 29 F. Yuan, L. Lv, Z. Li, N. Mi, H. Chen and H. Lin, Effect of transglutaminase-catalyzed glycosylation on the allergenicity and conformational structure of shrimp (*Metapenaeus ensis*) tropomyosin, *Food Chem.*, 2016, **219**, 215.
 - 30 L. Fu, C. Wang and Y. Wang, Seafood allergen-induced hypersensitivity at the microbiota-mucosal site: implications for prospective probiotic use in allergic response regulation, *Crit. Rev. Food Sci. Nutr.*, 2017, **58**, 1512–1525.
 - 31 I. Ahmed, L. Lv, H. Lin, Z. Li, J. Ma, C. Guanzhi, L. Sun and L. Xu, Effect of tyrosinase-aided crosslinking on the IgE binding potential and conformational structure of shrimp (*Metapenaeus ensis*) tropomyosin, *Food Chem.*, 2018, **248**, 287–295.
 - 32 F. Yuan, I. Ahmed, L. Lv, Z. Li, Z. Li, H. Lin, H. Lin, J. Zhao, S. Tian and J. Ma, Impacts of glycation and transglutaminase-catalyzed glycosylation with glucosamine on the conformational structure and allergenicity of bovine β -lactoglobulin, *Food Funct.*, 2018, **9**, 3944–3955.
 - 33 O. Palomares, The role of regulatory T cells in IgE-mediated food allergy, *J. Invest. Allergol. Clin. Immunol.*, 2013, **23**, 371.