Research Article

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Characterization of systemic allergenicity of tropomyosin from shrimp (*Macrobrachium nipponense*) and anaphylactic reactions in digestive tract

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Abstract

BACKGROUND: Tropomyosin (TM) is the major allergen of crustaceans. The allergenicity of TM from *Macrobrachium nipponense* (*Mn*TM) and the anaphylactic reaction in the digestive tract are still unclear. The aim of this study was to evaluate the allergenicity of *Mn*TM and the anaphylactic reaction in the digestive tract.

RESULTS: Serum IgE and IgG1 binding ability in the TM group were significantly higher than those in the PBS and CT groups ($P < 0.01$) and CP group ($P < 0.05$), while serum IgG and IgG2a binding ability showed no obvious difference between the four groups ($P > 0.05$). The levels of cytokines IL-4, IL-5 and IL-13 in TM and CP groups were significantly higher than those in PBS and CT groups. Histamine and β-hexosaminidase in the TM and CP groups from basophil cell models were significantly higher than those in the PBS group. The highest mRNA expression of IL-4 and IL-13 was in the jejunum from TM-sensitized mice. Histopathological analysis showed that more immune cells infiltrated into the jejunum than the duodenum and ileum from the TM-sensitized mice.

CONCLUSIONS: *Mn*TM could promote an allergic response in mice and lead to degranulation in basophil cells. The jejunum was more easily affected by *Mn*TM than duodenum and ileum, and the jejunum may be the major site of allergic response in the digestive tract.
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Supporting information may be found in the online version of this article.

Keywords: tropomyosin; *Macrobrachium nipponense*; allergenicity; mRNA expression; histopathology

INTRODUCTION

Crustaceans, including shrimps and crabs, are considered as high-quality foods with high nutritional value, providing abundant dietary proteins and omega fatty acids to the body. However, crustaceans are also one of the eight major sources of food allergens,[1] leading to food allergies in particular populations. Shrimp farming is popular worldwide, and *Macrobrachium nipponense*, one of the most important freshwater shrimps, is widely distributed and cultured in the area of the Yangtze River of China. With the increased consumption of shrimps, allergic diseases caused by *M. nipponense* are becoming more prevalent in China; however, up to now, few studies are available concerning the allergens from *M. nipponense*.

Tropomyosin (TM) has been identified as the major allergen in most crustacean species,[2,3] and as the major invertebrate pan-allergen found in all edible crustacean species, it has highly conserved amino acid sequences and protein structures. The homology of TM amino acid sequences ranges from 93% to 99% in crustaceans[4] with a molecular weight of approximately 35 kDa. To date, TM has been identified in numerous invertebrate species, such as crustaceans (lobster,[5] prawn,[6] shrimp,[7] crab[8]), mollusks,[9] cockroaches[10] and mites.[11] TM from *M. nipponense* (*Mn*TM) was previously purified and identified in our laboratory. To further explore the characteristics of *Mn*TM, the allergenicity of *Mn*TM was assessed in murine and cell models in the study reported here.

Currently, there are numerous methods for evaluating allergencity, including serological analysis, simulated gastric digestion, bioinformatics analysis and *in vitro* tests in cell and animal

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models.[12] Animal models can provide direct and powerful methods for the preliminary assessment of the potential allergenicity of food allergen. Murine strains, including BALB/c, C57BL/6, C3H/HeOuJ and A/J, are the most commonly used to build allergic models[13,14] to study the pathophysiology of food allergies, which showed similarities to food allergies of humans.[15] Changes in serum IgE levels, inflammatory cell recruitment and Th2 cytokine secretions could be detected in murine models.[15] Th2-related cytokines, such as IL-4, IL-5 and IL-13, are induced rapidly in food allergies. These cytokines can promote allergens to bind to the Fcε receptors on the surface of mast cells and basophils.[16,17] However, the binding process could be modulated by several factors. Firstly, the cytokines secreted by regulatory T cells, including IL-10 and TGF-β, could suppress the production of Th2-related cytokines. Secondly, IFN-γ produced by Th1 cells could relieve allergy symptoms since IFN-γ could suppress Th2 differentiation and proliferation.[17] Therefore, changes in cytokines may reflect the type of allergic response and severity of anaphylaxis.

Moreover, the degranulation of cell models *in vitro* could directly reflect the allergenicity of food allergens.[18] Cell models, such as human basophil and mast cells, have been successfully used to investigate the mechanisms of allergenicity. Basophils are important immune cells used to define food allergy by measuring the levels of β-hexosaminidase and histamine,[19,20] which are important indicators of the degranulation of basophils. The activity of basophils and the degree of degranulation are positively correlated with allergenicity.

In the work reported here, we focused on the characterization of systemic allergenicity of *Mn*TM *in vivo* and *in vitro* and the anaphylactic reactions in the digestive tract of TM-sensitized mice. Regarding the evaluation of the allergic response *in vivo*, BLAB/c mouse models were established, followed by analysis of the allergic symptoms, allergic-related antibodies and cytokines. The degranulation of basophil cells was used to assess the allergic response *in vitro*. In order to investigate the anaphylactic reactions in the digestive tract, the mRNA expression of allergic-related cytokines and the morphology of the digestive tract were analyzed in the TM-sensitized-mice.

MATERIALS AND METHODS

Preparation of crude protein and TM

TM and crude protein were extracted and purified from *M. nipponense*. The shrimps were peeled and minced quickly with a FastPrep-24 5G (MP, USA), and then immersed in 2-fold volume of 50% precooled acetone and stirred continuously for 4 h at 0 °C. Afterwards, the supernatant was collected at 10 000 × *g* for 20 min (4 °C), and then filtered through 0.45 μm membranes. Shrimp proteins were subjected to salting-out with 30% (w/v) ammonium sulfate, and then the mixture was centrifuged at 10 000 × *g* for 20 min at 4 °C. The pellet was re-suspended with 20 mmol L^{-1} Tris–HCl buffer. The crude proteins of *M. nipponense* were produced, followed by analysis with sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (supporting information, Fig. S1), and concentrated using PEG 20000.

Moreover, the crude proteins were further purified using Tris–HCl buffer DEAE Fast Flow (500 mL; General Electric Company, USA) anion-exchange chromatography column (3.5 × 100 cm), and eluted with a linear gradient of NaCl (0–0.5 mol L^{-1}) in phosphate buffer (PBS; 0.02 mol L^{-1}, pH 8.0). Afterwards, the target eluent was collected and dialyzed against distilled water for 48 h at 4 °C, followed by freeze-drying and stored at −20 °C until use. To identify the proteins of purified proteins, LC-MS/MS analysis (Applied Protein Technology, Shanghai, China) was performed using Easy nLC and Q Exactive (Thermo Scientific, USA). The results were searched and compared in the Uni-ProtKB database (www.uniprot.org) by MaxQuant, Mascot2.2 and Proteome Discoverer 1.4 (supporting information Fig. S2; supporting information Table S1). The purity and concentration were determined using SDS-PAGE and the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA, USA) (supporting information, Fig. S1).

Animals

Forty BALB/c mice with a body weight of 18.0 ± 2.0 g (female; 5–6 weeks old) were purchased from the Center of Laboratory Animal Science of Nanchang University. All experiments were supervised by the Animal Research Ethics Board of Nanchang University (permission number SYXK (Gan) 2015-0001). The mice were housed in a room with a controlled temperature of 22 ± 1°C, 53 ± 5% relative humidity, and a 12 h light–dark cycle. All mice were acclimatized to the conditions for 1 week before experimental manipulation. Enough synthetic mice feed without crustaceans was provided for the mice during the experiment, and water was supplied continuously by automatic ports.

Animal models of TM allergy

The 40 mice were randomly divided into four groups, namely PBS group, cholera toxin group (CT group), crude proteins group (CP group) and the prepared TM group (TM group). The mice of the CP and TM groups were sensitized by intragastric administration with 200 μg of crude proteins and purified TM per mouse and containing 10 μg of CT (ABsin, Shanghai, China) in a total of 300 μL of PBS, on days 0, 7, 14 and 21. The mice of the PBS group were intragastrically administered with 300 μL of PBS without CT, and the mice of the CT group were intragastrically administered with 300 μL of PBS containing 10 μg of CT. All mice were orally challenged on day 28 with 800 μg of prepared TM proteins per mouse in a total of 300 μL of PBS (Fig. 1). Then, 40 min after final challenge, the allergic symptoms were recorded using a validated anaphylactic scoring (Table 1) and the body weight measured (25.2 ± 2.0 g), and mice were sacrificed under carbon dioxide narcosis for collecting blood, spleen, duodenum, jejunum, ileum, etc. All these tissues were frozen for further use.

Measurement of serum antibody levels

Indirect enzyme-linked immunosorbent assay (ELISA) was used to analyze the serum allergen-specific antibody levels, including IgE, IgG, IgG1 and IgG2a. Microtiter plates were coated with purified TM (15 μg mL^{-1}) in 50 mmol L^{-1} sodium carbonate buffer (pH 9.6) for 12 h at 4 °C. Serum samples were diluted to different concentrations (1:20 for specific IgE; 1:200 for specific IgG; 1:50 for specific IgG1 and IgG2a) with PBS, and 100 μL of diluted serum was added in duplicate, followed by incubation for 1 h at 37 °C. The secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse IgE, IgG, IgG1 and IgG2a antibodies (1:6000; SouthernBiotech, USA). *o*-Phenylenediamine substrate was added in the plates for 15 min at 37 °C, and then were terminated with 2 mol L^{-1} H_2SO_4. The optical density was analyzed at 490 nm with a microplate reader (Benchmark 96, Bio-Rad Laboratories, Hercules, CA, USA).

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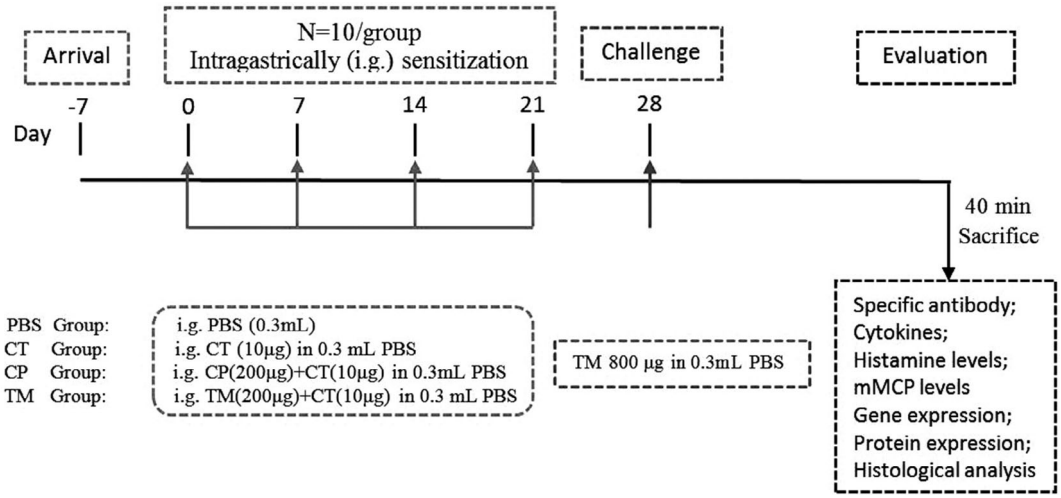


Figure 1. Oral immunization of BALB/c mice with PBS, CT, CP and TM. BALB/c mice were immunized weekly by intragastric administration with the crude proteins or the prepared TM using CT as an adjuvant. After challenge, bloods were taken for specific antibody, cytokine, histamine and mMCP level assays, and tissues were used to analyze the gene expression and histopathology.

Table 1. Anaphylactic symptom scoring

Score	Symptoms
0	No symptoms
1	Scratching nose and mouth
2	Swelling around the eyes and mouth; pillar erection; reduced activity; high breathing rate
3	Shortness of breath; blue rash around the mouth and tail; higher breathing rate
4	No activity after stimulation; shivering and muscle contractions
5	Death by shock

Measurements of mouse mMCP-1 and histamine levels

The levels of mMCP-1 in serum and histamine in plasma were measured using mouse mMCP-1 and histamine available ELISA Ready-SET-Go enzyme immunoassay kit (Affandi-e, Shanghai, China), according to the manufacturer's instructions.

Preparation of lymphocytes and *in vitro* stimulation

Spleens were collected from the mice, followed by being minced and filtered through sterile nylon filters. All cells were re-suspended in complete RPMI 1640 medium. Cells were lysed for 15 min on ice using Red Blood Cell Lysis (Solarbio, Beijing, China), followed by centrifugation to collect cells, and then re-suspended in complete RPMI 1640 medium. Spleen lymphocytes were divided to quadruplication, and 1 mL of medium (containing 2×10^6 cells) was seeded into each well of 12-well plates, followed by adding Con A (1 μg mL^{-1}; Sigma, USA) and the purified TM (50 μg mL^{-1}) to each well. Afterwards, cells were incubated at 37 °C in 5% CO_2 for 72 h, followed by collection of the supernatants for analysis of cytokines.

Analysis of cytokines

The supernatants of cell cultures in each group ($n = 6$) were used to analyze cytokine levels. The cytokines involved were IFN-γ, IL-4, IL-5, IL-13, IL-17A, IL-21, IL-22, IL-23, IL-10 and TGF-β.

An assay of a panel of cytokines was carried out with a Procarta-Plex™ Multiplex Immunoassay Kit (Invitrogen, CA, USA). Samples (25 μL) in duplicate were added to a 96-well filter plate pre-configured with a panel of anti-cytokine antibodies covalently linked to unique polystyrene beads. Standards (7-point dilutions) and controls were added at this time. The detailed steps are contained in the product specifications. Finally, the plate was transferred to a reader (Luminex, TX, USA) for quantitative analysis. TGF-β was measured with a TGF-β available ELISA Ready-SET-Go enzyme immunoassay kit (Affandi-e, Shanghai, China).

Assessment of degranulation induced by TM in basophil cells

A human basophil cell line, KU812 cells, was cultured in complete IMDM media, followed by measuring the cells and seeding the cells in 6-well plates (2×10^6 cells per well). The cells were incubated at 37 °C in 5% CO_2 overnight. Afterwards, the purified TM and crude proteins from *M. nipponense* (50 μg mL^{-1}) were added to stimulate cells for 12 h, while the control cells were stimulated by an equal volume of PBS. Finally, the culture supernatants were collected to measure histamine and the β-hexosaminidase. The methods were followed according to the manuals of the ELISA kits (Affandi-e, Shanghai, China).

Cytokine mRNA expression in digestive tract

Total RNA was extracted from the duodenum, jejunum and ileum of mice using a Multisource Total RNA Miniprep Kit (Axygen, Hangzhou, China). The quality and quantity of total RNA were assessed using a nucleic acid analyzer (PerkinElmer, USA). The cDNA was synthesized using HiScript II Q RT SuperMix for qPCR (Vazyme, Nanjing, China), and the samples were stored at −20 °C until use. The β-actin protein of mice (no. BC138614) was used as an internal control. All primers for real-time polymerase chain reaction (PCR) are presented in Table 2.

Real-time PCR was conducted using ChamQ™ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) in an ABI 7500 real-time PCR system (ABI, USA). The reaction volume was 20 μL. The specific methods were according to the manufacturer's protocols. The amplified results were analyzed using 7500 system SDS v 2.2

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Table 2. Primers for real-time PCR used in this study

Primer	Forward (5′–3′)	Reverse (5′–3′)
IL-4	CATCCTGCTCTTCTTTCTCG	CTGTGGTGTTCTTCGTTGCT
IL-13	CTCTTGCTTGCCTTGGTGGT	GGGGAGTCTGGTCTTGTGTGAT
TGF-β	ACCGCAACAACGCCATCTAT	ACCAAGGTAACGCCAGGAAT
IFN-γ	CTGGAGGAACTGGCAAAAG	TGGACCTGTGGGTTGTTGAC
β-Actin	CTGAGAGGGAAATCGTGCGT	TCCATACCCAAGAAGGAAGGC

software (ABI, USA). The relative expression level was evaluated based on the $2^{-\Delta\Delta Ct}$ method.[21]

Histopathological analysis in digestive tract

Segments of the digestive tract, including duodenum, jejunum and ileum, were excised immediately from mice in each group ($n = 5$ per group) after the mice were sacrificed. These tissues were washed with PBS and fixed in 4% paraformaldehyde with PBS, followed by being embedded in paraffin. Slides (4–5 μm) were sectioned and stained with hematoxylin and eosin for histological observation. The images were recorded with a CKX41 microscope (Olympus, Japan). The numbers of eosinophils, lymphocytes and goblet cells were counted in 25 random fields (×400 magnification) for 5 slides from 5 mice in the TM and PBS groups.

Statistical analysis

Statistical evaluation of the data was performed by one-way analysis of variance using SPSS.16.0 (IBM SPSS, Armonk, NY, USA). Data are expressed as mean ± standard deviation and were analyzed by one-way or two-way analysis of variance. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Anaphylactic shock score

The system anaphylactic reactions of mice of four groups ($n = 10$ per group) were assessed in the BALB/c mice. Anaphylactic symptoms were not detected in mice in the PBS and CT groups. Most of the mice in the CP and TM groups were found to have various anaphylactic symptoms. One mouse had a serious anaphylactic symptom with a higher breathing rate at a score of 3 in the TM group. Six out of ten mice in the CP and TM groups had symptoms, such as reducing activity and swelling around the mouth (score 2). Four out of ten mice in the TM group and two out of ten mice in the CP group had only slight cutaneous reaction with scratching nose and mouth (score 1). The allergic symptoms of the CP and TM groups were significantly different from those of the PBS and CT groups ($P < 0.01$) (Fig. 2).

Serum-specific IgE, IgG, IgG1 and IgG2a levels

The sensitive response to TM was evaluated by detecting serum-specific antibody levels *in vivo*. Serum-specific IgE was analyzed using ELISA kits. The TM-specific IgE levels in the TM and CP groups were significantly higher than those in the PBS and CT groups ($P < 0.01$), while there was no significant difference between the PBS and CT groups (Fig. 3(a)). Serum-specific IgGs were analyzed in the four groups. The IgG1 change trends were similar to those of IgE. The TM-specific IgG1 levels in the TM and CP groups were significantly higher than those in the PBS and CT groups ($P < 0.01$) (Fig. 3(b)). The IgG and IgG2a levels did not alter significantly among the four groups ($P > 0.05$) (Figs 3 (c) and (d)).

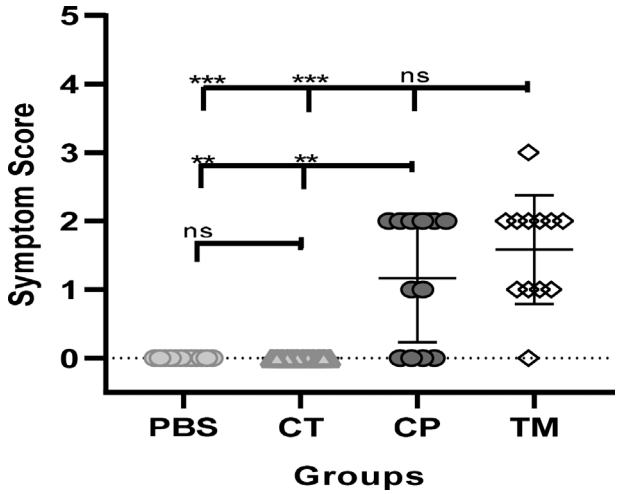


Figure 2. Score of hypersensitivity symptoms. The symptoms of hypersensitivity were scored on a scale from 0 (no symptoms) to 5 (death) as described in Table 1. One-way analysis was used to determine statistical significance. ***$P < 0.0001$, **$P < 0.01$, *$P < 0.05$, ns $P > 0.05$.

Measurements of mouse histamine and mMCP-1 levels

The concentration of plasma histamine in the TM group was 28.11 ± 3.67 μg L^{-1}, which was significantly higher than those of the PBS and CT groups ($P < 0.01$). The concentration in the CP group (23.43 ± 3.86 μg L^{-1}) was significantly higher than that in PBS and CT groups ($P < 0.05$), whereas there was no significant difference between PBS and CT groups nor between TM and CP groups ($P > 0.05$) (Fig. 4(a)). The mMCP-1 levels in the TM and CP groups were significantly higher than those in the PBS and CT groups ($P < 0.01$). However, there was no obvious difference between the CT group and the TM group ($P > 0.05$) (Fig. 4(b)). These results indicated that the prepared TM and CP could induce the production of histamine and mMCP-1 in serum.

Multiplex cytokine assay

Cytokines were measured in the supernatant of splenocyte culture media. It was found that the level of IFN-γ in the TM group was significantly lower than those in the PBS and CT groups ($P < 0.05$). Regarding the Th2-related cytokines, levels of IL-4, IL-5 and IL-13 in the CP and TM groups were significantly higher than those in the PBS and CT groups ($P < 0.05$). The levels of IL-17A, IL-21 and IL-22 were increased in the CP and TM groups compared with the PBS and CT groups, while the levels of IL-23 in the CP and TM groups were lower than those in the PBS and CT groups. Regarding regulatory T cell-related cytokines, levels of IL-10 and TGF-β in the CP and TM groups were lower than those in the PBS and CT groups, but there was no significant difference among the four groups ($P > 0.05$) (Table 3).

Degranulation of basophil cells

Basophils were stimulated by the prepared TM and crude proteins from *M. nipponense* for 12 h, and then histamine and β-hexosaminidase were measured using ELISA kits. The results showed that histamine and β-hexosaminidase could both be induced by the purified TM and crude proteins from *M. nipponense*. The concentrations of histamine in the TM and

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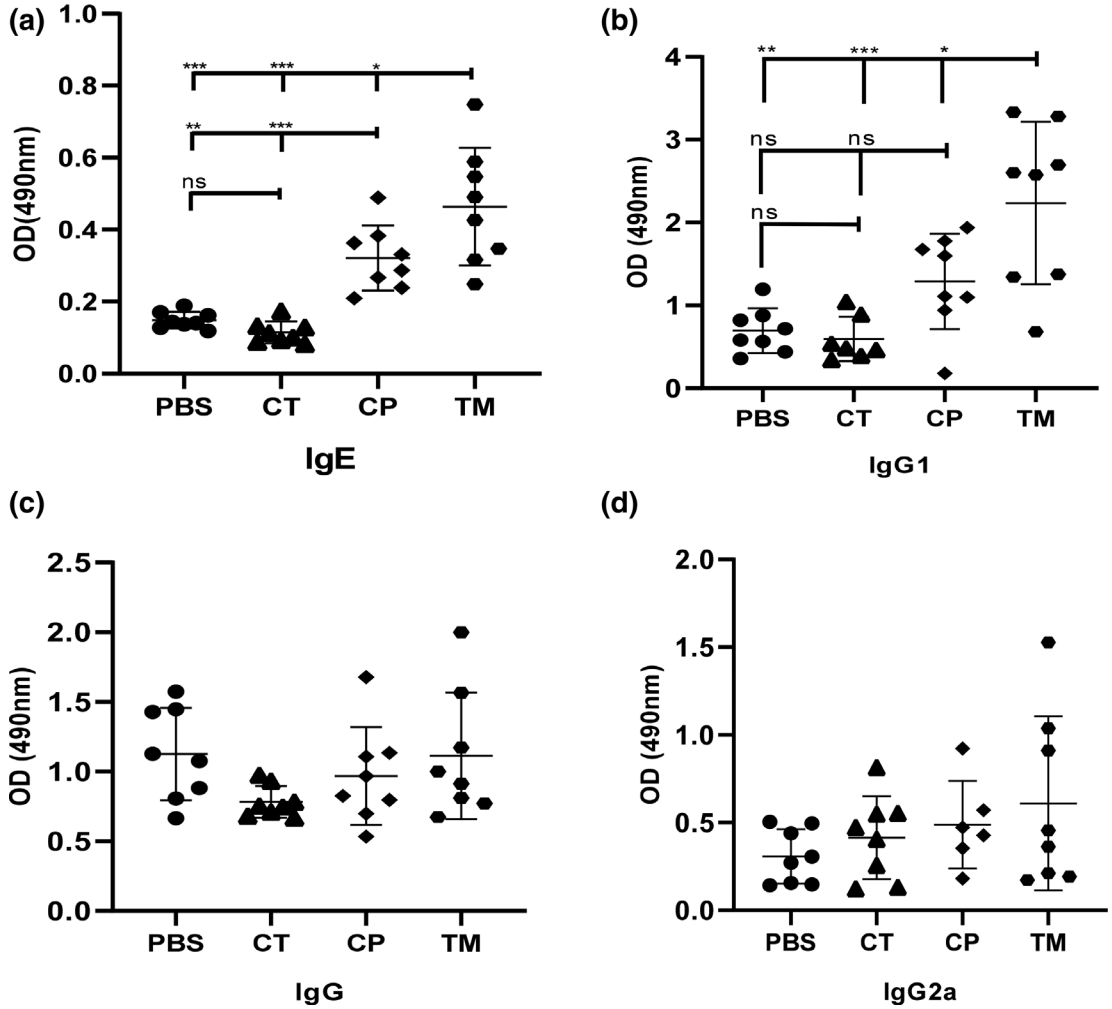


Figure 3. Levels of specific (a) IgE, (b) IgG1, (c) IgG and (d) IgG2a in sera from mice exposed to PBS, CT, CP and TM were detected using ELISA. One-way analysis was used to determine statistical significance. ***$P < 0.0001$, **$P < 0.01$, *$P < 0.05$, ns $P > 0.05$.

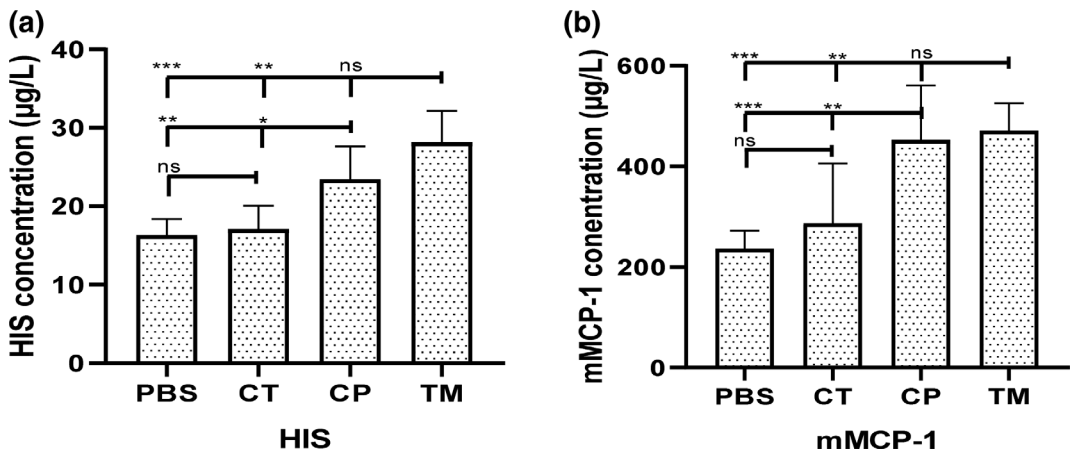


Figure 4. (a) Plasma histamine (HIS) level and (b) serum mMCP-1 level were determined using enzyme immunoassay kit. One-way analysis was used to determine statistical significance. ***$P < 0.0001$, **$P < 0.01$, *$P < 0.05$, ns $P > 0.05$.

CP groups (15.47 ± 1.05 and 12.91 ± 1.59 μg mL^{-1}, respectively) were significantly higher than that in the PBS group (9.53 ± 1.30 μg mL^{-1}) (Fig. 5(a)). The release rates of *β*-hexosaminidase in the TM and CP groups (63.88 ± 3.71% and 55.25 ± 2.27%, respectively) were significantly higher than that in the PBS group (36.24 ± 3.10%) (Fig. 5(b)). These observations indicated that the prepared TM and crude proteins from *M. nipponense* could induce basophils to release granules and promote an allergic response.

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Table 3. Multiplex cytokines (pg mL^{-1}) from supernatant of spleen cells

	Group			
Cytokine	PBS	CT	CP	TM
IFN-γ	5922 ± 127.3	5270 ± 134.1	3885 ± 159.3	1863 ± 83.43*
IL-4	15.84 ± 3.99	14.86 ± 2.71	36.20 ± 5.42*	39.26 ± 1.72*
IL-5	83.64 ± 28.49	78.30 ± 17.52	151.7 ± 12.45*	286.4 ± 81.78*
IL-13	228.7 ± 10.09	221.0 ± 9.84	469.2 ± 28.41*	697.9 ± 26.86*
IL-17A	907.5 ± 442.7	607.1 ± 314.1	1356 ± 555.7	1619 ± 432.5
IL-21	0.9725 ± 0.04	0.7540 ± 0.02	0.9140 ± 0.04	1.436 ± 0.08
IL-22	5554 ± 96.88	5136 ± 12.42	4624 ± 14.4	6614 ± 72.21
IL-23	31.88 ± 5.993	34.15 ± 4.260	30.01 ± 4.290	22.16 ± 3.575
IL-10	387.2 ± 218.5	398.7 ± 161.3	196.0 ± 55.23	193.8 ± 49.82
TGF-β	190.7 ± 13.80	172.2 ± 15.18	131.0 ± 6.805	142.7 ± 15.67

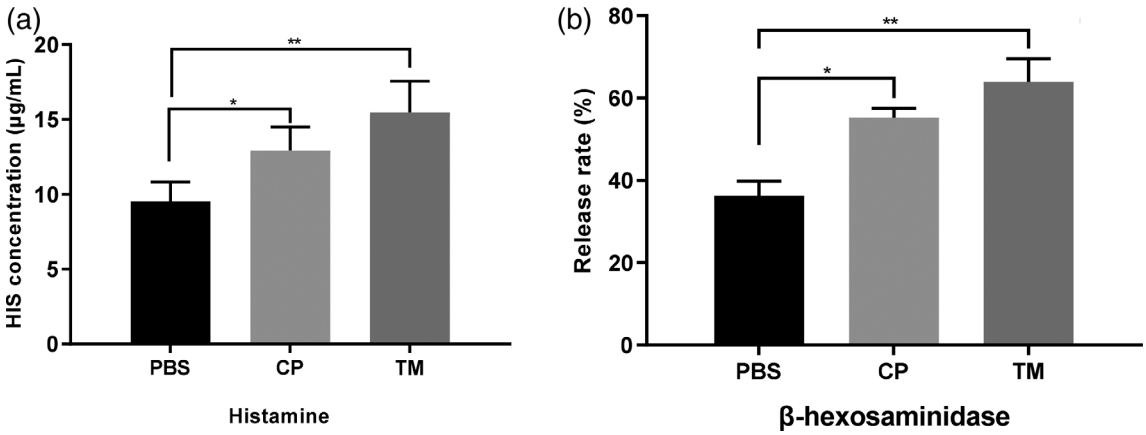


Figure 5. Concentrations of (a) histamine (HIS) and (b) β-hexosaminidase from basophils stimulated by prepared TM and crude proteins. One-way analysis was used to determine statistical significance. $^{**}P < 0.01$, $^{*}P < 0.05$.

mRNA expression of cytokines in digestive tract

In order to evaluate the allergic response in different parts of the digestive tract, we determined the IL-4, IL-13, IFN-γ and TGF-β mRNA expressions in duodenum, jejunum and ileum of mice. The four cytokines were expressed in duodenum, jejunum and ileum from four different groups. The highest expression levels of IL-4 and IL-13 were in the jejunum, the next were in the duodenum and the lowest levels were in the ileum (Figs 6(a) and (b)). However, IFN-γ and TGF-β mRNA expressions showed no significant differences in the digestive tract ($P > 0.05$). This indicated that jejunum exhibited a more severe allergic response than duodenum and ileum. Moreover, IL-4 and IL-13 expression levels in the TM and CP groups were higher than those in the CT and PBS groups, while IFN-γ and TGF-β expression levels in the TM and CP groups were lower than those in the CT and PBS groups. These results showed that TM and crude proteins from *M. nipponense* could induce an allergic response in the different parts of the digestive tract.

Histopathological analysis in digestive tract

In order to analyze the histopathology in the digestive tract, the duodenum, jejunum and ileum of mice were dissected and tissue sections were obtained. Histopathology of the duodenum revealed that the villi were regular and orderly and the intestinal crypts showed no evidence of swelling in the different groups. Immune cells, including lymphocytes and eosinophils, infiltrated into the villi and lamina propria in the TM and CP groups, while being less pronounced in the PBS and CT groups. Hyperplasia of goblet cells in the TM and CP groups was more obvious than in the other two groups (Figs 7(a)–(d)). Histopathology of jejunum showed more obvious variation among the four groups. The intestinal villi in TM-sensitized mice were irregular, while they were regular in the PBS and CT groups. The swelling of intestinal villi was observed from the section in the TM and CP groups. More immune cells infiltrated into the villi and lamina propria of the TM and CP groups than of the PBS and CP groups. The accumulation of goblet cells was obvious in the epithelium of the jejunum in the TM and CP groups compared to the PBS and CT groups (Figs 7(e)–(h)). This may be caused by allergen proteins, resulting in local allergic response and local inflammation in the jejunum. In the histopathology of ileum, the intestinal crypts and villi showed no obvious change in all groups, while a few immune cells were detected in the villi and lamina propria in the TM and CP groups. The increase of goblet cells was obvious in the TM and CP groups compared to PBS and CT groups (Figs 7(i)–(l)).

The number of goblet cells in the jejunum from TM-sensitized mice was higher than in the duodenum and ileum, showing a significant increase compared with the PBS group $P < 0.05$) (Figs 8 (a) and (b)). Eosinophils and lymphocytes obviously infiltrated into the jejunum in TM-sensitized mice (Fig. 8(a)). The numbers of

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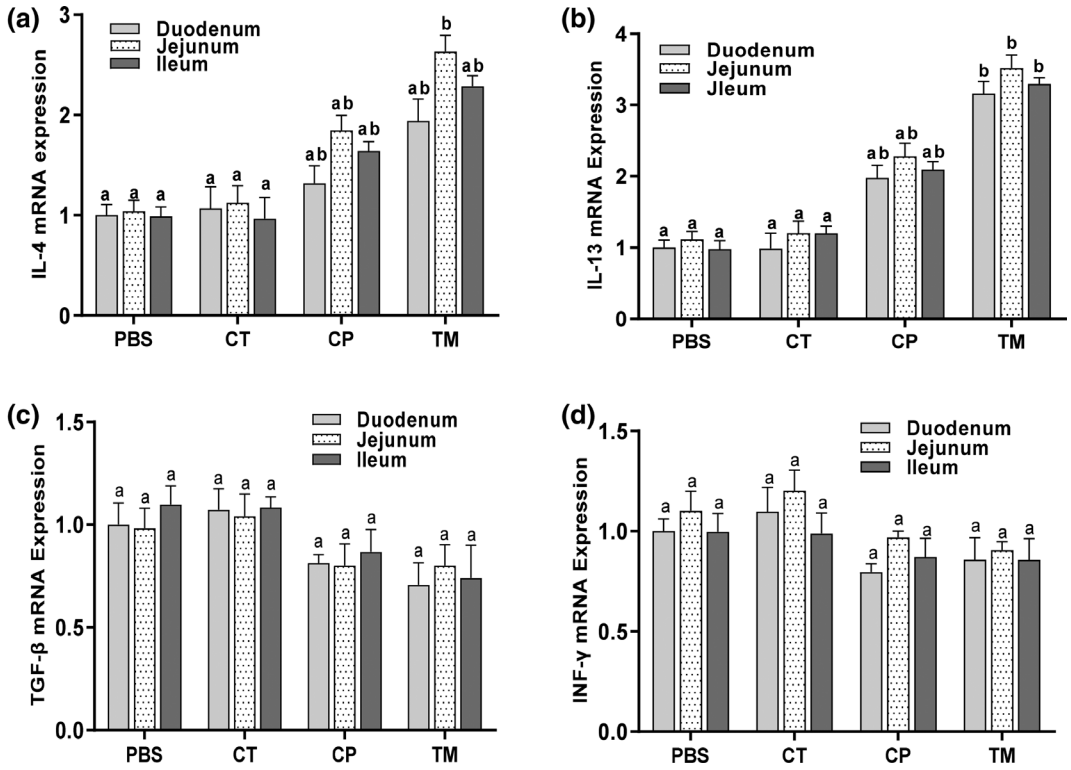


Figure 6. mRNA expression of cytokines in different parts of the digestive tract: (a) IL-4; (b) IL-13; (c) TGF-β; (d) IFN-γ. Means with different letters are significantly different at the $P < 0.05$ level.

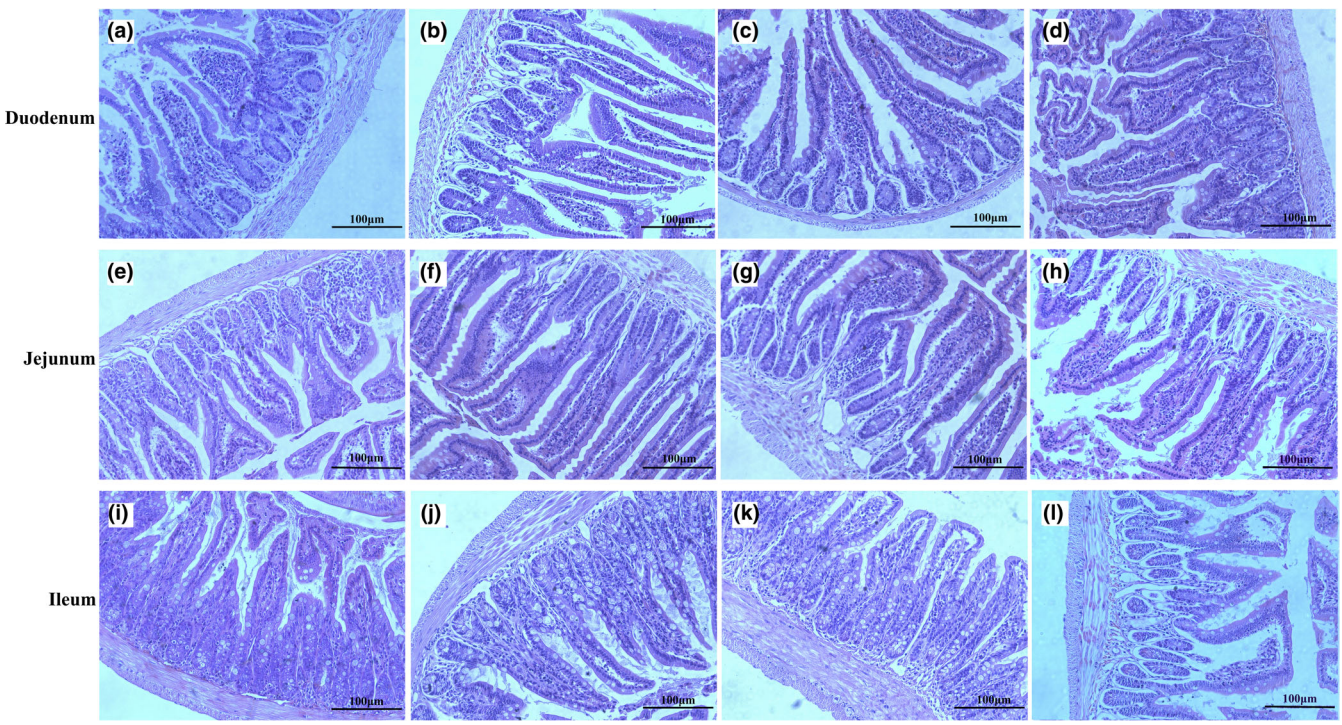


Figure 7. Histopathological analyses of different regions of digestive tract after challenge by TM sensitization. (a–d) Duodenum; (e–h) jejunum; (i–l) ileum. (a, e, i) Sections from PBS group; (b, f, j) sections from CT group; (c, g, k) sections from CP group; (d, h, l) sections from TM group.

eosinophils and lymphocytes were significantly higher than in the duodenum and ileum ($P < 0.05$) (Figs 8(c) and (d)); however, there was no significant difference between duodenum and ileum ($P > 0.05$). The infiltration of eosinophils and lymphocytes indicated that inflammation may occur in the digestive tract of the TM-sensitized mice, especially in the jejunum.

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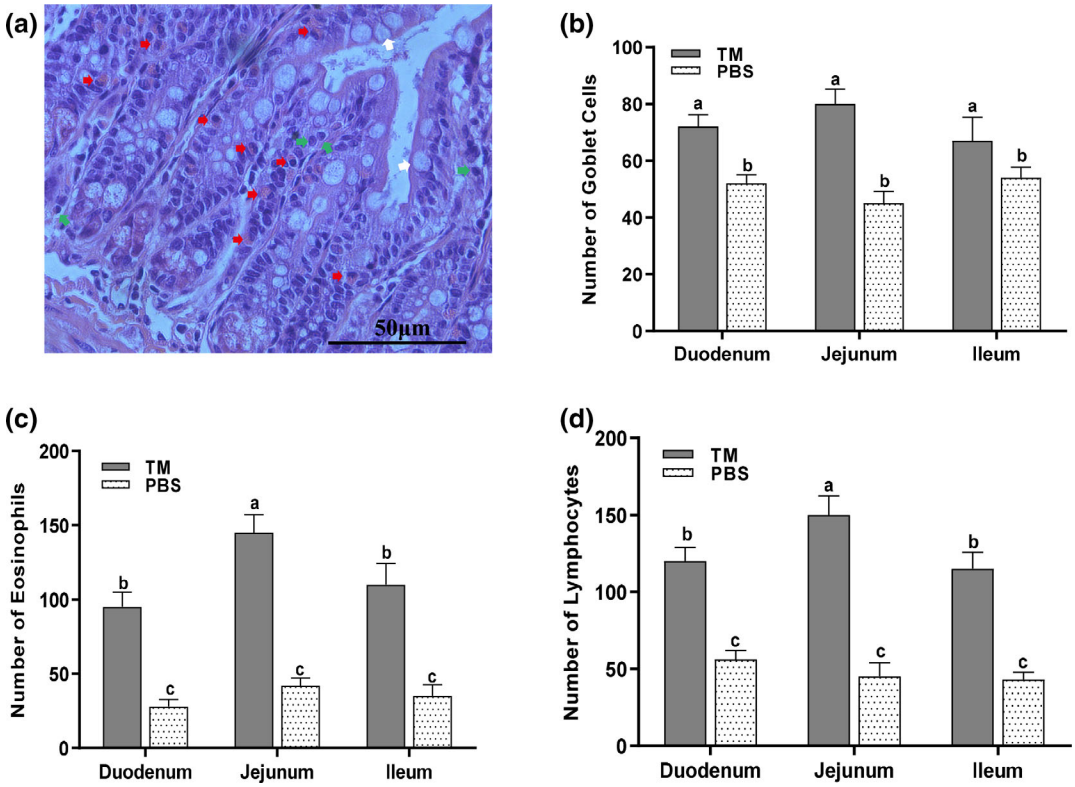


Figure 8. (a) Infiltration of immune cells in the jejunum from TM-sensitized mice. White arrows: goblet cells; red arrows: eosinophils; green arrows: lymphocytes. Statistics for (b) goblet cells, (c) eosinophils and (d) lymphocytes. Means with different letters are significantly different at the $P < 0.05$ level.

DISCUSSION

M. nipponense is one of the most consumed freshwater shrimp in China, and more shrimp-induced allergic cases have emerged recently. Here, murine and cell models were used to define allergic symptoms and characterize the underlying mechanism.

In this study, BALB/c mouse models were developed by intragastric administration with the adjuvant CT. Food allergies can always induce changes in serum antibodies, including IgE, IgG, IgG2, IgG2a, etc.[12,22] Of them, IgE is a crucial marker of type I hypersensitivity. It can bind to the high-affinity FcεI receptors on the surface of mast cells and basophils.[23] Inflammation mediators and cytokines are released quickly after IgE binds to the receptors on the surface of these cells. Therefore, serum-specific IgE was considered as a hallmark of Th2 immune response.[12] Moreover, the production of serum IgE and IgG1 in mice was detected in the Th2 immune response.[24] In this study, the serum IgE levels in TM-sensitized mice were significantly higher than those in the other groups ($P < 0.01$). Our findings were consistent with most previous results from other researchers.[14,23] The change of serum IgG1 level was similar to that of the serum IgE level, indicating it was induced by the prepared TM. However, the levels of IgG and IgG2a were not obviously induced by the prepared TM, and there was no significant difference among the groups ($P > 0.05$). Since IgG and IgG2a are important indicators of the immune response of Th1 cells,[25] it is suggested that the prepared TM could cause the Th2 immune response, instead of the Th1 response.

Moreover, we found that histamine levels in the TM group were significantly higher than those in the other groups ($P < 0.05$), while they showed no obvious difference in the PBS, CT and CP groups ($P > 0.05$). As an important marker of immediate-type hypersensitivity in the BALB/c mouse models, serum histamine level reflects the degree of degranulation of basophils and mast cells.[18,26] Reasonably, our findings revealed that mast cells in TM-sensitized mice were degranulated and promoted allergy, which is also supported by the work of other researchers.[19]

Regarding further assessment of the prepared TM allergenicity, allergic-related cytokines were tested by measuring the supernatants of cultured spleen cells and blood plasma using ELISA kits. Th2-related cytokines, including IL-4, IL-5 and IL-13, can induce B-cell growth and differentiation, as well as IgE and IgG1 production.[24] The results of this work showed that the levels of IL-4, IL-5 and IL-13 in the TM group were higher than those in the other groups. Moreover, the IL-4 and IL-13 mRNA expressions in the gut tissues from the TM and CP groups were also higher than those from the other groups. Similar results have also been reported by others.[23,27] The levels of IL-4, IL-5 and IL-13 showed that the prepared TM from *M. nipponense* could induce allergy in the mice. IFN-γ, one of the Th1-related cytokines, could suppress the IgE production from B cells[28] and IL-4 and IL-5 secretion by resting Th2 cells.[28,29] In this study, the levels of IFN-γ in the TM and CP groups were significantly lower than those in the PBS and CT groups ($P < 0.05$). Similar trends were also found for the mRNA expression in the gut tissues. IFN-γ was suppressed in the TM-sensitized mice; however, different results were reported by Capobianco *et al.*,[27] in which the levels of IFN-γ in shrimp TM-immunized mice were significantly higher than those of the control mice. Moreover, IL-10 and TGF-β are both secreted by T regulatory cells. IL-10 can indirectly inhibit the secretion of cytokines and the proliferation of antigen-specific $CD4^+$ T effector cells,[30] and regulate Th2 responses and allergic responses.[23,31] TGF-β has a potent regulatory capacity and could suppress immune responses, especially during induction and maintenance of

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peripheral tolerance to allergens.[32] In this study, IL-10 and TGF-β levels in the TM and CP groups were lower than those in the PBS and CT groups, indicating that the Th2 response was downregulated or suppressed in the PBS and CT groups, while there was an obvious Th2 response in the TM and CP groups. Taking together the change trends of the various cytokines, the prepared TM and crude proteins from *M. nipponense* could induce a shift from Th1 to Th2 response, leading to food allergy.

Basophils are some of the critical cells during allergy activation; once activated they can release allergic mediators, including histamine and β-hexosaminidase.[33] In this work, KU812 cells were used to evaluate the potential allergenicity of the prepared TM and crude proteins from *M. nipponense*. The degranulation of stimulated KU812 cells was evaluated in terms of the content of histamine and the release rate of β-hexosaminidase. Basophil histamine release was regarded as a more accurate indicator of clinical severity than antibody measurements.[34] The content of histamine was increased sharply after the challenge of the prepared TM (15.47 ± 1.05 μg mL^{-1}) when compared to the PBS group (9.53 ± 1.30 μg mL^{-1}), suggesting the prepared TM could activate KU812 cells and lead to higher release of histamine. Actually, the histamine increase of KU812 cells was previously reported in mice by stimulating with roasted peanut protein extracts comparing to raw sample.[35] The release rate of β-hexosaminidase induced by the prepared TM from *M. nipponense* was significantly higher than that of the PBS group, indicating that TM could promote KU812 cells to degranluate and cause an allergic response. Similar results were reported by Zhang *et al*.,[3] in which the release of β-hexosaminidase increased when cells were stimulated by different TM treated with deglycosylation and glucose glycation.

In order to better understand the profile of allergic responses at the gene level, the mRNA expressions of allergic-regulated cytokines in tissues of three parts of the gut, duodenum, jejunum and ileum, of mice were measured. The highest expressions of IL-4 and IL-13 were in the jejunum, followed by those in the ileum and in the duodenum, sequentially. However, there was no obvious difference between TGF-β and IFN-γ expressions in the gut segments ($P > 0.05$). Similar results were found in that IL-4 and IL-13 mRNA expressions in the junction between the duodenum and jejunum segments of TM-sensitized mice were higher than those of the control mice, and IL-10 and IFN-γ expression levels showed no difference between the two groups of mice.[36] IL-4 mRNA expression in the jejunum was increased after TM immunization compared with the control mice, whereas its expression in colon was not affected by immunization, and IL-13 was expressed only in the immune jejunum while absent in the colonic tissue from immune mice and control mice.[27] Such results were different from ours, in which IL-13 could be expressed in the duodenum, jejunum and ileum of TM-sensitized mice in this study.

Food intolerance and intestinal inflammation induced by food allergens are mainly related to gut-associated mucosal defense in lymphoid tissues.[37] In the histopathological examination, intestinal villi and crypts were regular and intact in the mice of the PBS and CT groups, while they became irregular and disordered in the TM-sensitized mice. Moreover, more immune cells, such as lymphocytes and eosinophils, were found to infiltrate into the jejunum compared with the duodenum and ileum in the TM-sensitized mice, indicating more obvious inflammation occurred in the jejunum than in the duodenum and ileum. The hyperplasia of goblet cells in the digestive tract in the TM group was more obvious than in the PBS group. Similar results have been described by other researchers. Mucus-secreting goblet cells accumulated in the gut epithelium from TM-sensitized mice[36] or other animal models of food allergy.[22] Rice allergy led to inflammation in the jejunum of mice, and the epitheliums in the small intestine were damaged by the inflammation.[38] The core of papillary structures and lamina propria in the duodenum showed diffuse infiltration of lymphocytes, plasma cells and histocytes, but not in the gland and villi, while there was no much variation between the TM-treated group and the control group in jejunum.[14] According to histological changes, it has been confirmed that duodenum was easier to be affected than the jejunum in sensitized mice.[14] However, different results were found in that severe inflammation was detected in the jejunum, while a slight inflammation was also found in the duodenum and ileum from Tg-mice.[39] The proximal jejunum of small intestine is the intestinal portion most affected by food allergy.[22] In our study, the histopathological changes among jejunum, duodenum and ileum showed that jejunum in the TM-sensitized mice had more infiltrated immune cells and intestinal villi and crypts were more irregular and disrupted compared with the duodenum and ileum. Moreover, IL-4 and IL-13 expressions in the jejunum were higher than those in the duodenum and ileum. Therefore, it is suggested that the jejunum may be more easily affected by TM than the duodenum and ileum, and the jejunum could be the major site for allergic response and inflammation when food allergy occurs.

In summary, this study demonstrated TM could induce a strong allergic response in mice and lead to degranulation in basophil cells. Moreover, the allergic reaction mainly occurs at the site of the jejunum, which may be more easily affected by TM than the duodenum and ileum.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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Supplementary Figures:

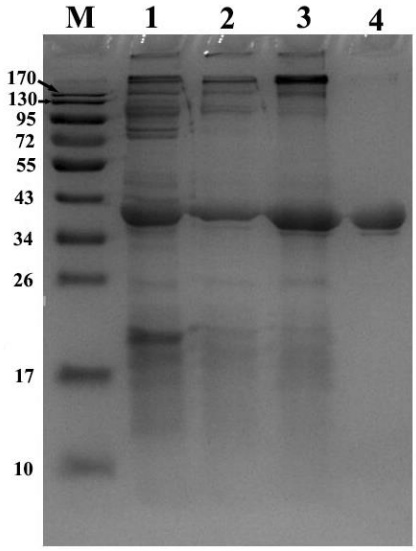


Figure 1S SDS-PAGE analysis of shrimp tissue protein (M: Protein marker; 1: Crude protein; 2: Boiled crude protein; 3: The concentrated solution from the supernatant extracted by 30% saturation of ammonium sulfate; 4: Purified TM)

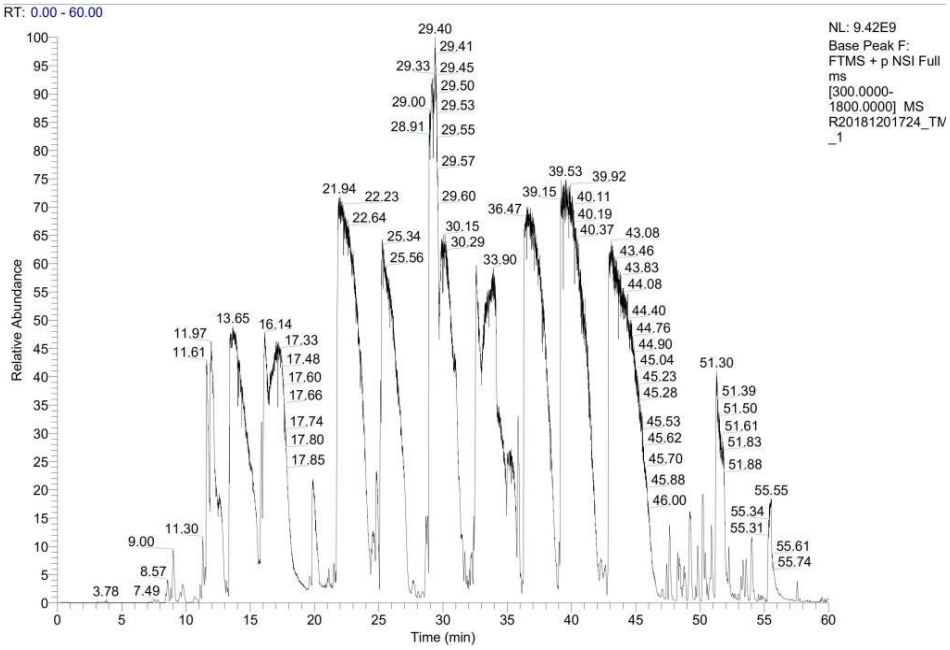


Figure 2S LC-MS/MS spectrometry profile of tropomyosin from *M. nipponense.*

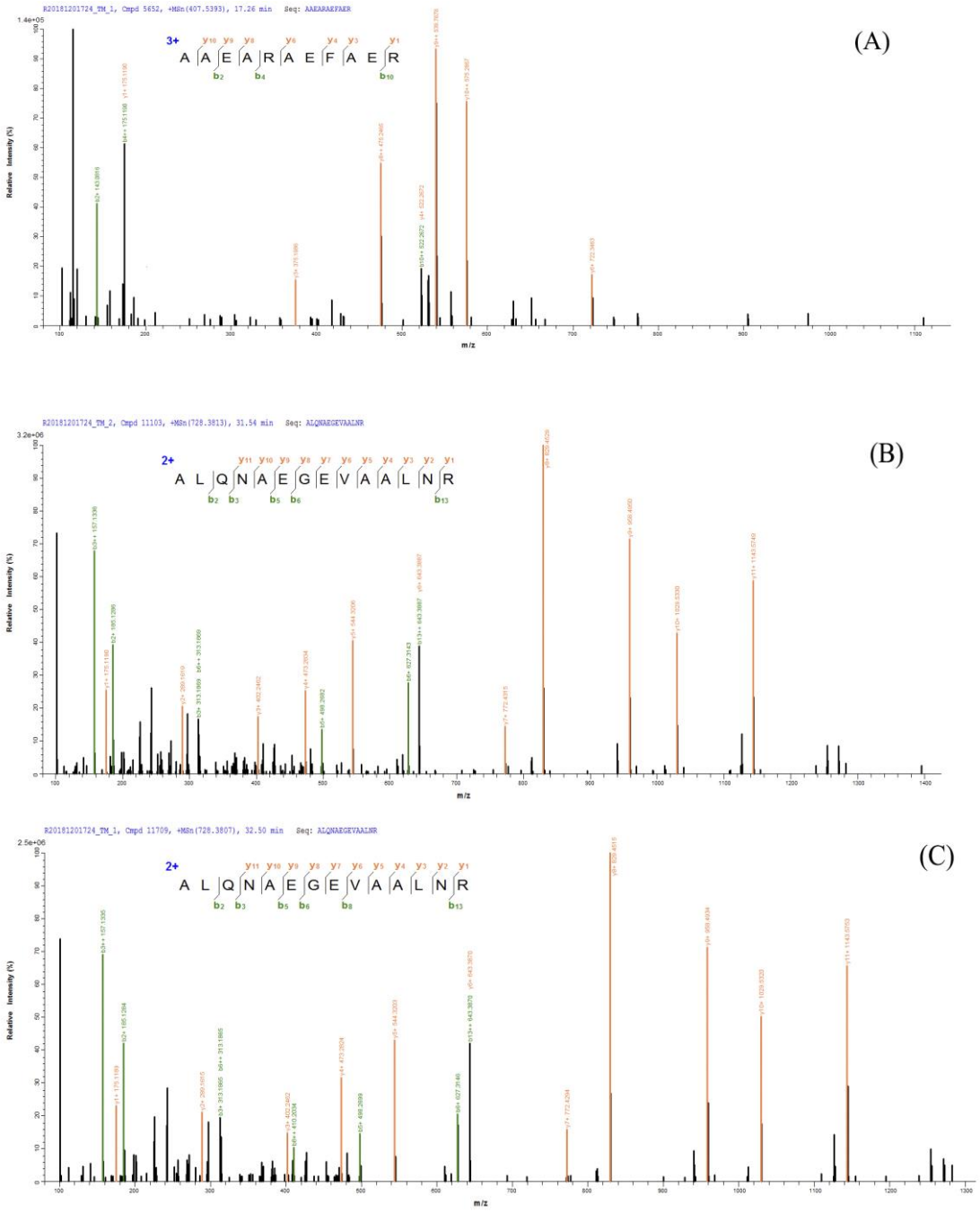


Figure 3S The MS2 spectra of TM at different time point, A:17.26 min；B: 31.54 min; C: 32.50 min.

Table 1S The identified peptide from LC-MS/MS

Name	Sequences	MW	pI
R20181201724_TM_1,5652	AAEARAEFAER	32930.13	4.71
R20181201724_TM_1,12754	ALQNAEGEVAALNR	32930.12	4.71
R20181201724_TM_1,4859	ANQREEAYKEQIK	32930.13	4.71
R20181201724_TM_1,8674	DNAMDRADTLEQQNK	32930.12	4.71
R20181201724_TM_1,853	EANNR	32930.12	4.71
R20181201724_TM_1,9377	EARFLAEEADR	32930.12	4.71
R20181201724_TM_1,5099	EQIKTLTNK	32930.12	4.71
R20181201724_TM_1,11240	EVDRLEDELVNEKEK	32930.14	4.71
R20181201724_TM_1,17997	IVELEEELRVVGNNLK	32930.13	4.71
R20181201724_TM_1,6385	LAEASQAADESERMRK	32930.13	4.71
R20181201724_TM_1,12975	LAMVEADLERAEER	32930.12	4.71
R20181201724_TM_1,4876	LEKDNAMDR	32930.13	4.71
R20181201724_TM_1,19585	MQQIENELDQVQEQLSLANTK	32930.13	4.71
R20181201724_TM_1,23509	SITDELDQTFSELSGY	32930.12	4.71
R20181201724_TM_1,5183	SLEVSEEKANQR	32930.12	4.71
R20181201724_TM_1,3507	TEEEIRTTQK	32930.12	4.71
R20181201724_TM_1,4777	TLTNKLK	32930.12	4.71
R20181201724_TM_1,7006	VLENRSLSDEER	32930.12	4.71
R20181201724_TM_1,22922	YDEVAR	32930.12	4.71
R20181201724_TM_1,21171	YKSITDELDQTFSELSGY	32930.12	4.71
R20181201724_TM_1,3596	ADTLEQQNKEANNR	32930.12	4.71
R20181201724_TM_1,17788	AETGESKIVELEEELR	32930.13	4.71
R20181201724_TM_1,4911	EEAYKEQIK	32930.12	4.71
R20181201724_TM_1,10694	FLAEEADRKYDEVAR	32930.14	4.71
R20181201724_TM_1,14999	IQLLEEDLERSEER	32930.13	4.71
R20181201724_TM_1,13561	KLAMVEADLERAEER	32930.12	4.71
R20181201724_TM_1,2478	KVLENR	32930.12	4.71
R20181201724_TM_1,16588	KYDEVAR	32930.12	4.71
R20181201724_TM_1,7922	LEDELVNEKEK	32930.12	4.71
R20181201724_TM_1,9838	LNTATTKLAEASQAADESER	32930.13	4.71
R20181201724_TM_1,11183	MDALENQLKEAR	32930.12	4.71
R20181201724_TM_1,3641	MRKVLENR	32930.13	4.71
R20181201724_TM_1,14336	RIQLLEEDLER	32930.12	4.71
R20181201724_TM_1,3590	SEERLNTATTK	32930.12	4.71
R20181201724_TM_1,15536	SLSDEERMDALENQLK	32930.13	4.71
R20181201724_TM_1,10582	VVGNNLKSLEVSEEK	32930.12	4.71