

Natural Shrimp (*Litopenaeus vannamei*) Tropomyosin Shows Higher Allergic Properties than Recombinant Ones as Compared through SWATH-MS-Based Proteomics and Immunological Response

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ABSTRACT: Tropomyosin (TM) is the major shrimp allergen that could trigger anaphylactic reactions. Recently, recombinant TM (rTM) has been accepted widely in the field of allergen-specific immunotherapy, but the allergenicity of rTM has not been compared with natural TM (nTM) based on an *in vitro* digestion profile. In this work, IgG-/IgE binding, allergen peptides, and degranulation ability of the digested samples in simulated gastric fluid/simulated intestinal fluid/gastrointestinal models from nTM and rTM were evaluated by immunoassays, proteomics, and basophil degranulation assay. Results showed that pepsin-digested and trypsin-digested samples of rTM exhibited lower IgG-/IgE binding and degranulation than those of nTM. More peptides of the digested samples from rTM (57.8%) matched shrimp allergic epitopes than those from nTM (33.3%). However, the peptide SITDELDQTF (269–278) appeared most frequently. These findings would supply foundation data for epitope-based immunotherapy to shrimp allergic individuals.

KEYWORDS: natural tropomyosin, recombinant tropomyosin, *in vitro* digestion, SWATH-MS based proteomics, antigenicity

INTRODUCTION

Food allergy is regarded as an immunologically mediated reaction that could adversely affect the quality of life, causing a burden for health systems as well as economic systems of individual persons.¹ Shrimp is highly consumed around the world for its delicate flavor and high nutrition. *Litopenaeus vannamei*, as one of the common species of shrimp, is a popular delicacy around the country, especially in coastal areas.^{2,3} According to the Food and Agriculture Organization, shellfish is described as the most frequent species that could cause food allergy.⁴ Numerous proteins have been deemed as allergens, such as tropomyosin (TM), arginine kinase, triosephosphate isomerase, and so forth. TM, which is composed of two identical subunits with 35–38 kDa, has been considered as the major allergen of shrimp.^{5,6} Currently, there are two different methods to obtain the purified protein. One is extraction and purification from the shrimp muscle (natural protein), and the other is by expression and purification (recombinant protein). As allergen-specific immunotherapy requires administration of multiple doses, natural allergen extraction makes treatment cumbersome, resulting in poor compliance and poor treatment effect. Compared with the natural allergen, the recombinant allergen and its derivatives have the advantage of shortening the buildup phase, which makes them to be widely used in the field of allergen-specific immunotherapy.⁷ As the recombinant protein could not thoroughly restore the same conformation structure as the natural one, it could exhibit slightly different three-dimensional structures in comparison with the natural ones. Thus, epitopes might have different levels of exposure and differences in allergenicity.

However, differences in allergenicity between natural TM (nTM) and recombinant TM (rTM) from shrimp species are still not clear.

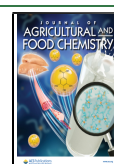
Individuals become sensitized to dietary food allergens via the gastrointestinal (GI) tract during ingestion.⁸ During the process of digestion, dietary proteins could be broken up and produce peptides that might exhibit the ability of potential antigenicity. Some of these peptides, named as epitopes, could pass through the passage and be taken up by intestinal epithelial cells to trigger allergic reactions.^{9,10} Moreover, based on the characteristics of human digestion, some proteins may be digested by pepsin or trypsin or both of them to produce different peptides. Among them, some peptides that may cause different degrees of allergic response in sensitive individuals are identified as allergenic epitopes. However, there are few literature studies about the changes of peptides during digestion and also the relationship between digestible fragments and epitopes. Mass spectrometry is widely used for its sensitivity and high throughput in the field of protein analysis. Sequential windowed acquisition of all theoretical fragment ion mass spectra (SWATH-MS) could parallelly analyze thousands of proteins to provide a comprehensive overview of the profile of enzymatic peptides.

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In comparison with other proteomics methods, SWATH-MS has the advantages of enhanced sensitivity, better repeatability, and the supply of more information.¹¹

This work was aimed to evaluate the effects of *in vitro* digestion on the allergenicity of nTM and rTM. By using different digestion models of the simulated gastric fluid (SGF), simulated intestinal fluid (SIF), and GI, IgG-/IgE binding, peptide sequences of digestion products in SGF/SIF/GI, and degranulation ability were assessed. This work would provide primary data of the allergenic potential of digestion products from natural and recombinant TM, which would be useful for diagnosis and clinical treatment of shrimp allergic patients.

MATERIALS AND METHODS

Materials. Shrimp (*L. vannamei*) was obtained from a local supermarket (Liqun supermarket, Qingdao, Shandong, China), and raw muscles were used to obtain proteins based on our laboratory method.¹² Briefly, the minced muscle was homogenized in Buffer A (50 mM KCl containing 2 mM NaHCO₃). After the centrifugation, contents were washed by cold absolute. Then, the shrimp dried powders were suspended in Buffer B (0.02 M Tris-HCl, 1 M KCl, and 0.1 mM DTT, pH 7.4). The mixture was fractionated with ammonium sulfate from 20 to 30% and subjected to isoelectric precipitation (pH 4.6). The precipitate was dissolved and dialyzed in phosphate-buffered saline (PBS) (0.01 M, pH 7.4) to obtain natural TM.

The recombinant TM was obtained from AtaGenix (Wuhan) Co., Ltd. (Wuhan, Hubei, China).¹³ Briefly, the amino acid sequence was obtained from the National Center of Biotechnology Information (Genbank accession number: ACB38288.1). The cDNA was chemically synthesized with optimization for expression in *E. coli*, and then, the cDNA sequence was cloned in the pET28b expression vector. The bacteria starter was obtained by incubation at 37 °C for 4 h. Then, the recombinant protein was obtained using a Ni-NTA Superflow column (Qiagen, Hilden, Germany).

Twelve individual patients' sera with a documented clinical history were obtained from the Affiliated Hospital of Qingdao University (Qingdao, Shandong, China) (Table 1). The total IgE and specific IgE levels were measured using the Phadia ImmunoCAP (Thermo Fisher Scientific, Waltham, MA, USA). These human sera were used in the indirect enzyme-linked immunosorbent assay (ELISA) to evaluate IgE binding of nTM and rTM. Additionally, the serum with a high specific IgE level was used in the mediator-release assay. These sera were kept at −80 °C before using. The clinical consent forms were signed by the serum donors and approved by the institution. The study protocol was approved by the medical ethics committee (File no. QD-PF-20190618685).

Antishrimp TM IgG was obtained from shrimp-TM-immunized mouse sera using the protein A sepharose column. Antishrimp TM IgG and immunized mouse sera containing antishrimp TM IgE antibodies were used as the primary antibodies. The secondary antibodies [Horseradish peroxidase (HRP)-labeled goat antimouse IgG-/IgE antibody and HRP-labeled goat antihuman IgE antibody] were purchased from Zhongshan Jinqiao Co. (Beijing, China). These primary antibodies and secondary antibodies were used in western blot and ELISA to evaluate the IgG-/IgE binding of natural TM and recombinant TM together with their digestion products. The mice sera were kept at −80 °C before using. All experiments were performed in accordance with the established guidelines of the Ethical Committee of Experimental Animal Care at the Ocean University of China (SPXY2019052001).

The human basophil cell line (KU812) was acquired from Procell Life Science & Technology Co. Ltd. (Wu Han, Hu Bei, China). Human myeloma IgE was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile (LC–MS grade) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Formic acid (LC–MS) was obtained from Fluka (Buchs, Switzerland). Ultrapure water was obtained from a Milli-Q system (EMD Millipore, Billerica, MA, USA). Other reagents used were all of analytical grades.

Table 1. Clinical Data from Shrimp-Allergic Patients

no.	sex ^a	age	symptoms ^a	clinical history	IgE levels (kUA/L)	
					total	shrimp
1	M	31	As	d1, d2, f23, f24, i6	982	4.77
2	F	40	U	f24	<60	0.83
3	F	18	Dr	d1, d2, e1, e5, f1, f2, f3, f10, f23, f24, i6, m3, m6	3947	2.56
4	F	5	U	d1, d2, f23, f24, i6	110.4	1.57
5	M	3	Dr	d1, d2, e5, f1, f2, f3, f4, f10, f13, f14, f23, f24, fx1, i6, m3, w1, w6	997	69.4
6	F	18	U	f23, f24		8.58
7	F	33	Ar	d1, d2, f23, f24, i6	195.0	2.7
8	M	24	U	d1, d2, f23, f24, i6		0.98
9	M	17	Ar	f3, f23, f24		1.53
10	F	24	Dr	f1, f2, f4, f10, f23, f24		1.73
11	F	30	U	f3, f23, f24,		0.09
12	M	36	ND	f3		0.01

^aF, female; M, male; As, anaphylactic shock; U, urticaria; Dr, dermatitis; Ar, anaphylactic reaction; ND, not determined; d1, dermatophagoides pteronyssinus; d2, dermatophagoides farina; e1, cat dander; e5, dog dander; f1, protein; f2, milk; f3, fish; f4, wheat; f10, sesame; f13, peanut; f14, soybean; f23, crab; f24, shrimp; fx1, food mixture; i6, German cockroach; m3, aspergillus fumigatus; m6, alternaria alternata; w1, artemisiifolia; w6, mugwort. A serum with specific IgE > 0.35 (kUA/L) is defined as positive.

SGF Digestion. The digestibility assay was performed as mentioned in the previous reports.^{14,15} The SGF was made by following the pharmacopeia of the United States,¹⁶ which consisted of porcine pepsin (380 U/mg protein) in 25 mM NaCl (pH 1.2). After the SGF (1:3 pepsin to TM, w/w) was preheated for 15 min at 37 °C, nTM and rTM were added into the solution, respectively. Then, the tube was kept shaking at 37 °C for 30 min in the incubator. Finally, the reaction was stopped by adding 30 μL of 0.2 M Na₂CO₃ (the final pH value was 7.5), and the product was collected for further analysis. All these experiments were carried out three times to obtain uniformity.

SIF Digestion. The SIF comprises trypsin (2.1 U/mg protein) in 5 mM KH₂PO₄ (pH 7.5). After the SIF (1:700 trypsin to TM, w/w) was preheated for 15 min at 37 °C, nTM and rTM were added into the digestion and incessantly shaken at 37 °C for 30 min in the incubator. Subsequently, SIF digestion was heated at 95 °C in the water bath for 5 min to end the reaction.¹⁷

GI Digestion. As SGF digestion stopped, the SIF was added into the same tube and subjected to incessant shaking at 37 °C for 30 min in the incubator. Before using, the SIF (1:700 trypsin to TM, w/w) was preheated for 15 min. The reaction was ended by heating at 95 °C in the water bath for 5 min.

SDS-PAGE and Western Blot Analyses. SDS-PAGE was carried out as the method described before.¹⁸ Samples mixed with the loading buffer were heated and then loaded into the pre-run gel (5% stacking gel and 12% resolving gel). Electrophoresis was set at 100 V for the sample to run through the whole gel. After staining with 0.125% Coomassie brilliant blue G-250 (Bio-Rad, Richmond, CA, USA), the gel was destained and pictured by the HP Scanjet G4050 Photo Scanner (China Hewlett-Packard Co, Beijing, China).

Western blot was carried out as described before.¹⁹ After electrophoresis, the pre-run gel was transferred onto a polyvinylidene fluoride (PVDF) membrane by using the iBlot 2 Gel Transfer Device (Life Technologies Co., Israel). Then, the membrane was incubated overnight with primary antibodies (antishrimp TM IgG, diluted 1:5000, or antishrimp TM IgE, diluted 1:10) and secondary antibodies (HRP-labeled goat antimouse IgG, diluted 1:5000, or HRP-labeled goat antimouse IgE, diluted 1:5000) by using the iBind Western Device (Life Technologies Co., Israel). Finally, images were photographed by

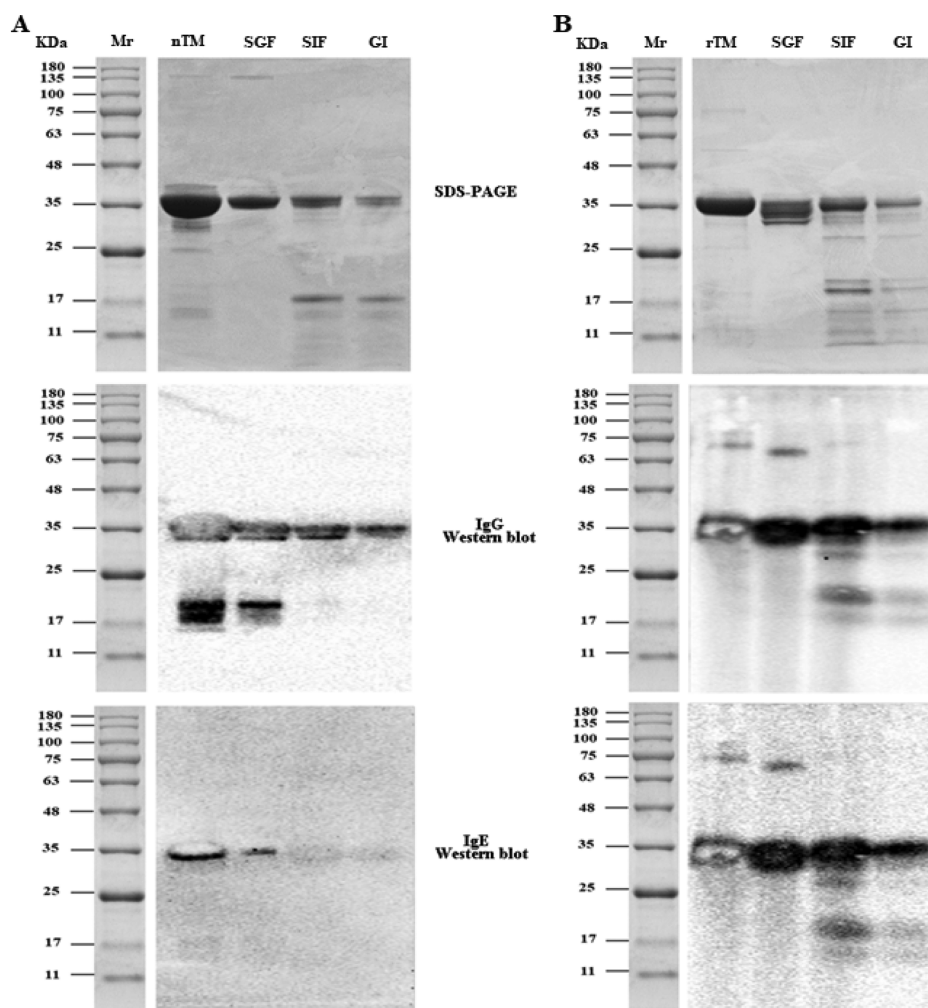


Figure 1. SDS-PAGE and IgG/IgE western blot of nTM and rTM in SGF/SIF/GI. (A) SDS-PAGE and IgG/IgE western blot of nTM in SGF/SIF/GI; (B) SDS-PAGE and IgG/IgE western blot of rTM in SGF/SIF/GI. Line Mr: protein marker. Protein digested by the SGF for 30 min, by the SIF for 30 min and by GI for 1 h (SGF: 30 min, SIF: 30 min). The protein content was all 7.5 μ g for each lane. Both mouse IgG and IgE were used as primary antibodies.

Tanon-4200SF (Tanon Science & Technology Co., Ltd, Shanghai, China) with an enhanced chemiluminescent (ECL) kit substrate.

Indirect ELISA. ELISA was carried out following the previous method.²⁰ Briefly, samples (5 μ g) dissolved in PBS (pH 7.4) were coated in the ELISA plate and incubated overnight at 4 °C. Then, the plate was incubated for 2 h at 37 °C by using a blocking buffer [1% bovine serum albumin and 5% skimmed milk powder dissolved in PBS containing 0.1% Tween-20 (PBST)]. Antishrimp TM IgG (diluted 1:5000), immunized mouse serum (diluted 1:10), or individual human serum (diluted 1:4) were used as the primary antibodies, and HRP-conjugated goat antimouse IgG (diluted 1:5000), HRP-conjugated goat antimouse IgE (diluted 1:5000), or HRP-conjugated goat antihuman IgE (diluted 1:2000) played as the secondary antibodies. Finally, the absorbance was obtained at 450 nm with a microplate reader (CMax Plus, Molecular Devices, LLC., San Jose, CA, USA).

Proteomic Analysis by SWATH-MS. The supernatant liquid of digestion products was put into 3 K ultrafiltration tubes (Merck, Whitehouse Station, NJ, USA) and centrifuged at 12,000g for 20 min. The filtrate was desalted by a ZipTip C18 column (EMD Millipore, Billerica, MA, USA) before instrumental analysis, following the method described as before with some modifications.²¹

The Triple TOF 5600 mass spectrometer system (SCIEX, Framingham, MA, USA) coupled with a Nexera X2 30 Å ultrahigh-performance liquid chromatography system (Shimadzu, Kyoto, Japan) was used to analyze the digested samples. An Agilent Advanced Bio Peptide Map C18 column (150 mm $\times$ 2.1 mm i.d. 2.7 μ m, Agilent,

Santa Clara, CA, USA) was used to separate the digested samples at 0.25 mL/min flow rate and 30 μ L injection volumes. The gradient elution was performed as follows: eluent A (water containing with 0.1% formic acid) and eluent B (acetonitrile containing with 0.1% formic acid); procedure is as follows: 0–2 min, 95% A, 5% B; 2–27 min, 95–80% A, 5–20% B; 27–37 min, 80–65% A, 20–35% B; 37–39 min, 65–20% A, 35–80% B; 39–42 min, 20% A, 80% B; and 42–46 min, 95% A, 5% B.

A “top 20” data-dependent acquisition (DDA) system using the positive ion mode was set, and an electrospray ionization source (ESI) was used (spray voltage: 5500 V) in a high sensitivity mode. The acquisition mode included a 200 ms survey scan (300–1500 m/z) and an MS/MS scan (100–1500 m/z) for the top 20 precursor ions. The intensities were higher than 150 counts/s from the survey scan. Singly charged ions were excluded from product ion scans. According to the precursor m/z frequency of DDA, the spectral ion library was obtained using a SWATH acquisition method (SWATH variable window, SCIEX). For SWATH-MS acquisition, a 50 ms full mass scan (350–1500 m/z) with all precursor ions was performed, following the scanning of 60 isolation windows (400–1250 m/z) with product ions.

Peptide Identification and Spectral Library Generation. The peptides were identified by ProteinPilot software (version 5.0.1, SCIEX, Framingham, MA, USA) using the National Center for Biotechnology Information (NCBI) protein database (downloaded in January 2020 and 632,350 proteins were searched) entries for all proteins of *L. vannamei*, and the false discovery rate (FDR) was 1%.

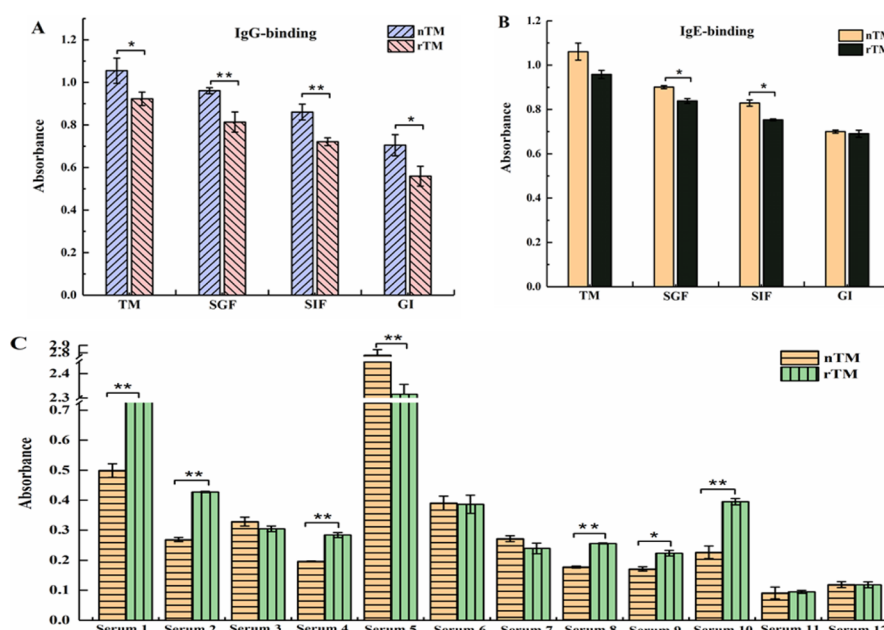


Figure 2. IgG/IgE binding by ELISA of nTM and rTM in SGF/SIF/GI. (A) IgG-binding of digestion products of nTM and rTM in SGF/SIF/GI; (B) IgE-binding of digestion products of nTM and rTM in SGF/SIF/GI. Protein digested by the SGF for 30 min, by the SIF for 30 min and by GI for 1 h (SGF: 30 min, SIF: 30 min); (C) IgE-binding of individual serum. Serum nos. 11–12 were used as the negative control. The data represent the mean $\pm$ SD ($n = 3$). “*” stands for significance at $p < 0.05$, and “**” stands for significance at $p < 0.01$. Immunized shrimp mouse sera and shrimp-allergic patients’ sera were used as primary antibodies.

Peak View software (SCIEX, Framingham, MA, USA) was employed to generate a spectral library for SWATH-MS peak extraction by using the identified peptide MS/MS fragment ions and analyzing the data-independent acquisition data from the library.²¹

Peptide Matching. Peptides were selected as follows: (1) with a minimum peptide confidence of 90%; (2) peptides that are not shared or modified; and (3) FDR value $\leq 1\%$. Sequences of these peptides were compared with allergen epitopes of seven shrimp species (*Penaeus indicus*, *Penaeus aztecus*, *L. vannamei*, *Metapenaeus ensis*, *Penaeus monodon*, *Penaeus chinensis*, and *Exopalaemon modestus*) reported previously (Table 3), and the matching degree was more than 50% for each protein fragment.²²

KU812 Mediator-Release Assay. KU812 cells were cultured in 1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37 °C for 24 h following the method described as before.²³ After culturing for several days, human myeloma IgE (0.5 $\mu\text{g/mL}$) was added into the cells and kept in the incubator for 7 days to stimulate Fc ϵ RI expression. Then, the grown cells (2×10^6 cells/mL) were seeded and passively sensitized with shrimp-allergic patients’ sera (1:15) for 12 h and then stimulated with digestion products. Cells stimulated with Tyrode’s buffer were used as the negative control, and those stimulated with compound 48/80 were used as the positive control. The cell supernatant was collected to determine the release of β -hexosaminidase. The levels of histamine and tryptase were also measured by using commercial kits (Fusheng Industry Co., Shanghai, China).

Statistical Analysis. Marker View software (version 1.2.1, SCIEX, Framingham, MA, USA) was used to make data normalization based on the total peak areas. Data of SWATH-MS was analyzed by the principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) model using SIMCA software (version 14.0, Umetrics), while a fold-change (ratio > 1.5) and $p < 0.05$ were satisfied with statistical significance. Data of others are presented as mean $\pm$ standard deviation SD ($n = 3$) and obtained using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA) and Origin 8. software (Origin Lab, Hampton, AK, USA). $p < 0.05$ was regarded as statistically significant.

RESULTS AND DISCUSSION

SDS-PAGE and Western Blot Analysis. The results of SDS-PAGE and western blot of nTM and its digestion products

are shown in Figure 1A. As shown in the SDS-PAGE profile, nTM was observed at 35 kDa with purity higher than 90%. Compared with GI, the protein was slightly degraded and showed higher resistance to pepsin digestion. When treated with trypsin, more protein fragments around 11–17 kDa appeared in the gel. In comparison with digestion products in the SIF, those samples in the GI model presented slight differences and shared almost similar protein fragments. The IgG-/IgE binding of nTM and its digested samples was analyzed by western blot using antishrimp TM IgG and shrimp TM-immunized mice sera. In both IgG and IgE western blot images, compared with the sample of nTM, the TM band (around 35 kDa) appeared to be shallower in the samples of SGF, SIF, and GI models, implying that the protein was partially destructed in the liquid of SGF, SIF, and GI models.

The results of SDS-PAGE and western blot of rTM and its digestion products are shown in Figure 1B. As observed in SDS-PAGE, rTM appeared around 35 kDa with purity higher than 90%. rTM and its digestion products showed a similar trend as nTM. More protein fragments were seen in the SIF and GI lanes than in the SGF lane. The recombinant *Coptotermes formosanus* TM, recognized by commercial antibodies and serum IgE, was also reported that a fragment at about 13 kDa showed resistance to both *in vitro* SGF and SIF digestion.²⁴ The IgG-/IgE binding of rTM and its digested samples was analyzed by western blot using antishrimp TM IgG and shrimp TM-immunized mice sera. In IgG and IgE western blot images, the overall trends of rTM and its digested samples were similar to those of nTM and its digested samples. Consistent with the results of SDS-PAGE, digestion samples of rTM showed new bands around 17–25 kDa, implying new protein fragments that formed during intestinal digestion and GI digestion as they could be recognized by antishrimp TM IgG and shrimp TM-immunized mice sera.

Shrimp Allergic Subjects and IgG-/IgE Binding by ELISA. As shown in Figure 2A, for IgG binding, the overall

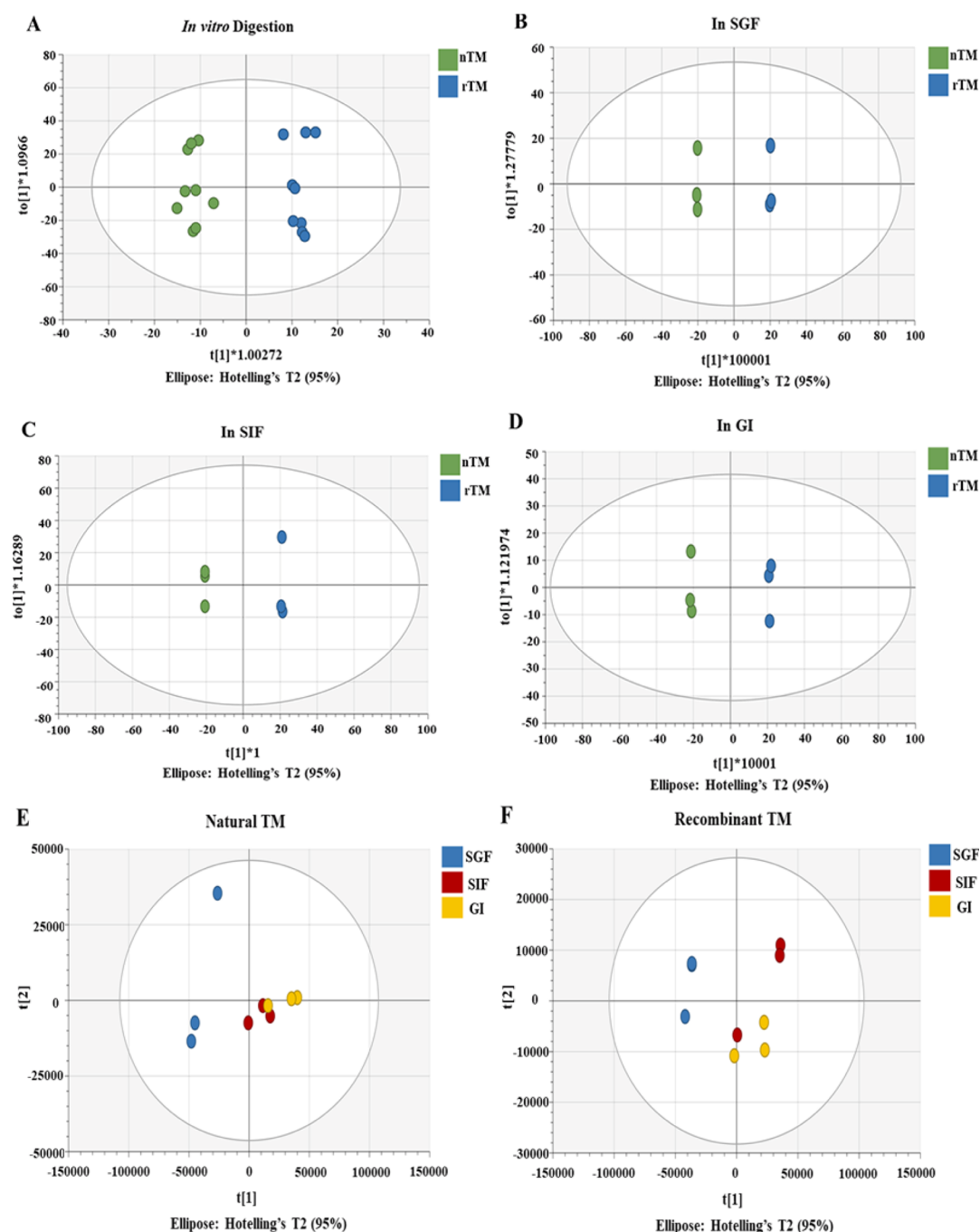


Figure 3. Score plots of the PCA model and OPLS-DA model of nTM and rTM in SGF/SIF/GI. (A) Score plots of the OPLS-DA model of nTM and rTM *in vitro* digestion ($R^2X_1 = 0.135$, $R^2X_0 [1] = 0.502$); (B) Score plots of the OPLS-DA model of nTM and rTM in the SGF ($R^2X_1 = 0.45$, $R^2X_0 [1] = 0.151$); (C) Score plots of the OPLS-DA model of nTM and rTM in the SIF ($R^2X_1 = 0.48$, $R^2X_0 [1] = 0.292$); (D) Score plots of the OPLS-DA model of nTM and rTM in GI ($R^2X_1 = 0.501$, $R^2X_0 [1] = 0.0914$); (E) Score plots of the PCA model in the PCA-X mode of nTM *in vitro* digestion ($R^2X_1 = 0.746$, $R^2X_2 = 0.139$); (F) Score plots of the PCA model in the PCA-X mode of rTM *in vitro* digestion ($R^2X_1 = 0.889$, $R^2X_2 = 0.0656$); Protein digested by the SGF for 30 min, by the SIF for 30 min, and by GI for 1 h (SGF: 30 min, SIF: 30 min).

trend of absorbance values of natural TM and its digestion products was higher than that of recombinant TM. Results showed that there was a significant difference between samples from nTM and rTM ($p < 0.01$) in two groups (SGF and SIF) and also a significant difference ($p < 0.05$) of those in two groups (TM and GI). It has been reported that the attachment of a His-tag could affect the physical properties of a protein by altering the structure, while the recombinant parvalbumin with the His-tag (MGSSHHHHHHSSGLVPRGS) has no significant difference influence on the IgG binding ability.^{13,25}

Meanwhile, the results of IgE binding of natural TM and recombinant TM as well as their digestion products are shown in Figure 2B. Two groups of digestion products in the SGF and SIF showed a significant difference ($p < 0.05$) between samples of nTM and rTM, while TM and GI groups showed approximate absorbance values, suggesting that IgE binding was similar to intact proteins and GI digestion products.

Twelve shrimp allergic sera including five male and seven female individuals with the age between 3 and 40 years were with subjective allergic history (Table 1). Compared with

Table 2. Matched Peptides of nTM and rTM in the Simulate Digestion

digestion	protein	no.	start-End	best Conf (peptide)	sequence	Obs M_w	Obs m/z
SGF	nTM	NG1	14–25	99	EKDNAMDRADTL	1377.613	689.8138
		NG2	275–284	90.5	DQTFSELSGY	1145.55	573.783
SGF	rTM	RG1	11–19	97	MKLEKDNAM	1078.52	540.266
		RG2	11–20	97	MKLEKDNAMD	1193.56	597.786
		RG3	72–83	98.1	VEKDKALSNAEG	1259.64	630.826
		RG4	72–84	98.1	VEKDKALSNAEGE	1388.67	695.341
		RG5	72–85	98.1	VEKDKALSNAEGEV	1487.74	744.877
		RG6	72–86	98.1	VEKDKALSNAEGEVA	1558.78	780.396
		RG7	72–87	93.3	VEKDKALSNAEGEVAA	1629.81	544.277
		RG8	72–88	97	VEKDKALSNAEGEVAAL	1742.91	581.976
		RG9	99–106	93.3	LERSEERL	1030.54	516.279
		RG10	145–153	97	ENQLKEARF	1133.58	567.797
		RG11	146–153	97	NQLKEARF	1004.54	503.277
		RG12	154–164	98.1	LAEEADRKYDE	1337.61	669.814
		RG13	154–166	98.1	LAEEADRKYDEVA	1507.71	754.862
		RG14	182–192	97	RAETGESKIVE	1217.63	609.824
		RG15	182–193	91.7	RAETGESKIVEL	1330.72	666.365
SIF	nTM	RG16	221–228	93.3	YKEQIKTL	1021.58	511.798
		RG17	221–229	97	YKEQIKTLT	1122.59	562.303
		NI1	22–30	99	ADTLEQQNK	1045.51	523.761
		NI2	39–48	99	SEEEVHNLQK	1211.57	404.865
		NI3	50–65	99	MQQLENDLDQVQESLL	1901.9	634.975
		NI4	50–66	99	MQQLENDLDQVQESLLK	2030	677.673
		NI5	66–74	99	KANIQLVEK	1041.62	521.816
		NI6	67–74	99	ANIQLVEK	913.522	457.768
		NI7	67–76	99	ANIQLVEKDK	1156.64	386.554
		NI8	77–88	99	ALSNAEGEVAAL	1143.58	572.796
		NI9	77–89	99	ALSNAEGEVAALN	1257.63	629.821
		NI10	91–101	99	RIQLLEEDLER	1412.76	471.926
		NI11	77–90	99	ALSNAEGEVAALNR	1413.72	707.866
		NI12	92–101	99	IQLEEDLER	1256.66	629.338
		NI13	106–112	99	LNTATTK	747.413	374.714
		NI14	113–125	99	LAEASQAADER	1375.61	688.815
		NI15	128–133	99	KVLENR	757.445	379.73
		NI16	134–140	99	SLSDEER	834.375	418.195
		NI17	141–149	99	MDALENQLK	1060.52	531.268
		NI18	153–160	99	FLAEEADR	949.448	475.731
		NI19	161–167	99	KYDEVAR	879.442	440.728
		NI20	168–178	99	KLAMVEADLER	1273.67	637.842
		NI21	169–178	99	LAMVEADLER	1145.58	573.797
		NI22	170–178	99	AMVEADLER	1032.49	517.253
		NI23	183–189	99	AETGESK	720.325	361.17
		NI24	190–198	99	IVELEELR	1128.6	565.308
		NI25	199–205	99	VVGNNLK	742.433	372.224
		NI26	206–213	99	SLEVSEEK	919.448	460.731
		NI27	206–217	95.6	SLEVSEEKANQR	1388.72	695.366
		NI28	214–221	99	ANQREEAY	979.437	490.726
		NI29	269–278	99	SITDELDQTF	1167.53	584.771
SIF	rTM	RI1	1–10	99	MDAIKKKMQA	1162.63	582.321
		RI2	22–30	99	ADTLEQQNK	1045.51	523.761
		RI3	39–48	99	SEEEVHNLQK	1211.57	404.865
		RI4	42–50	99	EVHNLQKRM	1153.6	577.808
		RI5	42–52	99	EVHNLQKRMQQ	1409.73	705.872
		RI6	42–53	99	EVHNLQKRMQQI	1522.8	508.607
		RI7	47–57	99	QKRMQQLENDL	1401.71	701.864
		RI8	168–178	99	KLAMVEADLER	1273.65	637.83
		RI9	48–57	99	KRMQQLENDL	1273.65	637.833
		RI10	50–65	99	MQQLENDLDQVQESLL	1901.9	634.975
		RI11	50–66	99	MQQLENDLDQVQESLLK	2030	677.673
		RI12	66–74	99	KANIQLVEK	1041.62	521.816
		RI13	67–74	99	ANIQLVEK	913.522	457.768

Table 2. continued

digestion	protein	no.	start-End	best Conf (peptide)	sequence	Obs M_w	Obs m/z
GI	nTM	RI14	67–76	99	ANIQLVKDK	1156.64	386.554
		RI15	75–88	99	DKALSNAEGEVAAL	1386.7	694.358
		RI16	77–88	99	ALSNAEGEVAAL	1143.58	572.796
		RI17	77–89	99	ALSNAEGEVAALN	1257.63	629.821
		RI18	91–101	99	RIQLLEEDLER	1412.76	471.926
		RI19	77–90	99	ALSNAEGEVAALNR	1413.72	707.866
		RI20	92–101	99	IQLEEDLER	1256.66	629.338
		RI21	106–112	99	LNTATTK	747.413	374.714
		RI22	113–125	99	LAEASQAADER	1375.61	688.815
		RI23	127–138	99	RKVLNRSLSDE	1444.77	482.596
		RI24	128–133	99	KVLENR	757.445	379.73
		RI25	134–140	99	SLSDEER	834.368	418.191
		RI26	141–149	99	MDALENQLK	1060.52	531.268
		RI27	153–160	99	FLAEEADR	949.448	475.731
		RI28	161–167	99	KYDEVAR	879.442	440.728
		RI29	165–171	99	VARKLAM	787.471	394.743
		RI30	169–178	99	LAMVEADLER	1145.58	573.797
		RI31	170–178	99	AMVEADLER	1032.49	517.253
		RI32	183–189	99	AETGESK	720.325	361.17
		RI33	190–198	99	IVELEELR	1128.6	565.308
		RI34	199–205	99	VVGNNLK	742.433	372.224
		RI35	206–213	99	SLEVSEEK	919.448	460.731
		RI36	206–217	96.3	SLEVSEEKANQR	1388.72	695.366
		RI37	214–221	99	ANQREEAY	979.445	490.73
		RI38	222–232	99	KEQIKTLTNKL	1314.76	439.262
		RI39	242–252	99	AERSVQKLQKE	1314.76	439.262
		RI40	242–254	99	AERSVQKLQKEVD	1528.82	510.615
		RI41	248–256	99	KLQKEVDRL	1127.66	376.893
		RI42	261–271	99	VNEKEYKSIT	1337.71	446.912
		RI43	261–273	99	VNEKEYKSITDE	1581.79	528.269
		RI44	269–278	99	SITDELDQTF	1167.53	584.771
		NGI1	50–66	93.9	MQQLENDLDQVQESLLK	2029.98	677.667
		NGI2	77–90	99	ALSNAEGEVAALNR	1413.71	707.864
		NGI3	113–125	99	LAEASQAADER	1375.6	688.806
		NGI4	267–284	99	YKSITDELDQTFSELGY	2094.97	1048.49
		NGI5	269–278	99	SITDELDQTF	1167.52	584.767
		NGI6	269–284	99	SITDELDQTFSELGY	1803.8	902.908
	rTM	RG11	221–232	99	YKEQIKTLTNKL	1477.84	493.622
		RG12	246–256	99	VQKLQKEVDRL	1354.79	452.604

nTM, six individual shrimp allergic sera showed higher IgE binding to rTM, three individuals showed lower IgE binding to rTM, and one individual showed a similar IgE binding to rTM. As for the negative control (serum 11 and serum 12), nTM and rTM showed similar IgE binding (Figure 2C). The results indicated that the IgE binding of nTM and rTM was highly dependent on the quality of shrimp allergic sera. It is documented that natural chicken serum albumin and recombinant chicken serum albumin showed similar IgE binding activity in nine individuals.²⁶ It has also been reported that 57% ($n = 8$) individual sera and 43% ($n = 6$) allergic patients' sera showed IgE bound to native and recombinant cockroach TM proteins, respectively.²⁷ The possible reason for the different situation mainly depended on the quality of allergic patients' sera.

SWATH-MS-Based Proteomics. One can perform uniform scanning by SWATH, and information of all precursor/secondary products was collected. In addition, in comparison with the DDA mode, this technique effectively avoided the problems of low sensitivity and poor reproducibility, which were caused by high-abundant signals and dynamic exclusion.^{28,29}

The OPLS-DA model, as a supervised chemometric method, could overcome the limitations of factors affecting the accuracy of the data in the actual operation, including environment or systematic errors. According to the information of groups, OPLS-DA could improve the priority of categorical variables and achieve good discrimination ability based on the information given in advance. It could distinguish different groups, especially those with larger intergroups. The PCA model, as an unsupervised method, could highlight similarities and differences in the underlying patterns in the data. By using the PCA model, the number of dimensions could be reduced, and the similarity between different classes based on SWATH-MA data using the unit variance scaling method could also be primary assessed.

As shown in Figure 3, in the groups of *in vitro* digestion (Figure 3A), SGF (Figure 3B), SIF (Figure 3C), and GI (Figure 3D), the digested samples of natural TM could all be separated from those of recombinant TM in the first principal component, implying the significant difference of digestion products between natural TM and recombinant TM. In Figure 3E,F, the spots of both nTM and rTM in the SGF were scattered in

Table 3. Epitopes of Shrimp-TM Reported in Literature

no.	location	sequence	length	species
E1	50–66	MQQLENDLDQVQESLLK	17	<i>P. indicus</i>
E2	153–161	FLAEEADRK	9	
E3	43–57	VHNLQKRMQQLENDL	15	<i>P. aztecus</i>
E4	85–105	VAALNRRIQLEEDLERSEER	21	
E5	133–148	RSLSDDEERMDALENQL	16	
E6	187–202	ESKIVELEELRVVGN	16	
E7	247–284	QKLQKEVDRLEDELVNEKEKYKSITDELDTFSELSGY	38	
E8	12–25	KLEKDNAMDRADTL	14	<i>L. vannamei</i>
E9	28–41	QNKEANNRAEKSEE	14	
E10	47–61	QKRMQQLENDLDQVQ	15	
E11	97–108	EDLERSEERLNT	12	
E12	117–132	SQAADSESRMRKVLEN	16	
E13	149–166	KEARFLAEEADRKYDEVA	18	
E14	178–191	RAEERAETGESKIV	14	
E15	209–224	VSEKANQREEAYKEQ	16	
E16	244–257	RSVQKLQKEVDRLE	14	
E17	261–274	VNEKEKYKSITDEL	14	
E18	25–30	LEQQNK	6	<i>M. ensis</i>
E19	43–60	VHNLQKRMQQLENDLDQV	18	
E20	87–103	ALNRRIQLEEDLERSE	17	
E21	146–154	NQLKEARFL	9	
E22	161–165	KYDEV	5	
E23	191–211	VELEELRVVGNLKSLEVSE	21	
E24	236–241	EARAEF	6	
E25	247–255	QKLQKEVDR	9	
E26	269–281	SITDELDTFSEL	13	
E27	89–105	NRRIQLEEDLERSEER	17	<i>P. monodon</i>
E28	115–128	EASQAADSESRMRK	14	
E29	131–142	ENRSLDEERMD	12	
E30	145–164	ENQLKEARFLAEEADRKYDE	20	
E31	177–190	ERAEEAETGESKI	14	
E32	210–224	SEKANQREEAYKEQ	15	
E33	243–259	ERSVQKLQKEVDRLEDE	17	
E34	263–280	EKEKYKSITDELDTFSE	18	
E35	12–25	KLEKDNAMDRADTL	14	<i>P. chinensis</i>
E36	47–60	QKRMQQLENDLDQV	14	
E37	87–105	ALNRRIQLEEDLERSEER	19	
E38	112–128	KLAEASQAADSESRMRK	17	
E39	131–147	ENRSLDEERMDALENQ	17	
E40	155–167	AAEADRKYDEVAR	13	
E41	187–203	ESKIVELEELRVVGN	17	
E42	221–239	YKEQIKTLTNKLKAAEARA	19	
E43	243–259	ERSVQKLQKEVDRLEDE	17	
E44	263–280	EKEKYKSITDELDTFSE	18	
E45	43–59	VHNLQKRMQQLENDLDS	17	<i>E. modestus</i>
E46	85–105	VAALNRRIQLEEDLERSEER	21	
E47	131–164	ENRSLDEERMDALENQLKEARFLAEEADRKYDE	34	
E48	187–201	ESKIVELEELRVVG	15	
E49	243–280	ERSVQKLQKEVDRLEDELVNEKEKYKSITDELDTFSE	38	

the first principal, which were far away from the spots of nTM and rTM in the SIF and GI models, implying the significant difference between the SGF as well as SIF and GI digestion profiles. Additionally, compared with rTM, nTM showed a higher correlation between the SIF digestion profile and the GI digestion profile.

Furthermore, these peptides were compared with a spectral library of all kinds of shrimp (*L. vannamei*) protein information from the NCBI protein database (632,350 proteins in total), and the results are listed in Table 2. As shown, “2”, “29”, and

“6” protein fragments of digestion products from natural TM appeared in SGF, SIF, and GI groups, respectively, while “17”, “44”, and “2” protein fragments of those from recombinant TM appeared in the groups SGF, SIF, and GI, respectively. All these fragments matched the sequence of TM from *L. vannamei*, and the best confidence of peptides was higher than 90%. It was obvious that much more peptides appeared in the digested samples from recombinant TM in comparison with those from natural TM. In contrast with the digestion models SGF and GI, there also existed numerous peptides in the

Table 4. Similarity of Fragments of nTM and rTM in SGF/SIF/GI with Allergen Epitopes of Shrimp

species	epitope	fragment	sequence ^a	location	similarity (%)	source
<i>P. indicus</i>	E1	NI3	MQQLENDLDQVQESLL	50–65	94.1	nTM-SIF
		NI4	MQQLENDLDQVQESLLK	50–66	100	nTM-SIF
		RI10	MQQLENDLDQVQESLL	50–65	94.1	rTM-SIF
		RI11	MQQLENDLDQVQESLLK	50–66	100	rTM-SIF
		NGI1	MQQLENDLDQVQESLLK	50–66	100	nTM-GI
	E2	NI18	FLAEEADR	153–160	88.9	nTM-SIF
		RI27	FLAEEADR	153–160	88.9	rTM-SIF
	E3	RI6	EVHNLQKRMQQL	42–53	80	rTM-SIF
		RI5	EVHNLQKRMQQ	42–52	73.3	rTM-SIF
		RI4	EVHNLQKRM	42–50	53.3	rTM-SIF
<i>P. aztecus</i>	E5	NI7	MDALENQLK	141–149	50	nTM-SIF
		RI26	MDALENQLK	141–149	50	rTM-SIF
	E6	NI24	IVELEEELR	190–198	56.3	nTM-SIF
		RI33	IVELEEELR	190–198	56.3	rTM-SIF
<i>L. vannamei</i>	E8	NG1	EKDNAMEADRADTL	14–25	85.7	nTM-SGF
		RG1	MKLEKDNAM	11–19	64.3	rTM-SGF
		RG2	MKLEKDNAMD	11–20	71.4	rTM-SGF
	E10	RI7	QKRMQQLENDL	47–57	73.3	rTM-SIF
	E11	RG9	LERSEERL	99–106	66.7	rTM-SGF
	E12	NI14	LAEASQAADER	113–125	56.3	nTM-SIF
		RI22	LAEASQAADER	113–125	56.3	rTM-SIF
		NGI3	LAEASQAADER	113–125	56.3	nTM-GI
	E13	RG13	LAEADRKYDEVA	154–166	72.2	rTM-SGF
		RG12	LAEADRKYDE	154–164	61.1	rTM-SGF
	E14	RG14	RAETGESKIVE	182–192	78.6	rTM-SGF
		RG15	RAETGESKIVEL	182–193	78.6	rTM-SGF
		NI22	AETGESK	183–189	50	nTM-SIF
		RI32	AETGESK	183–189	50	rTM-SIF
	E15	NI27	SLEVSEKANQR	206–217	56.3	nTM-SIF
		RI36	SLEVSEKANQR	206–217	56.3	rTM-SIF
	E16	RI39	AERSVQKLQKE	242–252	64.3	rTM-SIF
		RI40	AERSVQKLQKEVD	242–254	78.6	rTM-SIF
		RI41	KLQKEVDRL	248–256	64.3	rTM-SIF
		RG12	VQKLQKEVDRL	246–256	78.6	rTM-GI
		RI42	VNEKEKYKSIT	261–271	78.6	rTM-SIF
	E17	RI43	VNEKEKYKSITDE	261–273	92.9	rTM-SIF
		NGI4	YKSITDELDTFSELSGY	267–284	57.1	nTM-GI
<i>M. ensis</i>	E18	NI1	ADTLEQQNK	22–30	100	nTM-SIF
		RI2	ADTLEQQNK	22–30	100	rTM-SIF
	E19	NI3	MQQLENDLDQVQESLL	50–65	61.1	nTM-SIF
		NI4	MQQLENDLDQVQESLLK	50–66	61.1	nTM-SIF
		RI5	EVHNLQKRMQQ	42–52	55.6	rTM-SIF
		RI6	EVHNLQKRMQQL	42–53	61.1	rTM-SIF
		RI7	QKRMQQLENDL	47–57	61.1	rTM-SIF
		NGI1	MQQLENDLDQVQESLLK	50–66	61.1	nTM-GI
	E20	NI10	RIQLEEDLER	91–101	64.7	nTM-SIF
		NI12	IQLEEDLER	92–101	58.8	nTM-SIF
		RI18	RIQLEEDLER	91–101	64.7	rTM-SIF
		RI20	IQLEEDLER	92–101	58.8	rTM-SIF
	E21	RG10	ENQLKEARF	145–153	88.9	rTM-SGF
		RG11	NQLKEARF	146–153	88.9	rTM-SGF
	E22	RG12	LAEADRKYDE	154–164	80	rTM-SGF
		RG13	LAEADRKYDEVA	154–166	100	rTM-SGF
		RI28	KYDEVAR	161–167	100	rTM-SIF
		NI19	KYDEVAR	161–167	100	nTM-SIF
		RI39	AERSVQKLQKE	242–252	66.7	rTM-SIF
	E25	RI40	AERSVQKLQKEVD	242–254	88.9	rTM-SIF
		RI41	KLQKEVDRL	248–256	88.9	rTM-SIF
		RG12	VQKLQKEVDRL	246–256	100	rTM-GI
		NG2	DQTFSELSGY	275–284	53.8	nTM-SGF
	E26	NI29	SITDELDTQTF	269–278	76.9	nTM-SIF
		RI44	SITDELDTQTF	269–278	76.9	rTM-SIF
		NGI5	SITDELDTQTF	269–278	76.9	nTM-GI

Table 4. continued

species	epitope	fragment	sequence ^a	location	similarity (%)	source
<i>P. monodon</i>	E27	NGI6	SITDELDQTFSELGY	269–284	76.9	nTM-GI
		NI10	RIQLLEEDLER	91–101	64.7	nTM-SIF
		NI12	IQLEEDLER	92–101	58.8	nTM-SIF
		RI18	RIQLLEEDLER	91–101	64.7	rTM-SIF
		RI20	IQLEEDLER	92–101	58.8	rTM-SIF
	E28	NI14	LAEASQAADER	113–125	78.6	nTM-SIF
		RI22	LAEASQAADER	113–125	78.6	rTM-SIF
		NGI3	LAEASQAADER	113–125	78.6	nTM-GI
	E29	NI16	SLSDEER	134–140	58.3	nTM-SIF
		RI25	SLSDEER	134–140	58.3	rTM-SIF
	E30	RG12	LAEEADRKYDE	154–164	55	rTM-SGF
		RG13	LAEEADRKYDEVA	154–166	55	rTM-SGF
	E31	RG14	RAETGESKIVE	182–192	64.3	rTM-SGF
		RG15	RAETGESKIVEL	182–193	64.3	rTM-SGF
		NI23	AETGESK	183–189	50	nTM-SIF
	E32	RI32	AETGESK	183–189	50	rTM-SIF
		NI28	ANQREEAY	214–221	53.3	nTM-SIF
		RI37	ANQREEAY	214–221	53.3	rTM-SIF
	E33	RI39	AERSVQKLQKE	242–252	58.8	rTM-SIF
		RI40	AERSVQKLQKEVD	242–254	70.6	rTM-SIF
		RI41	KLQKEVDRL	248–256	52.9	rTM-SIF
	E34	RG12	VQKLQKEVDRL	246–256	64.7	rTM-GI
		NI29	SITDELDQTF	269–278	55.6	nTM-SIF
		RI42	VNEKEKYKSIT	261–271	50	rTM-SIF
		RI43	VNEKEKYKSITDE	261–273	61.1	rTM-SIF
		RI44	SITDELDQTF	269–278	55.6	rTM-SIF
		NGI4	YKSITDELDQTFSELGY	267–284	77.8	nTM-GI
		NGI5	SITDELDQTF	269–278	55.6	nTM-GI
		NGI6	SITDELDQTFSELGY	269–284	66.7	nTM-GI
<i>P. chinensis</i>	E35	NG1	EKDNAMDRADTL	14–25	85.7	nTM-SGF
		RG1	MKLEKDNAM	11–19	57.1	rTM-SGF
		RG2	MKLEKDNAMD	11–20	64.3	rTM-SGF
	E36	NI3	MQQLENDLDQVQESLL	50–65	78.6	nTM-SIF
		NI4	MQQLENDLDQVQESLLK	50–66	78.6	nTM-SIF
		RI7	QKRMQQLENDL	47–57	78.6	rTM-SIF
		RI9	KRMQQLENDL	48–57	71.4	rTM-SIF
		RI10	MQQLENDLDQVQESLL	50–65	78.6	rTM-SIF
		RI11	MQQLENDLDQVQESLLK	50–66	78.6	rTM-SIF
		NGI1	MQQLENDLDQVQESLLK	50–66	78.6	nTM-GI
		NI10	RIQLLEEDLER	91–101	57.9	nTM-SIF
	E37	NI12	IQLEEDLER	92–101	52.6	nTM-SIF
		RI18	RIQLLEEDLER	91–101	57.9	rTM-SIF
		RI20	IQLEEDLER	92–101	52.6	rTM-SIF
		NI14	LAEASQAADER	113–125	76.5	nTM-SIF
	E38	RI22	LAEASQAADER	113–125	76.5	rTM-SIF
		NGI3	LAEASQAADER	113–125	76.5	nTM-GI
		RG12	LAEEADRKYDE	154–164	76.9	rTM-SGF
	E40	RG13	LAEEADRKYDEVA	154–166	92.3	rTM-SGF
		NI24	IVELEEELR	190–198	52.9	nTM-SIF
	E41	RI33	IVELEEELR	190–198	52.9	rTM-SIF
		RI38	KEQIKTLTNKL	222–232	57.9	rTM-SIF
	E42	RG11	YKEQIKTLTNKL	221–232	63.2	rTM-GI
		RI39	AERSVQKLQKE	242–252	58.8	rTM-SIF
	E43	RI40	AERSVQKLQKEVD	242–254	70.6	rTM-SIF
		RG12	VQKLQKEVDRL	246–256	64.7	rTM-GI
		NI29	SITDELDQTF	269–278	55.6	nTM-SIF
	E44	RI44	SITDELDQTF	269–278	55.6	rTM-SIF
		NGI5	SITDELDQTF	269–278	55.6	nTM-GI
		NGI6	SITDELDQTFSELGY	269–284	66.7	nTM-GI
		NI3	MQQLENDLDQVQESLL	50–65	58.8	nTM-SIF
<i>E. modestus</i>	E45	NI4	MQQLENDLDQVQESLLK	50–66	58.8	nTM-SIF
		RI5	EVHNLQKRMQQ	42–52	64.7	rTM-SIF
		RI6	EVHNLQKRMQQL	42–53	64.7	rTM-SIF

Table 4. continued

species	epitope	fragment	sequence ^a	location	similarity (%)	source
		RI7	QKRMQQLENDL	47–57	64.7	rTM-SIF
		RI10	MQQLENDLDQVQESLL	50–65	58.8	rTM-SIF
		RI11	MQQLENDLDQVQESLLK	50–66	58.8	rTM-SIF
		NG11	MQQLENDLDQVQESLLK	50–66	58.8	nTM-GI
	E46	NI10	RIQLLEEDLER	91–101	52.4	nTM-SIF
		RI18	RIQLLEEDLER	91–101	52.4	rTM-SIF
	E48	NI24	IVELEEEELR	190–198	60	nTM-SIF
		RI33	IVELEEEELR	190–198	60	rTM-SIF

^aAmino acids matching shrimp-TM epitopes are shown in bold.

trypsin-based digestion model. These results implied that natural TM had a stronger ability to resist digestion, which was in agreement with the results of SDS-PAGE.

Tyr or Phe residues and peptide bonds could be cleaved by pepsin, which was also known as hydrolyzing aromatic amino acids.³⁰ Besides, aromatic amino acids located at the protein surface were susceptible to oxidation, which could cause the change of the protein structure. A possible reason for TM being slightly degraded in the SGF might be related to the modified or masked available digestion sites, and a similar phenomenon was also observed by Sante-Lhoutellier *et al.*³¹ Lys and Arg residues could be specifically cleaved by trypsin at a position, and lysine was easily modified or destroyed in the process of oxidation reactions. As several cleavage sites of pepsin and trypsin were contained in the primary protein structure, it might be possible for trypsin to attack the protein more easily.³² Different conformation structures between natural TM and recombinant TM might also be one possible reason for more protein fragments in digested samples of recombinant TM. As published before, the α -helical structures were found for both native shrimp (*P. monodon*) TM and recombinant shrimp TM, which was reflected by circular dichroism curves with minima at 208 nm.³³ Additionally, circular dichroism data showed about 3.13% α -helix and 49.32% β -sheets for native Per a 10 (*Periplaneta americana*), while, 19.92% α -helix and 27.52% β -sheets for recombinant Per a 10.³⁴

Matching Allergenic Epitopes. The allergenic epitopes are able to bind with IgE, which in turn bind with the receptor on the immune cell surface, triggering anaphylactic reactivities. Additionally, TM has a high homology in different shrimp species. Thus, we compared the sequences of digestion products from nTM and rTM with allergen epitopes of seven shrimp species (*P. indicus*, *P. aztecus*, *L. vannamei*, *M. ensis*, *P. monodon*, *P. chinensis*, and *E. modestus*), which were reported previously (Table 3).^{35–42} As shown in Table 4, the protein fragments were matched with allergen epitopes, and a matching degree of more than 50% was selected.

Among the total 49 epitopes, only 7 epitopes were not matched with our data. The summary of the peptides matching shrimp allergen epitopes is listed in Table 5. The matched numbers of peptides in the digested samples in the SGF were “2” and “3” of nTM and rTM, respectively, while “13” and “19” peptides in the SIF of nTM and rTM, respectively and “3” and “2” peptides in the GI group of nTM and rTM, respectively. The peptide numbers of digestion products in the GI group declined significantly, implying that the protein structure might be changed and the cleavage sites of trypsin might be covered or destroyed after pepsin digestion. The protein fragment SITDELDTQTF (269–278) appeared four times in the groups nTM-SGF, nTM-SIF, rTM-SIF, and nTM-GI, implying that

Table 5. Summary of Fragments of nTM and rTM in SGF/SIF/GI-Matched Shrimp Allergen Epitopes

source	no.	sequence	location	length
nTM-SGF	1	EKDNAMDRADTL	14–25	12
	2	YKSITDELDTQTFSELSGY	267–284	18
rTM-SGF	1	MKLEKDNAMD	11–20	10
	2	LERSEERL	99–106	8
	3	ENQLKEARF	145–153	9
	4	LAEADRKYDEVA	154–166	13
	5	RAETGESKIVEL	182–193	12
nTM-SIF	1	ADTLEQQNK	22–30	9
	2	MQQLENDLDQVQESLLK	50–66	17
	3	RIQLLEEDLER	91–101	11
	4	LAEASQAADER	113–125	13
	5	SLSDEER	134–140	7
	6	MDALENQLK	141–149	9
	7	FLAEADR	153–160	8
	8	KYDEVAR	161–167	7
	9	AETGESK	183–189	7
	10	IVELEEEELR	190–198	9
	11	SLEVSEKANQR	206–217	12
	12	ANQREEAY	214–221	8
	13	SITDELDTQTF	269–278	10
rTM-SIF	1	ADTLEQQNK	22–30	9
	2	EVHNLQKRMQQQL	42–53	12
	3	QKRMQQLENDL	47–57	11
	4	MQQLENDLDQVQESLLK	50–66	17
	5	RIQLLEEDLER	91–101	11
	6	LAEASQAADER	113–125	13
	7	SLSDEER	134–140	7
	8	MDALENQLK	141–149	9
	9	FLAEADR	153–160	8
	10	KYDEVAR	161–167	7
	11	AETGESK	183–189	7
	12	IVELEEEELR	190–198	9
	13	SLEVSEKANQR	206–217	12
	14	ANQREEAY	214–221	8
	15	KEQIKTLTNKL	222–232	11
	16	AERSVQLQKEVD	242–254	13
	17	KLQKEVDRL	248–256	9
	18	VNEKEYKSITDE	261–273	13
	19	SITDELDTQTF	269–278	10
nTM-GI	1	MQQLENDLDQVQESLLK	50–66	17
	2	LAEASQAADER	113–125	13
	3	YKSITDELDTQTFSELSGY	267–284	8
rTM-GI	1	YKEQIKTLTNKL	221–232	12
	2	VQKLQKEVDRL	246–256	11

the sequence was stabilized in the process of simulated digestion. These peptides were highly matched allergen epitopes

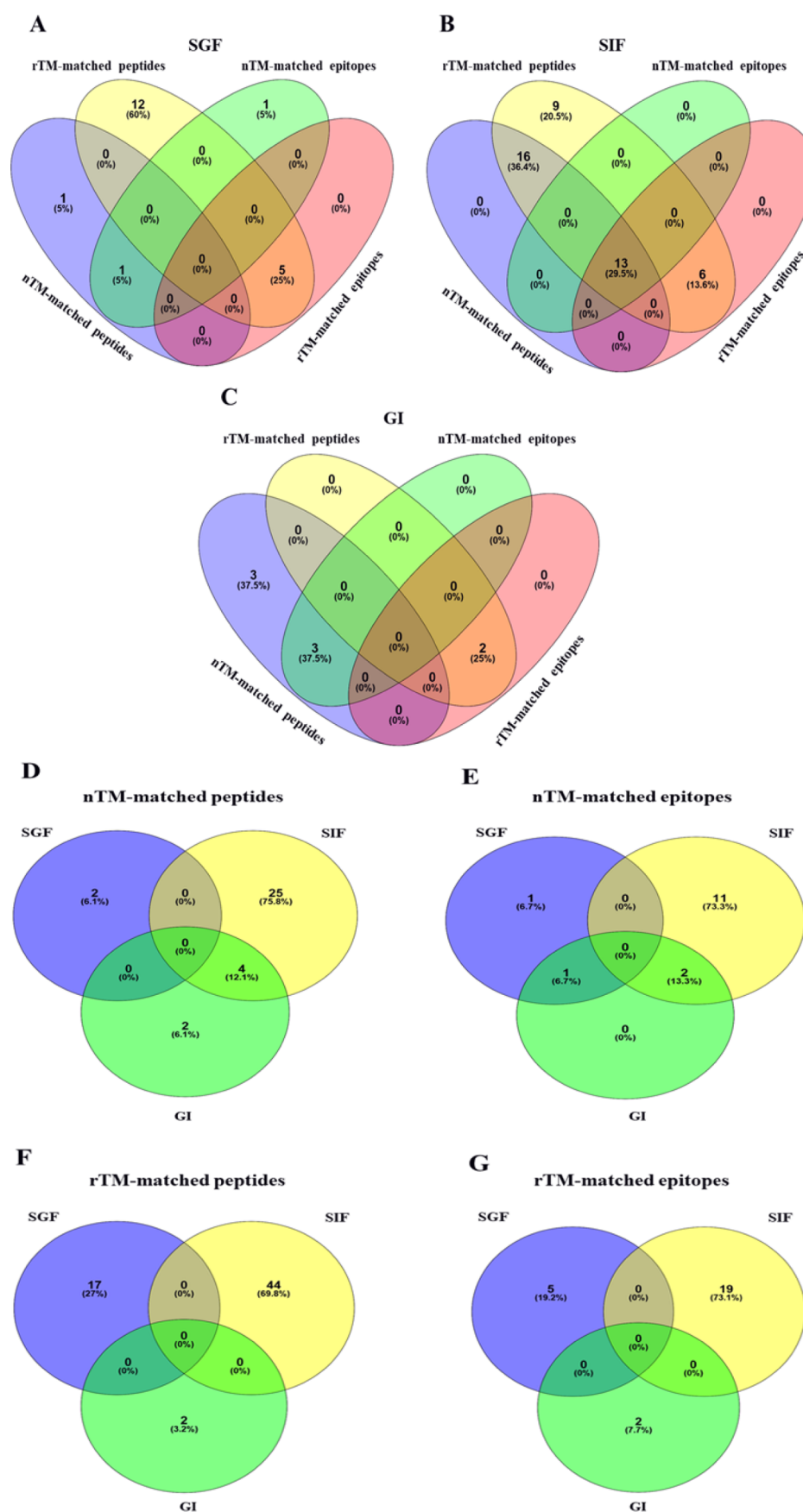


Figure 4. Correlation between fragment-matched peptides and fragment-matched epitopes of nTM and rTM in SGF/SIF/GI as well as in different digestion profiles. (A–C) Matched peptides and epitopes *in vitro* digestion (A) in the SGF, (B) in the SIF, (C) in GI; (D–G) matched peptides and epitopes in nTM and rTM, (D) matched peptides of nTM, (E) matched epitopes of nTM, (F) matched peptides of rTM, (G) matched epitopes of rTM.

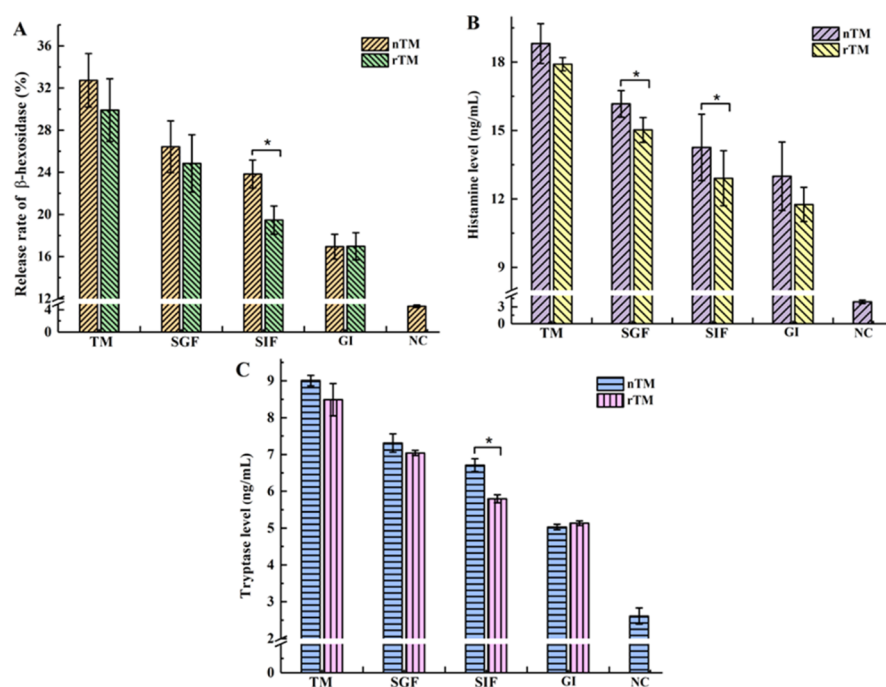


Figure 5. KU812 cells assay of nTM and rTM in SGF/SIF/GI. (A) Releases of β -hexosaminidase; (B) levels of histamine; (C) levels of tryptase. The data represent the mean $\pm$ SD ($n = 3$). Protein digested by the SGF for 30 min, by the SIF for 30 min, and by GI for 1 h (SGF: 30 min, SIF: 30 min). Tyrode buffer was used as a negative control. The data represent the mean $\pm$ SD ($n = 3$). "*" stands for significance at $p < 0.05$, and "***" stands for significance at $p < 0.01$.

from other shrimp species and might be one reason for the high allergy cross-reactivity among them, implying the close relationship between cross-reactivity and common allergen epitopes.⁴³

Additionally, the correlation between the fragment-matching peptides and the fragment-matching epitopes of different protein sources (nTM and rTM) as well as of different digestion profiles (SGF, SIF, and GI) was analyzed by a Venn diagram (Figure 4). As shown in the digestion profiles of the SGF and GI groups, there were no similar fragments that could match the peptides nor the epitopes between nTM and rTM, whereas "1" and "5" fragments could match both peptides and epitopes of nTM and rTM in the SGF (Figure 4A), respectively, and "3" and "2" fragments both belonged to groups of peptides and epitopes of nTM and rTM in the GI group (Figure 4C), respectively. Besides, in the digestion profile of the SIF, thirteen fragments belonged to these four groups (namely nTM-matched peptides, nTM-matched epitopes, rTM-matched peptides, and rTM-matched epitopes), while all the fragments of the nTM-matched peptide group ($n = 29$) overlapped with 65.9% fragments of the rTM-matched peptide group ($n = 44$) (Figure 4B). It was revealed that the digestion profile of the SIF was different from the other two digestion profiles (SGF and GI) in the view of fragment-matched peptides and epitopes.

Alternatively, a correlation between the fragment-matching peptides and the fragment-matching epitopes of nTM and rTM *in vitro* digestion was also analyzed (Figure 4D–G). As observed, four fragments from the nTM-matched peptides group were shared in SIF and GI digestion profiles, while, "1" and "2" fragments from the nTM-matched epitopes group were shared in the SGF and GI digestion profiles as well as the SIF and GI digestion profiles, respectively. However, there was no common fragment between the matched peptides and matched epitopes of rTM, implying that different source proteins exhibited different results of *in vitro* digestion.

Evaluation Degranulation Capacity. In comparison with immunochemical methods, cell models have the advantage of relatively little matrix influence on allergenicity assessment.¹³ Allergen proteins could work on the effector cells, including mast cells, basophils, and eosinophilia. With the released mediators, such as β -hexosaminidase, histamine, and leukotriene, mast cells or basophils are commonly used to identify protein anaphylaxis of allergen proteins. Currently, KU812 cell lines were used as an important model to evaluate the capability of degranulation. Thus, intact and digested samples of natural TM and recombinant TM were evaluated by basophil degranulation assay.

As shown in Figure 5, the overall trends of the release of β -hexosaminidase (Figure 5A), histamine contents (Figure 5B), and tryptase contents (Figure 5C) were similar. The data of natural TM and its digestion products were higher than those of recombinant TM and its digested samples. Only in the SIF group, there was a significant difference ($p < 0.05$) between digested samples of nTM and rTM based on the data of β -hexosaminidase release, histamine level, and tryptase level, which indicated that potential allergenicity of trypsin-based peptides might be higher than that of pepsin-based peptides. The reason might be that more relatively complete allergen epitopes were retained in natural TM and the relatively intact protein was easier to get accessibility to bind with IgE on the basophil cells.

In conclusion, the allergen characteristics of natural TM and recombinant TM as well as their *in vitro* digestion products were assessed by using immunology assays, SWATH-MS-based proteomics, and KU812 cell lines. In comparison with natural TM, pepsin-digested and trypsin-digested samples of recombinant TM showed lower IgG-/IgE binding, more protein fragments that matched allergen epitopes, and lower degranulation. However, the samples of pepsin- and trypsin-digested products from natural TM and recombinant TM showed similar numbers

of peptide-matching allergen epitopes and similar capability of degranulation. It could be speculated that there might be slightly different effects between natural TM and recombinant TM in the treatment of oral immunotherapy for shrimp-allergic patients. As natural TM is more complex than recombinant TM, more work should be done in the aspect of *in vivo* digestion profiles of natural TM and recombinant TM.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

TM, tropomyosin; PVDF, polyvinylidene fluoride; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; GI, gastrointestinal; SWATH-MS, sequential windowed acquisition of all theoretical fragment ion mass spectra; DDA, data-dependent acquisition; FDR, false discovery rate; PCA, principal component analysis; OPLS-DA, orthogonal partial least-squares discriminant analysis; ESI, electrospray ionization source; PBS, phosphate-buffered saline; KU812, human basophil cell line; HRP, horseradish peroxidase; ECL, enhanced chemiluminescent kit; ELISA, indirect enzyme-linked immunosorbent assay; NCBI, national center for biotechnology information; FBS, fetal bovine serum

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