

IgE-binding epitope mapping of tropomyosin allergen (Exo m 1) from *Exopalaemon modestus*, the freshwater Siberian prawn

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ABSTRACT

Exopalaemon modestus (EM) is a shrimp delicacy that could cause food allergy, the major allergen of EM is Exo m 1. The amino acid (AA) sequence, IgE-binding epitopes and allergenic peptides in gastrointestinal (GI) digests of Exo m 1, and their effects on basophil function were investigated. Exo m 1 has an AA-sequence of high similarity with other shrimp tropomyosins, while not 100% matching. The IgE-binding epitopes of Exo m 1 are epitope 1 (43–59, VHNLQKRMQQLLENDLS), epitope 2 (85–105, VAALNRRIQLLEEDLERSEER), epitope 3 (131–164, ENRSLDEERMDALENQLKEARFLAEEDRKYDE), epitope 4 (187–201, ESKIVELEELRVVG) and epitope 5 (243–280, ERSVQKLQKEVDRLDELVNEKEKYKSITDELDTQTFSE). Among the thirty-three peptides of Exo m 1 identified in GI digests, two were highly recognized by IgE, twenty-four moderately or weakly bound IgE, and seven had no IgE-reactivities. These IgE-binding epitopes and GI digestion induced-allergenic peptides could activate basophil degranulation, and CD63 and CD203c expression, they could be potential peptide-based immunotherapy for shrimp allergic individuals.

1. Introduction

Shrimp is among the most common food allergen sources (Faber et al., 2016; Sicherer & Sampson, 2018). *Exopalaemon modestus* (EM, the freshwater Siberian prawn) is a popular shrimp delicacy in Asia, while could cause food allergy. Recently it has been introduced to the Western Coast of North America (Washington, Oregon and California, Emmett, Hinton, Logan, & McCabe, 2002; Oh, Suh, Park, Ma, & Lim, 2002; Young, Berridge, O'Rear, Moyle, & Durand, 2017), making it a potentially relevant new food allergen. Exo m 1, a 38 kDa tropomyosin, is the major potent allergen of EM (Zhang, Zhang, Chen, & Zhou, 2018; Zhang, Xiao, & Zhou, 2019). With the increase of EM consumption, the incidence of allergic reactions also increased in China. However, the AA-sequence and IgE-binding epitopes of Exo m 1 were still unknown.

Food allergens are proteins with multiple IgE-binding epitopes (Matsuo, Yokooji, & Taogoshi, 2015). Binding of these epitopes with their corresponding receptors on immune cell surface is a necessary step for initiation of allergic reactions. Therefore, precise determination of IgE-binding epitopes is crucial for developing novel therapies for food

allergy. Immunoinformatics, a newly emergent branch, has become a potent tool for selecting epitopes from allergens (Ramesh et al., 2016; Sun, Shan, Yan, Zhang, & Guan, 2013). Moreover, an overlapping peptide library spanning the whole AA-sequence could be employed to improve the precision of epitope determination (Ayuso, Lehrer, & Reese, 2002; Ramesh et al., 2016). After computational immunoinformatics prediction of epitopes, the confirmatory experiments can be carried out based on the overlapping peptide assays of allergen-epitope peptides (Ayuso et al., 2002).

For dietary food allergen to sensitize an individual, it often goes via the GI tract (Wickham, Faulks, & Mills, 2009). After GI digestion, there might be potential allergenic peptides produced in GI digests, which could pass through GI passage and trigger allergic reactions (Astwood, Leach, & Fuchs, 1996; Wickham et al., 2009; Zhang et al., 2018). However, the allergenic peptides of Exo m 1 in GI digests and their IgE-reactivities are unknown and require further characterization.

Basophils are pivotal cells in triggering food allergy, once activated they release mediators such as bioactive histamine (Han et al., 2011; Mukai et al., 2005). KU812 human basophil cell line was used to

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Table 1
Information of shrimp allergy patients' sera.

Patient	Gender	Age	Clinical symptoms	IgE (kU _A /L)
1	Male	5 Years	AS	8.7
2	Female	2 Years	AS	6.3
3	Female	55 Years	HI, EC	6.8
4	Male	4 Years	AS, HI, DI	10.2
5	Female	42 Years	HI, CW	11.4
6	Female	24 Years	HI, NC	19.3
7	Male	37 Years	EC, RH	8.2
8	Male	2 Years	AS, NC	28.2
9	Female	10 Months	AS, EC	22.4
10	Male	5 Years	HI, EC, RH	65.5
11	Male	5 Years	AS, NC	35.9
12	Female	6 Years	AS, HI	69.6

Note: AS, asthma; CW, chest wheezing; EC, eczema; HI, hives; NC, nasal congestion; RH, rhinitis.

evaluate the allergenicity of epitope-peptides and GI digestion-induced allergenic peptides of Exo m 1.

The objectives of this work are purpose to investigate the AA-sequence of Exo m 1, IgE-binding epitopes and allergenic peptides in GI digests and their effects on IgE-reactivities and basophil function, to provide primary data for developing peptide-based immunotherapy for large cohorts of shrimp allergic individuals.

2. Materials and methods

2.1. Materials

Exopalaemon modestus (the freshwater Siberian prawn) was purchased from Nanquan fishery market (Wuxi, Jiangsu, China). The raw shrimp muscles were used to purify Exo m 1 according to a previous description (Zhang et al., 2018). Sera from twelve shrimp allergic patients with a clinical history served as positive controls (Table 1) and sera from eight non-food allergic and non-atopic healthy volunteers served as negative controls. Human basophil cell line (KU812) was obtained from the Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). 4-nitrophenyl *N*-acetyl- β -D-glucosaminide (PNAG, CAS: 3459-18-5) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Amino acid sequencing and sequence alignment of Exo m 1

2.2.1. Deglycosylation

Shrimp tropomyosin is a glycosylated protein (Hoffman, Day, & Miller, 1981; Zhang, Xiao, Zhang, & Zhou, 2019a), the existence of glycans renders AA-sequencing more challenging. Before AA-sequencing, Exo m 1 was enzymatically deglycosylated. Firstly, Exo m 1 was concentrated using ultrafiltration units (Amicon Ultra-4, 10 kDa, Merck Millipore Ltd., Tullagreen, Ireland), then *N*-deglycosylated using PNGase F (New England BioLabs, Ipswich, MA, USA) overnight at 37 °C. The *N*-deglycosylated TM were collected and washed with ultrapure water. Afterwards, the *N*-deglycosylated Exo m 1 was further O-deglycosylated using O-glycosidase & α 2-3,6,8 Neuraminidase Bundle (New England BioLabs, Ipswich, MA, USA). Finally, the *N*-O-deglycosylated Exo m 1 was washed with ultrapure water and lyophilized. The deglycosylated Exo m 1 was determined according to the instructions of Glycoprotein Staining kit (Thermo Fisher Scientific, Rockford, IL, USA).

2.2.2. Amino acid sequencing

The deglycosylated Exo m 1 was digested using sequencing grade trypsin (V511A, Promega Inc., Madison, WI, USA). Exo m 1 was hydrolyzed by trypsin (pH 7.5) under nitrogen gas at 37 °C for 22 h with the ratio of trypsin to Exo m 1 at 1:100 (w/w). Hydrolysis was

immediately terminated by lowering the pH to 2.0 with 1 M hydrochloric acid. The hydrolysates were desalted using Waters Sep-Pak C₁₈ SPE column (Waters Corp., Milford, MA, USA). The AA-sequence of Exo m 1 was determined by analyzing the tryptic digests of deglycosylated Exo m 1 using a quadrupole time-of-flight (Q-TOF) mass spectrometry (MS) (MALDI SYNAPT MS, Waters Corp., Manchester, UK). The mass detector was coupled to an electrospray ionization source (ESI).

A Waters Acquity Ultra Performance Liquid Chromatography (UPLC) equipped with an Acquity BEH C₁₈ column (2.1 × 50 mm, 1.7 μ m, Waters Corp., Milford, MA, USA) was applied to separate the peptides in tryptic digests. An aliquot of 10 μ L of sample was applied to the Waters Acquity UPLC system at a temperature of 45 °C. Solvent A was 100% formic acid, and solvent B was 100% acetonitrile. The elution was performed at a flow rate of 300 μ L/min using a gradient from 0 to 40% B within 55 min, to 100% B in the next 5 min, maintaining at 100% B for another 5 min, then back to 0% B within 1 min, and preserving for another 4 min. The mass measurement was performed in a positive ionization mode with scan *m/z* range started from 100 to 3000. The low collision energy was set at 6 V, and the high collision energy was set at 25 V. The capillary and sampling cone voltages were 3.5 kV and 30 V, respectively. The source and desolvation temperature were 100 °C and 400 °C, respectively. The cone gas flow and the desolvation gas flow were 50 and 500 L/h, respectively. The results were analyzed using MassLynx V4.1 and MassEnt (Waters Corp., Manchester, UK) to process MS spectra and perform peptide sequencing. The complete AA-sequence was obtained by combination of the overlapping peptides in tryptic digests of Exo m 1 according to a previous approach (Seo, L'Hocine, & Karboune, 2014).

2.2.3. Amino acid sequence alignment

The AA-sequence alignment was carried out by comparing the AA-sequence of Exo m 1 with representative shrimp tropomyosin allergens: Cra c 1 (Genbank accession number: ACR43473), Hom a 1 (Genbank accession number: AAC48287), Lit v 1 (Genbank accession number: ACB38288), Mac r 1 (Genbank accession number: ADC55380), Mel l 1 (Genbank accession number: AGF86397), Met e 1 (Genbank accession number: AAA60330), Pan b 1 (Genbank accession number: CBY17558), Pan s 1 (Genbank accession number: AAC38996) and Pen a 1 (Genbank accession number: AAZ76743), Pen i 1 (Shanti, Martin, Nagpal, Metcalfe, & Rao, 1993), Pro c 1 (Genbank accession number: ACN87223), using DNAMAN software (Version 8, Lynnon Corp., Vandreuil, Quebec, Canada).

2.3. Epitope identification

2.3.1. Epitope prediction

In order to improve the veracity of epitope prediction, the complete AA-sequence of Exo m 1 was analyzed using three immunoinformatics based computational approaches, including DNASTar Protean system (DNASTar Inc., Madison, WI, USA), AntheProt software (Version 6.9.3, PRABI-Lyon-Gerland, Lyon, France), BepiPred 2.0 Server (<http://www.cbs.dtu.dk/services/BepiPred/>), ABCpred Server (<https://webs.iitd.edu.in/raghava/abcpred/dataset.html>) and Immunomedicine Group Tools Server (<http://imed.med.ucm.es/Tools/antigenic.pl>).

In the DNASTar Protean system, the hydrophilicity, flexibility, accessibility and antigenicity of Exo m 1 AA-sequence were chosen as parameters for epitope prediction as previously described (Hopp & Woods, 1981; Sun et al., 2013). Based on the predictive results, peptide regions with high hydrophilicity, flexibility, surface accessibility and antigenic index were chosen as candidate epitopes of DNASTar prediction for further investigation.

The BepiPred 2.0 Server, ABCpred Server and Immunomedicine Group Tools Server were also applied to predict the epitopes of Exo m 1. The ultimate predicted epitopes were developed by combining the data of all immunoinformatics tools using a previously published method (Yang & Yu, 2008; Zheng, Lin, Pawar, Li, & Li, 2011). The combined

Table 2A

Location of predicted epitopes on Exo m 1 by immunoinformatics tools.

Tools	Location of the predicted epitopes
DNAStar Protean	23–40, 45–59, 89–105, 115–142, 145–165, 177–190, 210–225, 240–259, 263–280
AntheProt	25–37, 89–105, 115–125, 131–142, 145–164, 177–190, 243–260, 263–280
ABCpred Server	10–25, 19–34, 27–42, 38–53, 44–59, 50–65, 58–93, 64–79, 70–85, 82–97, 93–108, 100–105, 106–121, 117–132, 123–138, 132–147, 153–168, 162–177, 169–184, 179–194, 191–206, 215–230, 221–236, 232–247, 248–263, 267–282
Immunomedicine	56–69, 83–96, 110–118, 149–155, 161–174, 187–193, 195–202, 242–255
PepiPred 2.0 Server	16–40, 55–59, 76–84, 99–108, 112–128, 131–143, 158–164, 176–188, 209–223, 236–241, 243–259, 261–276

Table 2B

Predicated epitopes of Exo m 1 by combined immunoinformatics methods.

Number	Sequence	Source (position)	Length of sequence
pre-epitope 1	LTCEQQNKEANNRAEKSE	23–40	18
pre-epitope 2	NLQKRMQQLENDLDQ	45–59	15
pre-epitope 3	NRRIQLLEEDLERSEER	89–105	17
pre-epitope 4	EASQAADSESRMRK	115–128	14
pre-epitope 5	ENRSLSDERMD	131–142	12
pre-epitope 6	ENQLKEARFLAEADRKYDE	145–164	20
pre-epitope 7	ERAEEAETGESKI	177–190	14
pre-epitope 8	SEEKANQREEAYKEQ	210–224	15
pre-epitope 9	ERSVQKLQKEVDRLDE	243–259	17
pre-epitope 10	EKEKYKSITDELDTQTFSE	263–280	18

results of immunoinformatics tools (Table 2A) were applied to predict potential epitope regions. Finally, ten predicated epitope peptides (Table 2B) were synthesized using standard Fmoc solid phase peptide synthesis by Ontores Biotech (Hangzhou, Zhejiang, China).

2.3.2. Inhibitory dot-blot assay

The inhibitory dot-blot experiment was performed according to Fu, Wang, Ni, Wang, and Wang (2018) and Zheng et al. (2011), to determine the IgE-reactivities of predicted epitope peptides. Nitrocellulose membranes (0.45 µm) were immersed in ultrapure water for 15 min. When the membranes were damp-dry, Exo m 1 samples that dissolved in carbonate buffered saline (CBS, 0.015 mol/L Na₂CO₃, 0.035 mol/L NaHCO₃, pH 9.6) were spotted (4 µg for each dot) on the membranes, left to dry at 25 °C and blocked for 2 h using 3% BSA in TBST (0.1 mol/L Tris, 0.15 mol/L NaCl, 0.05% Tween-20, pH 7.5). Then the membranes were incubated with diluted human sera (1:10) for 1.5 h at 37 °C, which had been preincubated with predicted epitope peptides (2 mg/mL) for 1 h at 37 °C, followed by further incubating with HRP-conjugated goat anti-human IgE serum (1:5000) for 1 h at 37 °C. The membranes were then incubated in DAB (3,3'-Diaminobenzidine) luminous (Beyotime Biotech, Shanghai, China) for 1 min and detected using the ChemiDoc XRS⁺ image scanner with Image Lab software (Version 4.0, Bio-Rad, Hercules, CA, USA). The membranes were washed for 5 min with TBST for three times between each step. For interpretation of the results, IgE-reactivities were graded into four categories and scored according to their intensity: negative intensity (score: 0, black dot-blot), weak intensity (score: 1, dark gray dot-blot), medium intensity (score: 2, light gray dot-blot) and strong intensity (score: 3, white dot-blot), the black-white color code system was coded as follows: negative intensity = white; weak intensity = light gray; medium intensity = dark gray; strong intensity = black (Ayuso et al., 2002). The intensity of IgE reactivities was determined by both ChemiDoc XRS⁺ image scanner and visually by agreement between five different investigators, who graded the reactivities independently, assigning the above scores.

2.3.3. Overlapping peptide library for epitope mapping

A library of 46 overlapping peptides spanning the whole AA-sequence of Exo m 1 was synthesized by Ontores Biotech (Hangzhou, Zhejiang, China). Each peptide had a length of 15 AA residues with an

offset of 6 in relation to the previous and following peptides, with the exception of peptide 46, which had an offset of 5 AA residues (Table 3A). The inhibitory dot-blot experiment was performed to determine the IgE-reactivities of overlapping epitope peptides with IgE.

2.4. Allergenic peptides of Exo m 1 in GI digests

Native Exo m 1 was firstly gastric digested using pepsin (Sigma-Aldrich, St. Louis, MO, USA) with the ratio of pepsin to Exo m 1 at 1:50 (w/w), conducted by incubation at 37 °C (pH 1.2) for 1 h and terminated by adjusting pH to 7.5. Subsequently, trypsin (Sigma-Aldrich, St. Louis, MO, USA) was added with the ratio of trypsin to Exo m 1 at 1:100 (w/w) and incubated at 37 °C for 3 h, digestion was immediately terminated by lowering the pH to 2.0 with 1 M HCl. Analysis of peptides in GI digests was performed on Waters SYNAPT MS System (Waters Corp., Manchester, UK). Each peptide identified in GI digests of Exo m 1 was subsequently synthesized by Ontores Biotech (Hangzhou, Zhejiang, China). The inhibitory dot-blot assay was performed to assay the IgE-reactivities of peptides in GI digests.

2.5. Human basophil model assay

2.5.1. Passive KU812 basophil immunization

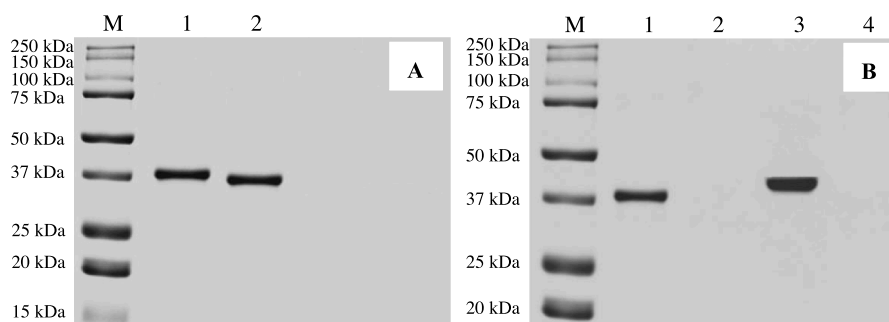
IgE-immunization of basophil is according to a previous method (Zhang et al., 2019). Basophils were cultured in IMDM culture medium at 37 °C for 24 h, then washed with PBS and sensitized by diluted human sera of shrimp-allergic subjects (1:10 diluted in culture medium without fetal bovine serum (FBS)). After incubating for 24 h, cells were centrifuged and washed with PBS. Allergy activation was induced by incubating with Exo m 1, epitope-peptides or GI digestion-induced peptides (37 °C for 1 h).

2.5.2. Histamine release and degranulation test

The IgE-immunized basophils were centrifuged with supernatant collected to determine histamine content using the histamine ELISA kit (IBL, Hamburg, Germany). KU812 degranulation was measured by determining the enzymatic activity of β-hexosaminidase in supernatant. The supernatant was transferred to a 96-well plate (30 µL/well), and pipetted 1 mmol/L PNAG (50 µL/well). After incubation (37 °C for 1 h), 0.2 mol/L glycine solution (100 µL/well, pH 10.7) was pipetted, the absorbance (OD_{450 nm}) was measured using a plate reader (SpectraMax M5, Molecular Devices, CA, USA). The results were presented as the percentage of β-hexosaminidase activity measured in supernatant samples compared to the total levels in corresponding cell lysates. For determining the total β-hexosaminidase activity, the basophils were lysed with 0.1% Triton-X 100 prior to removing the supernatant.

2.5.3. CD63 and CD203c antigens

IgE-immunized basophils were washed with PBS for three times. Then pipetting flow cytometry staining buffer (Thermo Fisher Scientific, Rockford, IL, USA), and separated basophils to two equal parts. Afterwards, pipetting FITC Mouse Anti-Human CD63 and PE Mouse Anti-Human CD203c (BD Bioscience, San Diego, CA, USA), respectively, incubated in dark for 30 min. Then, washing basophils and resuspended cells using staining buffer for flow cytometry analysis



Exo m 1 MDAIKKKMQAMKLEKDNAMDRADTLEQONKEANNRAEKSEEEVFSLQKRMOOLENDLPSV 60 **C**
 Cra c 1 MDAIKKKMQAMKLEKDNAMDRADTLEQONKEANNRAEKSEEEVFSLQKRMOOLENDLPSV 60
 Hom a 1 MDAIKKKMQAMKLEKDNAMDRADTLEQONKEANNRAEKSEEEVFSLQKRMOOLENDLPSV 60
 Lit v 1 MDAIKKKMQAMKLEKDNAMDRADTLEQONKEANNRAEKSEEEVFSLQKRMOOLENDLPSV 60
 Mac r 1 MDAIKKKMQAMKLEKDNAMDRADTLEQONKEANNRAEKSEEEVFSLQKRMOOLENDLPSV 60
 Mel l 1 MDAIKKKMQAMKLEKDNAMDRADTLEQONKEANNRAEKSEEEVFSLQKRMOOLENDLPSV 60
 Met e 1 MKLEKDNAMDRADTLEQONKEANNRAEKSEEEVFSLQKRMOOLENDLPSV 50
 Pan b 1 MDAIKKKMQAMKLEKDNAMDRADTLEQONKEANNRAEKSEEEVFSLQKRMOOLENDLPSV 60
 Pan s 1 MKLEKDNAMDRADTLEQONKEANNRAEKSEEEVFSLQKRMOOLENDLPSV 50
 Pen a 1 MDAIKKKMQAMKLEKDNAMDRADTLEQONKEANNRAEKSEEEVFSLQKRMOOLENDLPSV 60
 Pen i 1 MDAIKKKMQAMKLEKDNAMDRADTLEQONKEANNRAEKSEEEVFSLQKRMOOLENDLPSV 60
 Pen m 1 MDAIKKKMQAMKLEKDNAMDRADTLEQONKEANNRAEKSEEEVFSLQKRMOOLENDLPSV 60
 Pro c 1 MDAIKKKMQAMKLEKDNAMDRADTLEQONKEANNRAEKSEEEVFSLQKRMOOLENDLPSV 60

Exo m 1 QBALKKANAHLEKQKALSNAEGEVAALNRRIOLEEDLERSEERINTATTKLAEASQAA 120
 Cra c 1 QBALKKANAHLEKQKALSNAEGEVAALNRRIOLEEDLERSEERINTATTKLAEASQAA 120
 Hom a 1 QBALKKANAHLEKQKALSNAEGEVAALNRRIOLEEDLERSEERINTATTKLAEASQAA 120
 Lit v 1 QBALKKANAHLEKQKALSNAEGEVAALNRRIOLEEDLERSEERINTATTKLAEASQAA 120
 Mac r 1 QBALKKANAHLEKQKALSNAEGEVAALNRRIOLEEDLERSEERINTATTKLAEASQAA 120
 Mel l 1 QBALKKANAHLEKQKALSNAEGEVAALNRRIOLEEDLERSEERINTATTKLAEASQAA 120
 Met e 1 QBALKKANAHLEKQKALSNAEGEVAALNRRIOLEEDLERSEERINTATTKLAEASQAA 110
 Pan b 1 QBALKKANAHLEKQKALSNAEGEVAALNRRIOLEEDLERSEERINTATTKLAEASQAA 120
 Pan s 1 QBALKKANAHLEKQKALSNAEGEVAALNRRIOLEEDLERSEERINTATTKLAEASQAA 110
 Pen a 1 QBALKKANAHLEKQKALSNAEGEVAALNRRIOLEEDLERSEERINTATTKLAEASQAA 120
 Pen i 1 QBALKKANAHLEKQKALSNAEGEVAALNRRIOLEEDLERSEERINTATTKLAEASQAA 120
 Pen m 1 QBALKKANAHLEKQKALSNAEGEVAALNRRIOLEEDLERSEERINTATTKLAEASQAA 120
 Pro c 1 QBALKKANAHLEKQKALSNAEGEVAALNRRIOLEEDLERSEERINTATTKLAEASQAA 120

Exo m 1 DESERMRKVLENRSLSDEERMDALENQLKEARFLAEADRKYDEVARKLAMVEADLERAE 180
 Cra c 1 DESERMRKVLENRSLSDEERMDALENQLKEARFLAEADRKYDEVARKLAMVEADLERAE 180
 Hom a 1 DESERMRKVLENRSLSDEERMDALENQLKEARFLAEADRKYDEVARKLAMVEADLERAE 180
 Lit v 1 DESERMRKVLENRSLSDEERMDALENQLKEARFLAEADRKYDEVARKLAMVEADLERAE 180
 Mac r 1 DESERMRKVLENRSLSDEERMDALENQLKEARFLAEADRKYDEVARKLAMVEADLERAE 180
 Mel l 1 DESERMRKVLENRSLSDEERMDALENQLKEARFLAEADRKYDEVARKLAMVEADLERAE 180
 Met e 1 DESERMRKVLENRSLSDEERMDALENQLKEARFLAEADRKYDEVARKLAMVEADLERAE 170
 Pan b 1 DESERMRKVLENRSLSDEERMDALENQLKEARFLAEADRKYDEVARKLAMVEADLERAE 180
 Pan s 1 DESERMRKVLENRSLSDEERMDALENQLKEARFLAEADRKYDEVARKLAMVEADLERAE 170
 Pen a 1 DESERMRKVLENRSLSDEERMDALENQLKEARFLAEADRKYDEVARKLAMVEADLERAE 180
 Pen i 1 DESERMRKVLENRSLSDEERMDALENQLKEARFLAEADRKYDEVARKLAMVEADLERAE 180
 Pen m 1 DESERMRKVLENRSLSDEERMDALENQLKEARFLAEADRKYDEVARKLAMVEADLERAE 180
 Pro c 1 DESERMRKVLENRSLSDEERMDALENQLKEARFLAEADRKYDEVARKLAMVEADLERAE 180

Exo m 1 ERAETGESKIVLEEEELRVVGNLKSLEVSEEEKANQREERAYKBOIKTLTNKLKAAEARAE 240
 Cra c 1 ERAETGESKIVLEEEELRVVGNLKSLEVSEEEKANQREERAYKBOIKTLTNKLKAAEARAE 240
 Hom a 1 ERAETGESKIVLEEEELRVVGNLKSLEVSEEEKANQREERAYKBOIKTLTNKLKAAEARAE 240
 Lit v 1 ERAETGESKIVLEEEELRVVGNLKSLEVSEEEKANQREERAYKBOIKTLTNKLKAAEARAE 240
 Mac r 1 ERAETGESKIVLEEEELRVVGNLKSLEVSEEEKANQREERAYKBOIKTLTNKLKAAEARAE 240
 Mel l 1 ERAETGESKIVLEEEELRVVGNLKSLEVSEEEKANQREERAYKBOIKTLTNKLKAAEARAE 240
 Met e 1 ERAETGESKIVLEEEELRVVGNLKSLEVSEEEKANQREERAYKBOIKTLTNKLKAAEARAE 230
 Pan b 1 ERAETGESKIVLEEEELRVVGNLKSLEVSEEEKANQREERAYKBOIKTLTNKLKAAEARAE 240
 Pan s 1 ERAETGESKIVLEEEELRVVGNLKSLEVSEEEKANQREERAYKBOIKTLTNKLKAAEARAE 230
 Pen a 1 ERAETGESKIVLEEEELRVVGNLKSLEVSEEEKANQREERAYKBOIKTLTNKLKAAEARAE 240
 Pen i 1 ERAETGESKIVLEEEELRVVGNLKSLEVSEEEKANQREERAYKBOIKTLTNKLKAAEARAE 240
 Pen m 1 ERAETGESKIVLEEEELRVVGNLKSLEVSEEEKANQREERAYKBOIKTLTNKLKAAEARAE 240
 Pro c 1 ERAETGESKIVLEEEELRVVGNLKSLEVSEEEKANQREERAYKBOIKTLTNKLKAAEARAE 240

Exo m 1 FAERSVQKLQKEVDRLDELVNEKEKYKSTIDBLDQTFSELGSY. 284
 Cra c 1 FAERSVQKLQKEVDRLDELVNEKEKYKSTIDBLDQTFSELGSY. 284
 Hom a 1 FAERSVQKLQKEVDRLDELVNEKEKYKSTIDBLDQTFSELGSY. 284
 Lit v 1 FAERSVQKLQKEVDRLDELVNEKEKYKSTIDBLDQTFSELGSY. 284
 Mac r 1 FAERSVQKLQKEVDRLDELVNEKEKYKSTIDBLDQTFSELGSY. 284
 Mel l 1 FAERSVQKLQKEVDRLDELVNEKEKYKSTIDBLDQTFSELGSY. 284
 Met e 1 FAERSVQKLQKEVDRLDELVNEKEKYKSTIDBLDQTFSELGSY. 274
 Pan b 1 FAERSVQKLQKEVDRLDELVNEKEKYKSTIDBLDQTFSELGSY. 284
 Pan s 1 FAERSVQKLQKEVDRLDELVNEKEKYKSTIDBLDQTFSELGSY. 274
 Pen a 1 FAERSVQKLQKEVDRLDELVNEKEKYKSTIDBLDQTFSELGSY. 284
 Pen i 1 FAERSVQKLQKEVDRLDELVNEKEKYKSTIDBLDQTFSELGSY. 285
 Pen m 1 FAERSVQKLQKEVDRLDELVNEKEKYKSTIDBLDQTFSELGSY. 284
 Pro c 1 FAERSVQKLQKEVDRLDELVNEKEKYKSTIDBLDQTFSELGSY. 284

(caption on next page)

Fig. 1. Deglycosylation of Exo m 1 determined by SDS-PAGE (A) and glycoprotein staining kit (B), multiple AA-sequence alignment of shrimp tropomyosins (C) and DNASTAR prediction results of the hydrophilicity, flexible regions, antigenic index and surface probability of Exo m 1 (D). Note: A: M: marker; lane 1: Exo m 1; lane 2: N- & O-deglycosylated Exo m 1. B: M: marker; lane 1: Exo m 1; lane 2: N- & O-deglycosylated Exo m 1; lane 3: Horseradish peroxidase (HRP), lane 4: Soybean trypsin inhibitor (STI).

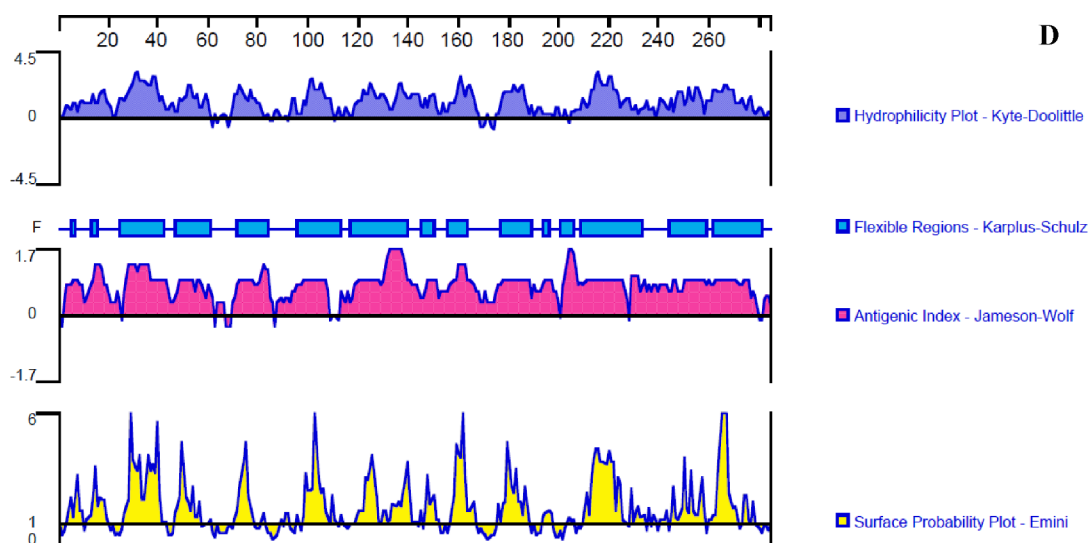


Fig. 1. (continued)

Table 2C

IgE reactivities of twelve shrimp-allergic subjects to the ten predicted epitopes of Exo m 1.

Sera	Predicted Epitopes										PN
Subjects No.	1	2	3	4	5	6	7	8	9	10	
Control sera											0
1											2
2											3
3											4
4											6
5											4
6											6
7											4
8											5
9											8
10											7
11											7
12											8
Positive subjects, n	3	10	10	2	10	8	4	3	7	7	
Intensity	5	18	19	3	21	13	9	3	16	15	
≥50% subjects		+	+		+	+			+	+	
>Mean intensity+SD			+		+						

Note: The color-coded system is carried out as follows: strong (3): black; medium (2): dark gray; weak (1): light gray; negative (0): white. PN = Positive number.

(FACSCalibur flow cytometer, BD Bioscience, NJ, USA). The expression of CD63 and CD230c antigens was determined using the MFI (mean fluorescence intensity).

2.6. Statistical analysis

All data were shown as means $\pm$ standard deviation (SD). Statistical analysis was carried out using SPSS software (SPSS Inc., Version 19, Chicago, IL, USA). The tropomyosin AA-sequence alignment was performed on DNAMAN 8 software (Lynnon Corp., Vandreuil, Quebec, Canada).

3. Results and discussion

3.1. Amino acid sequence of Exo m 1 and sequence alignment

Shrimp tropomyosin is a glycoprotein (Hoffman et al., 1981; Zhang et al., 2019a). The Glycoprotein Staining kit (Thermo Fisher Scientific, USA) was used to detect the carbohydrate moieties of glycoproteins. When treated with Oxidizing Reagent (periodic acid), glycols present in glycoproteins are oxidized to aldehydes. After completing the procedure, the glycols are stained by staining solution, yielding visible bands of glycoprotein (Suvarna, Layton, & Bancroft, 2018), therefore only glycoprotein with carbohydrate moieties has visible bands. As shown in Fig. 1A, compared with Exo m 1 (lane 1), after N- & O-deglycosylation, there was a decreased molecular weight, which suggested there should be glycans in Exo m 1. The deglycosylation of Exo m 1 was further confirmed by detecting glycoprotein saccharide moieties (Fig. 1B). Compared with the positive control (HRP, lane 3) and a negative control (STI, lane 4), there was invisible band in lane 2, which further confirmed the existence of glycans in Exo m 1 (lane 1), and the high efficiency to remove carbohydrate moieties for N- & O-deglycosylated Exo m 1 (lane 2). Our previous data showed that after enzymatically N- & O-deglycosylation, the carbohydrate content decreased from 3.031% to 0.059% ($\approx 0\%$), which further confirmed the high deglycosylation of PNGase F, O-glycosidase & $\alpha 2$ -3,6,8 Neuraminidase Bundle on Exo m 1 (Zhang, Xiao, Zhang, & Zhou, 2019b).

The AA-sequence of Exo m 1 was established by combination of the overlapping peptides identified in the tryptic digests of N- & O-

deglycosylated Exo m 1 (Table 3B). Through the multiple AA-sequence alignment (Fig. 1C), the AA-sequence of Exo m 1 had high sequence similarity with other shrimp tropomyosins, while not 100% matching: *Crangon crangon* (Cra c 1, 98.59%), *Homarus americanus* (Hom a 1, 94.37%), *Litopenaeus vannamei* (Lit v 1, 97.54%), *Macrobrachium rosenbergii* (Mac r 1, 98.59%), *Melicertus latisulcatus* (Mel l 1, 95.42%), *Metapenaeus ensis* (Met e 1, 96.83%), *Pandalus borealis* (Pan b 1,

97.18%), *Panulirus stimpsoni* (Pan s 1, 96.72%), *Penaeus aztecus* (Pen a 1, 97.54%), *Penaeus indicus* (Pen i 1, 79.58%), *Penaeus monodon* (Pen m 1, 97.53%) and *Procambarus clarkii* (Pro c 1, 95.77%). [Motoyama, Suma, Ishizaki, Nagashima, and Shiomi \(2007\)](#) also found the high AA-sequence similarity of crustacean tropomyosins by AA-sequence alignment.

Table 3A

Forty-six overlapping peptides spanning the entire amino acid sequence of Exo m 1.

		1	10	20	30	40	50	60	70	80	
		MDAIKKKMQAMKLEKDNAMDRLTCEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDSVQEALLKANAHLEEKDKALSNA									
1	1-15	MDAIKKKMQAMKLEK									
2	7-21	KMQAMKLEKDNAMDR									
3	13-27	LEKDNAMDRLTCEQ									
4	19-33	MDRLTCEQQNKEAN									
5	25-39	CEQQNKEANNRAEKS									
6	31-45	EANNRAEKSEEEVHN									
7	37-51	EKSEEEVHNLQKRMQ									
8	43-57	VHNLQKRMQQLENDL									
9	49-63	RMQQLENDLDSVQEA									
10	55-69	NDLDSVQEALLKANA									
11	61-75	QEALLKANAHLEEKD									
12	67-81	ANAHLEEKDKALSNA									
		80	90	100	110	120	130	140	150		
		EGEVAALNRRRIQLEEDLERSEERLNTATTKLAEASQAADSESRMRKVLENRSLSDERMDALENQLKEARF									
13	73-87	EKDKALSNAEGEVAA									
14	79-83	SNAEGEVAALNRRRIQ									
15	85-99	VAALNRRRIQLEEDL									
16	91-105	RIQLEEDLERSEER									
17	97-111	EDLERSEERLNTATT									
18	103-117	EERLNTATTKLAEAS									
19	109-123	ATTKLAEASQAADSE									
20	115-129	EASQAADSESRMRKV									
21	121-135	DESERMRKVLENRSL									
22	127-141	RKVLENRSLSDERMD									
23	133-147	RSLSDERMDALENQ									
24	139-153	ERMDALENQLKEARF									
		150	160	170	180	190	200	210	220		
		LAEEADRKYDEVARKLAMVEADLERAEERAETGESKIVELEELRVVGNLKSLEVSEEKANQREEAYKEQI									
25	145-159	ENQLKEARFLAEAD									
26	151-165	ARFLAEADRKYDEV									
27	157-171	EADRKYDEVARKLAM									
28	163-177	DEVARKLAMVEADLE									
29	169-183	LAMVEADLERAEERA									
30	175-189	DLRAEERAETGESK									
31	181-195	ERAETGESKIVELEE									
32	187-201	ESKIVELEELRVVG									
33	193-207	LEELRVVGNLKS									
34	199-213	VVGNLKSLEVSEEK									
35	205-219	KSLEVSEEKANQREE									
36	211-225	EEKANQREEAYKEQI									
		220	230	240	250	260	270	280			
		KTLTNKLKAAEARAEFAERSVQKLQKEVDRLEDELVNEKEYKSITDELDTFSELSGY									
37	217-231	REEAYKEQIKTLTNK									
38	223-237	EQIKTLTNKLKAAEA									
39	229-243	TNKLKAAEARAEFAE									
40	235-249	AEARAEFAERSVQKL									
41	241-255	FAERSVQKLQKEVDR									
42	247-261	QKLQKEVDRLEDELV									
43	253-267	VDRLEDELVNEKEY									
44	259-273	ELVNEKEYKSITDE									
45	265-279	EKYSITDELDTFSE									
46	270-284	ITDELDTFSELSGY									

Note: length: 15 amino acid residues; offset: 6 amino acids.

Table 4A
The matched peptides of Exo m 1 in the in vitro gastrointestinal digests.

No.	Start-End	Mr (obs)	Mr (calc)	Sequence
1	1–5	577.3180	577.3014	MDAIK
2	1–7	833.3044	833.4913	MDAIKKK
3	6–12	864.5101	864.4794	KKMQAMK
4	7–12	736.2449	736.3844	KMQAMK
5	14–23	1162.5271	1162.5521	EKDNAMEADRA
6	16–23	721.2834	721.2933	DNAMDR
7	22–30	1034.2908	1034.4935	ALTCEQQNK
8	31–38	931.4177	931.4592	EANNRAEK
9	39–49	1368.6563	1368.6866	SEEEVHNLQKR
10	72–78	832.2731	832.4410	EEKDKAL
11	79–88	960.3810	960.4632	SNAEGEVAAL
12	89–93	686.3531	686.4056	NRRIQ
13	100–106	918.3748	918.4639	ERSEERL
14	102–112	1249.9255	1249.6382	SEERLNTATTK
15	106–112	748.4188	748.4199	LNTATTK
16	107–113	748.3612	748.4199	NTATTKL
17	125–130	802.3698	802.5080	RMKRVL
18	126–133	1045.5482	1045.5935	MRVLENR
19	128–133	758.3832	758.4519	KVLENR
20	129–133	630.1393	630.3569	VLENR
21	150–161	1434.6431	1434.7335	EARFLAEADRK
22	162–167	752.2972	752.3573	YDEVAR
23	170–176	748.3511	748.3545	AMVEADL
24	183–189	721.3152	721.3362	AETGESK
25	190–198	1129.4746	1129.6099	IVELEEELR
26	199–205	743.4606	743.4410	VVGNNLK
27	218–226	1137.4258	1137.5786	EEAYKEQIK
28	223–231	1074.3914	1074.6153	EQIKTLTNK
29	227–231	576.3004	576.3351	TLTNK
30	232–238	758.3832	758.4519	LKAAEAR
31	232–248	1904.1882	1904.0348	LKAAEARAEFAERSVQK
32	234–238	517.2539	517.2729	AAEAR
33	268–273	692.2830	692.3461	KSITDE

by serum IgE from more than 6 out of 12 subjects (50%) and/or with an intensity score of IgE binding larger than the mean pre-epitope score plus one standard deviation ($12.2 + 6.76$, Ayuso et al., 2002). Based on the frequency and intensity of IgE-reactivities, six major predicted IgE-binding epitopes were identified as: pre-epitope 2 (45–59), pre-epitope 3 (89–105), pre-epitope 5 (131–142), pre-epitope 6 (145–164), pre-epitope 9 (243–259) and pre-epitope 10 (263–280), the intensity scores were 18, 19, 21, 13, 16 and 15, respectively.

3.2.3. Epitope mapping

Table 3C shows the IgE recognition frequency and intensity of 46 overlapping peptides by 12 subjects' sera. As shown in Table 3C, the pooled control sera had no IgE-reactivity with the 46 overlapping peptides. 34 out of the 46 overlapping peptides were recognized by serum IgE, and 12 out of 46 overlapping peptides (6, 7, 9, 10, 14, 26, 34, 35, 39–41) had no immunological response with the 12 shrimp allergic sera. Each serum IgE recognized overlapping peptides varied from 4.3% (2 out of 46) to 50% (23 out of 46). As the IgE-binding epitope was defined as a region recognized by serum IgE from more than half subjects (50%) and/or with an intensity score of IgE-binding higher than the mean peptide score plus one standard deviation ($5.65 + 5.98$, Ayuso et al., 2002), the IgE-binding regions were identified as follows: region 1 (43–57), region 2 (85–105), region 3 (133–159), region 4 (187–201) and region 5 (247–279). Region 1 was recognized by 10 out of 12 subjects (83.33%), region 2 by 11 out of 12 subjects (91.67%), region 3 by 11 out of 12 subjects (91.67%), region 4 by 7 out of 12 subjects (58.33%), and region 5 by 7 out of 12 subjects (58.33%). The intensity scores of IgE recognition for these IgE-binding regions were 18, 17.5, 13.3, 14 and 12, respectively.

The immunoinformatics predicted epitopes were highly consistent with the overlapping peptides determined epitopes. Specifically, the predicted epitopes 2, 3, 5, 6, 9 and 10 almost covered the overlapping epitopes 8, 16, 23, 25, 42 and 45, respectively. Comprehensively

considering the results of predicted epitopes and overlapping epitopes, five IgE-binding epitopes of Exo m 1 came out: epitope 1 (43–59), epitope 2 (85–105), epitope 3 (131–164), epitope 4 (187–201) and epitope 5 (243–280). Moreover, each IgE-binding epitope contained a repeated AA-sequence of LEXXL, where L is leucine, E is glutamic acid, and X is D, E, N or Q. The five tandem motifs were: 53–57 (LENDL, motif 1), 95–99 (LEEDL, motif 2), 144–148 (LENQL, motif 3), 193–197 (LEEEL, motif 4) and 256–260 (LEDEL, motif 5). The presence of tandem amino acid repeats contained within food allergens was also reported in other studies (Ayuso et al., 2002, 2010; Beezhold, Hickey, Slater, & Sussman, 1999; Pomes et al., 1998).

Previous literatures also had similar epitope results for shellfish tropomyosins. Ayuso et al. (2002) found the IgE-binding epitopes of Pen a 1 (a shrimp tropomyosin allergen) were region 1 (43–57), region 2 (85–105), region 3 (133–148), region 4 (187–202) and region 5 (247–284). Rao, Rajagopal, and Ganesh (1988) reported the T-cell epitopes of Met e 1 (a shrimp tropomyosin) were in regions: 26–66, 86–110, 105–132, 151–171, 236–261 and 261–281. The IgE-binding epitopes of Exo m 1 were highly similar with those of Pen a 1 and Met e 1. Moreover, Exo m 1 had a high AA-sequence similarity with Pen a 1 and Met e 1 (97.54% for Pen a 1, 96.83% for Met e 1, Fig. 1C), this might be the reason why the tropomyosins of different shrimp species had high allergy cross-reactivity. Previous reports indicated that the cross-reactivity among crustacean tropomyosins can be derived on the basis of the common IgE-binding epitopes proposed for the brown shrimp tropomyosin (Ayuso et al., 2002; Ayuso, Reese, Leong-Kee, Plante, & Lehrer, 2002), which implied that the cross-reactivity among tropomyosins is realized by the common IgE-binding epitopes.

3.3. Sequencing the allergenic peptides of in vitro GI digests

The digestibility of allergens has long been considered as a critical prerequisite for the induction of food allergy (Hantusch et al., 2006; Pali-Schöll, Untersmayr, Klems, & Jensen-Jarolim, 2018). After being GI digested in digestive system, there would be allergenic peptides of food allergens generated in passage and trigger allergy response (Wickham et al., 2009). The digestion-induced peptides (dig-peptides) in GI digests were analyzed using UPLC-Q-TOF-MS^E, the IgE reactivities of dig-peptides were characterized using inhibitory dot-blot immunoassay. Table 4A showed the AA-sequences of thirty-three identified dig-peptides. As shown in Table 4B, the pooled control sera from healthy volunteers had no IgE-reactivity with the 33 dig-peptides, two digestion-induced peptides (dig-peptide 21 and dig-peptide 31) could highly bind with IgE (7 out of 12 sera), dig-peptide 1–8, 10, 12–23, 25–27 and 33 could moderately or weakly bind with IgE (≤ 5 out of 12 sera), whilst dig-peptide 9, 11, 24 and 28–30, 32 could not react with any patient's serum. Leung et al. (1994) and Shanti et al. (1993) reported that the AA-sequence of FLAEADRK in shrimp tropomyosin allergens contained IgE-reactive epitopes. As shown in Table 4A, dig-peptide 21 and dig-peptide 31 contained the AA-sequence of FLAEADRK, this might be the reason why they performed high IgE-reactivities. The 26 allergenic peptides in GI digests were consistent with a previous report that the GI digests of TM still had IgE-reactivities due to the existence of allergenic peptides (Zhang et al., 2018).

3.4. Basophil degranulation assay

As shown in Fig. 2A, compared with the control (15.09 ng/mL, PBS activated basophil), the histamine content increased sharply after incubated with Exo m 1, epitope-peptides and GI digestion-induced allergenic peptides ($p < 0.05$). Compared to Exo m 1 (117.21 ng/mL), the basophil incubated with IgE-binding epitope 1–5 and allergenic dig-peptides became weaker with less histamine release (51.56–96.85 ng/mL). This may be due to that Exo m 1 had multiple epitopes to get higher accessibility to bind with the IgE on basophil, while single epitope peptide or dig-peptide had lower possibility to bind the IgE on

Table 4BIgE reactivities of the twelve shrimp allergic subjects to the thirty-three peptides of Exo m 1 in *in vitro* gastrointestinal digests.

Sera	The peptides of Exo m 1 in <i>in vitro</i> gastrointestinal digests																																	PN
Subjects No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	
Control sera																																		0
1																																		3
2																																		2
3																																		4
4																																		3
5																																		7
6																																		11
7																																		3
8																																		7
9																																		6
10																																		14
11																																		4
12																																		6
Positive subjects, n	1	1	3	2	3	3	2	3	—	1	—	3	5	2	2	1	1	2	1	1	7	2	4	—	5	4	1	—	—	—	7	—	3	
Intensity	1	1	3	2	5	3	2	3	—	1	—	3	5	2	2	1	1	2	1	1	9	2	4	—	5	4	1	—	—	—	11	—	3	
≥50% subjects																					+											+		
>Mean intensity+SD																					+											+		

Note: The color-coded system is carried out as follows: strong (3): black; medium (2): dark gray; weak (1): light gray; negative (0): white. PN = Positive number.

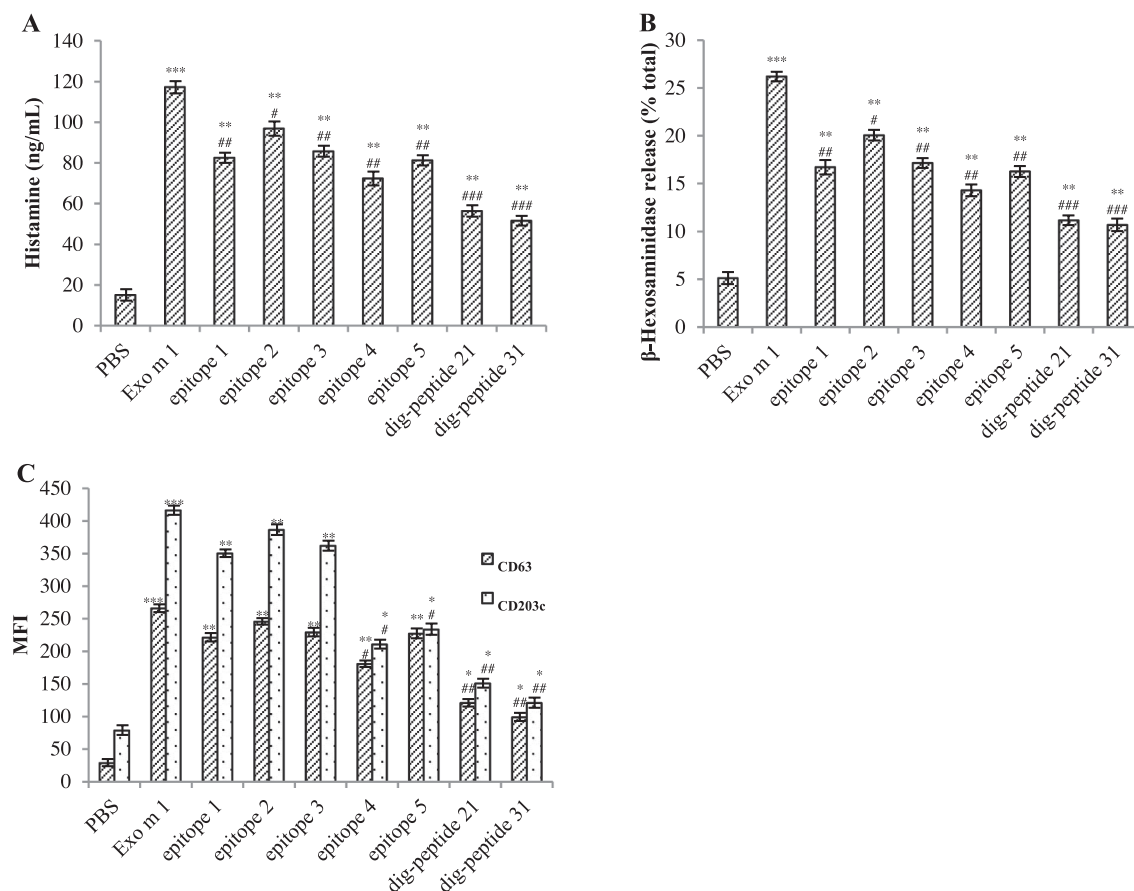


Fig. 2. Effects of Exo m 1, epitope-peptides and GI digestion-induced allergenic peptides of Exo m 1 on histamine release (A), degranulation (B) and expression of CD63 and CD203c antigens (C) of IgE-immunized human basophil cell line (KU812). * showed significant differences as compared with the PBS group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; # showed significant differences as compared with the Exo m 1 group, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$.

basophil, resulting in weaker basophil allergy response.

Basophil degranulation was determined by measuring the β -hexosaminidase content of KU812 basophils. As shown in Fig. 2B, compared with the control (5.12%, PBS activated basophil), Exo m 1, IgE-binding epitopes and dig-peptides had significant degranulation on basophils ($p < 0.05$), while single epitope or dig-peptide could trigger weaker degranulation than Exo m 1, due to the synergetic effects of multiple epitopes on Exo m 1 contributed to higher IgE recognition, therefore led to higher degranulation.

Basophil activation is typically associated with up-regulation of certain activation-linked cell-surface antigens, and CD63 and CD203c belong to the most important antigens (Cassard et al., 2018; Liu et al., 2016). As shown in Fig. 2C, the MFI of CD63 and CD203c of KU812 basophils stimulated by Exo m 1 was 265.86 and 416.19, respectively, significantly higher than the PBS activated basophils, while single epitope-peptide was found to be less positive in up-regulation of CD63 and CD203c on basophils than Exo m 1, which might be due to the multiple epitopes on Exo m 1 that had higher possibility to bind IgE, led to higher expression of CD63 and CD203c antigens. In addition, the upregulation of CD203c was accompanied by upregulation of CD63, and positively correlated with the histamine release and degranulation of basophils. The activation of human basophils is commonly associated with the up-secretion of histamine and degranulation, and up-expression of CD63 and CD203c antigens (Kneidinger et al., 2008; Liu et al., 2016), which suggested basophil histamine and degranulation might be associated with the upregulation of CD63 and CD203c.

4. Conclusion

To the best of our knowledge, we report the AA-sequence of Exo m 1 for the first time. Exo m 1 has a high AA-sequence similarity with other shrimp tropomyosins, while not 100% matching, therefore Exo m 1 should be regarded as a new tropomyosin allergen. Five IgE-binding epitopes of Exo m 1 were identified: epitope 1 (43–59, VHNLQKRMQ-QLNDLDS), epitope 2 (85–105, VAALNRRIQLLEEDLERSEE), epitope 3 (131–164, ENRSLSEERMDALENQLKEARFLAEEADRKYDE), epitope 4 (187–201, ESKIVELEELRVVG) and epitope 5 (243–280, ERSVQKLQ-KEVDRLDELVNEKEKYKSITDELDTQTFSE). Among the 33 identified peptides of Exo m 1 in GI digests, 2 out of 33 were highly recognized by IgE, 24 out of 33 moderately or weakly bound IgE, and 7 out of 33 had no IgE-reactivities. These epitopes should be further evaluated as potential targets for immunotherapy for larger cohorts of shrimp allergic individuals.

Declaration of Competing Interest

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

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