

Identification and Amino Acid Analysis of Allergenic Epitopes of a Novel Allergen Paramyosin (Rap v 2) from *Rapana venosa*

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ABSTRACT: Besides tropomyosin (TM) that is widely recognized as a major allergen in molluscs, a 99-kDa novel allergen (Rap v 2) was recently found in the sea snail *Rapana venosa* and identified as paramyosin (PM). However, the allergenic epitopes of PM in any molluscs have not been identified yet. In the present study, seven allergenic epitopes of Rap v 2 were identified by immunoinformatics tools, dot-blot inhibition assay, and basophil degranulation assay. Based on the analysis of PM and allergenic epitope amino acids, it was found that highly hydrophobic and positively charged amino acid residues play an important role in the formation of Rap v 2 epitopes. In addition, three potential critical amino acids that may account for TM and PM cross-reactivity in molluscs were found by sequence- and structure-based methods. These findings could be of major importance for improving the understanding of relevant paramyosin epitopes and the prevention and therapy of mollusc allergy.

KEYWORDS: sea snail, paramyosin, Rap v 2, allergenic epitope, mollusc allergy

INTRODUCTION

Shellfish allergies are one of the most commonly encountered food allergies in the Asia-Pacific region, which are predominantly IgE-mediated type I hypersensitivity reactions. Like all other food allergens, it develops through ingestion, inhalation, or contact and typically persists throughout life, often associated with severe reactions, including life-threatening and even fatal anaphylactic reactions.^{1,2} Edible crustaceans and molluscs are commonly discussed as shellfish. The crustacean shellfish includes shrimp, crab, prawn, crawfish, etc. The commonly consumed molluscan shellfish consists of three classes: gastropods (sea snails and abalones), bivalves (clams, scallops, and oysters), and cephalopods (squid and octopus). However, the allergic reaction to molluscs has not been studied earlier and in-depth like crustaceans, although the significance of mollusk allergy is becoming generally recognized.^{3,4} As a result, there are neither unified standardized test substances nor effective treatments for molluscan allergic reactions.

To date, only four proteins have been fully identified by proteomics, immunology, and molecular biological approaches as elicitors of allergic reactions to mollusc species, including tropomyosin (TM), arginine kinase (AK), sarcoplasmic-calcium-binding protein (SCP), and paramyosin (PM).⁵ Among them, TM was considered to be a pan-allergen in molluscs, but studies have also indicated that the allergenicity of allergenic components may vary by region and individual.^{6,7} PM, an important structural component of thick filaments in molluscan muscle, has been identified as an allergen in molluscs such as abalone (Allergome Code: 8826), octopus (Allergome Code: 8828), and pacific oyster.^{8–10} It was not surprising since PM has been also confirmed as a major allergen in several other nonfood sources, including house dust mite, storage mite, and herring worm. Furthermore, the

immunogenic properties of PM have made it a prominent vaccine candidate for *Schistosoma japonicum*, *Clonorchis sinensis*, and *Dermanyssus gallinae*.^{11–13} Meanwhile, our previous studies have confirmed that *Rapana venosa* PM has obvious IgE-binding capacity with sea-snail-allergic patients' sera, and it is a highly conserved protein that can expose more epitopes to bind IgE of allergic subjects.¹⁴ In addition, the newly identified allergen PM in *R. venosa* was designated as Rap v 2 by the official database of allergen nomenclature that is approved by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Subcommittee. *R. venosa* is an economically important aquaculture species, and it is also one of the most popular seafood delicacies in China and hence may be an important cause of mollusc allergy.¹⁵ Moreover, paramyosins are large macromolecular assemblies linked with molecular evolution as well as important allergens relating to innate immune responses with potential biotechnological and therapeutic applications.¹⁶ For this reason, research on Rap v 2 is of great necessity for mollusc allergy in the Chinese population.

It is well known that antigenic epitopes are special chemical groups that activate allergic immune responses and are recognized by corresponding receptors on the immune cell surface to initiate allergic reactions. Thus, it is essential to identify the allergenic epitopes to characterize the allergenicity and cross-reactivity of allergen and to serve for diagnosis,

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vaccine design, disease prevention, and treatments of food allergy.^{17,18} Nevertheless, as an important allergen in molluscan food, the allergenic epitopes of PM have not been reported yet. Clarifying the allergenic epitopes of PM could provide more information for cross-reactivity among different mollusc species and clinical diagnosis of mollusc allergy. At present, identification and analysis of allergenic epitopes and critical amino acids in target epitopes can be implemented through bioinformatics prediction combined with immunological methods. Based on computer technology and biological databases, immunoinformatics has become a potent tool to predict epitopes from immunological proteins, which may decrease the number of synthesized potential epitope candidates and working time.¹⁹ After computational immunoinformatics prediction of epitopes, confirmatory experiments can be performed based on the peptide microarray IgE-binding and mediator release assays of antigen-epitope peptides.^{20,21}

In the present study, we aimed to screen the allergenic epitopes of Rap v 2 and to examine its amino acid sequence and critical amino acids. The potential epitopes were predicted and analyzed with the immunoinformatic tools, and we validated the allergenic epitopes of Rap v 2 by inhibition dot-blot and KU812 mediator release assays with specific sea-snail-allergic patients' sera. In addition, an integrated strategy was employed by combining results from sequence- and structure-based methods and protein–protein interaction tools to observe the characterization of critical amino acids of the allergenic epitopes. These results may enable us to better understand the cross-reactivity of allergens in different molluscan species, while the paramyosin allergenic epitopes can be used as diagnostic and immunotherapeutic agents to reduce the risk of sea-snail-induced allergic reactions.

MATERIALS AND METHODS

Materials. Sea snails (*R. venosa*) were purchased from the Liquan supermarket (Qingdao, Shandong, China). Goat anti-human IgE antibody was purchased from Zhongshan Jiaojiao Co. (Beijing, China). The enhanced chemiluminescent kit (ECL) was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The human basophil cell line (KU812) was obtained from Procell Life Science & Technology Co. Ltd. (Wu Han, Hu Bei, China). Human myeloma IgE was acquired from Sigma Chemical Co. (St. Louis, MO, USA). The ELISA kits of histamine and trypsin for the KU812 cell assay were procured from R & D Systems (Minneapolis, MN, USA), and other reagents used were all of analytical grade.

Human Sera. Sera from six sea-snail-allergic patients served as positive controls, sera from two clam-allergic patients served as other molluscan serum controls, and sera from a shrimp-allergic patient and two healthy volunteers served as negative controls, which were provided by the Affiliated Hospital of Qingdao University (Qingdao, Shandong, China) and approved by the medical ethics committee (File No. QD-PF-20190618685). Table S1 shows the detailed information on the sera from all shellfish- and sea-snail-allergic patients and healthy volunteers used in the study. All sera were stored at -80°C until further use.

Sequence Retrieval and Alignment Analysis. The amino acid sequences of Rap v 2 have been obtained in previous studies and submitted to the GenBank/EBI databases (accession number: MN784956).¹⁴ The complete amino acid sequence of Rap v 2 was used as a query to search for homologous sequences through the Swiss-Prot/TrEMBL (Uniprot) and tBLASTn in NCBI.²² Different numbers of protein sequences could be retrieved for PM with 30 protein sequences. The selected sequences represent 18, 8, and 4 different sea molluscs, Platyhelminthes, and Arthropoda species, respectively. The homologous amino acid sequences were aligned using the Multiple Sequence Alignment in Clustal Omega (EMBL-

EBI). The subsequent phylogenetic analyses of relatedness of proteins were obtained using the Neighbor-Joining method (1000 replicates) in MEGA 6.²³

Immunoinformatics Prediction of the Epitope. To improve the veracity of epitope prediction, the complete amino acid sequence of the paramyosin was analyzed using five immunoinformatics-based computational approaches including the DNASTar Protean system, AntheProt, BepiPred 2.0, ABCpred, and Immunomedicine Group. In the DNASTar and AntheProt Protean system, peptide regions that have good hydrophilicity and antigenic index, proper polarity, and exposed surface together with a high accessibility and flexibility in the amino acid sequence of Rap v 2 were chosen as candidate epitopes.^{24–26} In addition, the BepiPred 2.0 Server, ABCpred Server, and Immunomedicine Group Tools Server were also applied to predict the epitopes of Rap v 2 based on physicochemical properties of amino acids.^{21,27} The ultimate predicted peptide regions were developed by combining the data of five immunoinformatics tools using the method described earlier, and the repeat probability of the ultimate epitope predicted by three or more software ranged from 60 to 100%.²⁰

Peptides and Proteins. The peptides were synthesized by Sangon Biotech (Shanghai) Ltd. using the Fmoc solid-phase method. The purity and molecular weight of these peptides were determined by high-performance liquid chromatography (HPLC) and electrospray ionization-mass spectrometry (ESI-MS), respectively. The purity of the peptides was greater than 95%, and they were stored at -20°C until used. The sea snail paramyosin allergen Rap v 2 was purified and obtained by removing the sarcoplasmic protein and then using 10–20% gradient ammonium sulfate precipitation and the final hydroxyapatite chromatography as previously described.¹⁴

IgE-Reactivity Assay of Predicted Epitope Peptides. A dot-blot inhibition immune array assay was performed to determine the IgE binding of predicted epitope peptides according to the method described previously with a slight modification.²¹ For the allergen Rap v 2 samples, which were dissolved in carbonate buffer saline (CBS, pH 9.6), 4 μg of the Rap v 2 sample was spotted on the nitrocellulose membranes (Millipore, USA), left to dry in a fume cupboard at room temperature, and blocked for 2 h using 5% nonfat dry milk and 1% BSA in PBST. After three washes with PBST (15 min each time), the membranes were incubated with human sera (1:10 dilution), which were separately preincubated with the predicted 12 candidate antigen epitopes at room temperature for 1 h. After washing three times with PBST, the membranes were incubated with HRP-labeled goat anti-human IgE serum (Sigma, USA) at a dilution of 1:10,000 in PBST for 2 h at room temperature. Finally, the membranes were washed three times, incubated in ECL substrate for 2 min, and then detected by ChemiDoc XRS+ (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For interpretation of the results, IgE reactivities were graded and scored according to their intensity into four categories, i.e., negative (score: 0, black dot-blot), weak (score: 1, dark gray dot-blot), medium (score: 2, light gray dot-blot), and strong (score: 3, white dot-blot), and the color-coded system as follows: negative (white), weak (light gray), medium (dark gray), and strong (black). The intensity of the IgE reactivities was evaluated by the ImageJ software to grade the reactivities and assign the above scores.²⁸

Mediator and Cytokine Release Assays of KU812 Cells. KU812 cells were cultured in a complete RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin and placed at 37°C in a 5% CO_2 humidified incubator according to the method described previously.²⁹ Then, 0.5 $\mu\text{g}/\text{mL}$ human myeloma IgE was added into the cells and kept in the incubator for 7 days to stimulate the Fc ϵ R1 expression. Next, these cells were seeded and sensitized in a 96-well microtiter plate with diluted sera of sea-snail-allergic subjects (1:10 dilution) for 12 h. Following that, cells were stimulated with purified PM and predicted epitopes for 1 h at room temperature. The IgE-immunized KU812 cells were centrifuged with the supernatant collected to determine the release of trypsin, histamine, IL-4, and IL-13 cytokines using a commercial ELISA kit (Fusheng Industry Co., Shanghai, China), and

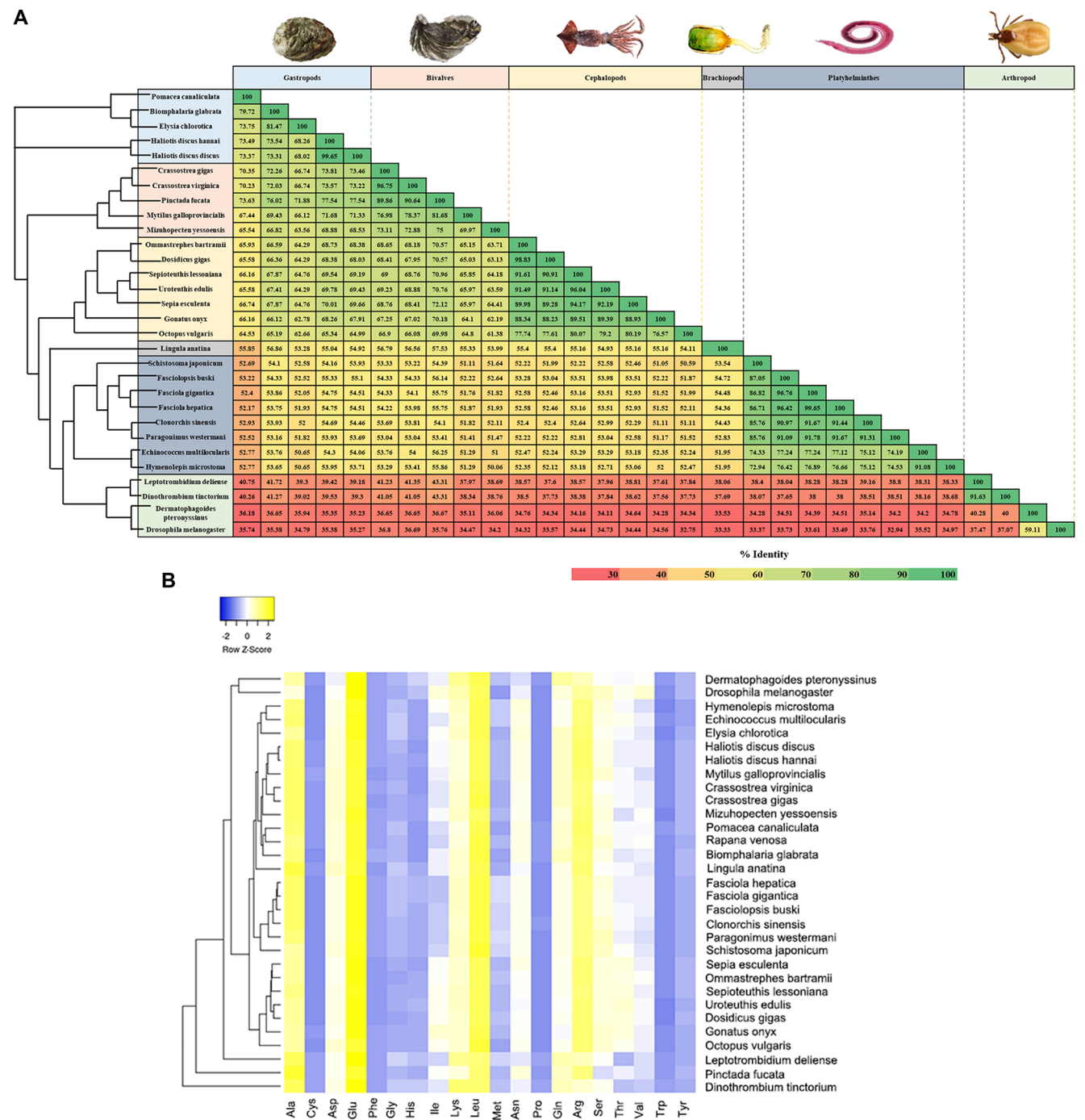


Figure 1. Analysis of the paramyosin sequences from different species used in this study. (A) Comparison of amino acid sequence identities (%) of paramyosin from different species. The sequence identities were calculated using the Multiple Sequence Alignment in Clustal Omega (EMBL-EBI). Molecular phylogenetic tree based on published amino acid sequences of PM from gastropod, bivalve, cephalopod, and brachiopod species and platyhelminth and arthropod species. The phylogenetic tree inferred from 1000 replicates taken to represent the evolutionary history of the taxa analyzed and constructed using the Neighbor-Joining method in MEGA6. (B) Heatmap representing the number of matches in specific residues of PM amino acid composition in different species. The heatmap was generated using Heatmapper and clustered using the Euclidean distance metric method. The color grading as indicated in the top left represents the number of amino acid matches found in the specific residues of paramyosins. Yellow indicates matches, and blue indicates maximum mismatches.

cell degranulation was measured by determining the enzymatic activity of β -hexosaminidase in the supernatant.

Molecular Modeling of Allergen Protein. To further analyze epitope location, the secondary structure was predicted using PSIPRED online servers;³⁰ the three-dimensional (3D) structure model of allergens was generated by the SWISS-MODEL homology-modeling and Robetta comparative-modeling server.^{31,32} PRO-

CHECK, ERRAT, and VERIFY 3D programs in the Structural Model Analysis and Verification Server (SAVES) were used to check the initial structural model of allergens. A Ramachandran plot for all of the models was generated, and it showed that the majority of the protein residues were in the favored, allowed, and/or disallowed regions.³³ The images of allergenic proteins and positional distribution of each allergenic epitope were obtained and rendered

Table 1. Location of Predicted Epitopes on Rap v 2 by Immunoinformatics Tools

software	epitope position
DNAstar	10–22, 25–34, 64–81, 107–129, 129–146, 229–241, 280–289, 364–374, 384–394, 490–500, 646–658, 670–678, 811–826, 843–852
AntheProt	17–25, 66–77, 106–116, 127–140, 234–246, 265–273, 289–294, 361–368, 380–387, 491–502, 665–673, 687–697, 772–778, 823–828
BepiPred	21–29, 43–47, 70–78, 104–120, 130–145, 172–176, 230–242, 272–286, 362–374, 380–391, 483–497, 664–680, 724–745, 818–824
ABCpred	9–24, 44–59, 67–76, 89–104, 110–124, 133–151, 167–182, 227–233, 276–286, 356–371, 379–394, 399–414, 487–499, 544–559, 658–673, 806–821
Immunomedicine Group	5–22, 55–68, 113–126, 131–145, 226–239, 279–292, 364–371, 378–390, 495–508, 668–674, 767–773, 781–796, 818–825

Table 2. Predicted Epitopes of Rap v 2 by Combined Immunoinformatics Methods

peptide	sequence	position	length of sequence	secondary structure
Peptide 1	VRKVSRTYNVYRGSSPST	10–27	18	100% helix
Peptide 2	ESRIRELEDALDSERD	32–47	16	100% helix
Peptide 3	DQLQDSLDEQGTSTAQM	64–81	18	100% helix
Peptide 4	EATESSMRKRH	106–116	11	100% helix
Peptide 5	VDYLNKNKNRVEKE	127–140	14	100% helix
Peptide 6	QASNDLKRQLDD	229–241	13	100% helix
Peptide 7	TSTNLRNQLSKVNADYS	273–289	17	100% helix
Peptide 8	DLTKRNRQLENENA	361–374	14	100% helix
Peptide 9	TGENNTRLRNEK	384–394	11	100% helix
Peptide 10	DMENRLREKDEE	491–502	12	100% helix
Peptide 11	AINNQRGAEERAER	665–678	14	100% helix
Peptide 12	RKAQALIDEAD	818–828	11	100% helix
Rap v 2	--	--	859	98.02% helix, 1.51% coil

by using the PyMOL software (DeLano Scientific, San Carlos, CA, USA).

Poisson–Boltzmann Electrostatic Potential Analysis. Electrostatic Poisson–Boltzmann (PB) potentials of the Rap v 2 model structure were obtained by using the APBS molecular modeling software implemented in PyMOL. AMBER99 in the PDB2PQR server was assigned the charges and radii to all of the atoms.³⁴ Fine grid spaces of 0.35 Å were used to solve the linearized PB equation in sequential focusing multigrid calculations in a mesh of 150 points per dimension at 290.00 K. The dielectric constants were 2.0 and 79.0 for the protein and water. The output mesh was processed in the scalar OpenDX format to render isocontours and mappings onto the surfaces with PyMOL. Potential values are given in units of kT per unit charge (k , Boltzmann's constant; T , temperature).

Structural Epitope Analysis of the Known Allergen PM and TM. Known allergen TM and PM sequences were retrieved from the WHO/IUIS Allergen Nomenclature database (www.allergen.org) and aligned using the Clustal W multiple sequence alignment tool with default parameters. Known PM allergenic epitopes were collected from the Immune Epitope Database (IEDB) and calculated using the conservancy analysis tool on the IEDB website (<https://www.iedb.org/>).²² The subsequent molecular modeling and epitope mapping of allergenic proteins were displayed using the method described in the previous molecular modeling of allergen protein section.

Statistical Analysis. All data were presented as means $\pm$ standard deviation (SD). Analysis of variance was used to assess significant differences between the groups using the SPSS 22.0 and GraphPad Prism8 software. A p value of <0.05 was considered statistically significant.

RESULTS

Analysis of the Amino Acid Sequence of PM from Different Species. PM in *R. venosa* was identified as a molluscan allergen, given the name Rap v 2 in the official database of allergen nomenclature (WHO/IUIS), and obtained the amino acid sequence of Rap v 2 in our previous research.¹⁴ Thirty paramyosin sequences were obtained from Uniprot and tBLASTn by searching the homologous sequences

of Rap v 2. The phylogenetic analysis was performed and inferred through the NJ method in MEGA. As shown in Figure 1A, paramyosin has a relatively conserved amino acid sequence among molluscs and Platyhelminthes ranging from 61.38 to 99.65% and 72.92 to 99.65% identity. However, the sequence identity of arthropods ranges only from 37.07 to 91.63%. Furthermore, the phylogenetic tree demonstrated that the sequence identity of paramyosin in the same species was higher than that in different species. Similar to TM, published amino acid sequences of molluscan PM are highly associated with each other. Therefore, the high sequence identity of paramyosin may explain another inducement for the frequency of clinical cross-reactivity in molluscs. In addition, the amino acid compositions of these paramyosins also have many common characteristics. Matching and clustering analysis of each amino acid residue using heat map analysis revealed that the typical characteristics of paramyosin are high proportions of Glu, Leu, Ala, and Arg (Figure 1B).

Prediction of the Allergenic Epitopes in Rap v 2. The paramyosin Rap v 2 potential epitopes were predicted by amino acid sequence and five structure-based immunoinformatics tools, including the DNAstar Protean system, AntheProt system, ABCpred, BepiPred 2.0 server, and Immunomedicine Group Server, and the predicted results of potential epitope regions from different immunoinformatic tools were shown in Table 1. Furthermore, the overlapped consensus was taken among the commonly predicted epitope regions by various prediction tools used in the study. Finally, 12 predicted peptides from 11 to 18 residues in length, named peptide 1 (P1) to peptide 12 (P12), were chosen to be the potential epitope regions of Rap v 2 and synthesized according to the peptide sequences for further validation (Table 2). Spatial distribution and analysis of these predicted epitopes on a 3D model of Rap v 2 were done using the PyMOL software; P1 to P12 epitopes all occurred in α -helix regions (Table 2).

Sera	Peptide No.												PR
No.	1	2	3	4	5	6	7	8	9	10	11	12	
Control 1													0
Control 2													0
Control 3													1
Control 4													2
1													8
2													5
3													5
4													6
5													5
6													4
Positive subjects, n	6	4	4	4	1	-	3	3	-	6	1	1	
Intensity	10	8	8	6	2		8	7		12	2	2	
>50% of subjects	+	+	+	+			+	+		+			
>Mean intensity + SD	+									+			

Figure 2. The IgE reactivities of pooled sera of two healthy individuals and three shellfish- and six sea-snail-allergic subjects to the 12 predicted epitopes of Rap v 2. Peptide nos. 1–12 (P1–P12) were the 12 potential antigenic epitopes of Rap v 2 predicted by using immunoinformatics. Human sera (1:10 dilution) and HRP-conjugated goat anti-human IgE (1:3000 dilution). Sera nos. 1–6 were the six different sea-snail-sensitive individual sera, and the profiles are shown in Table S1. The pooled sera of two healthy individuals, a shrimp-allergic patient as the negative control (Controls 1 and 2), and two clam-allergic patients as other molluscan serum controls (Controls 3 and 4), respectively. Note: PR: the number of predicted epitopes for each serum response. Positive subjects, *n*: the number of positive serum for each predicted epitope. IgE reactivity of each predicted epitope was graded into four categories and scored according to their intensity by using a color-coded system: strong (score: 3): black squares, medium (score: 2): dark gray squares, weak (score: 1): light gray squares, and negative (score: 0): white squares. “+”: predicted epitopes that can be recognized by more than 50% allergic sera and/or with an intensity score of IgE binding larger than the mean epitope score (6.5 ± 3.5).

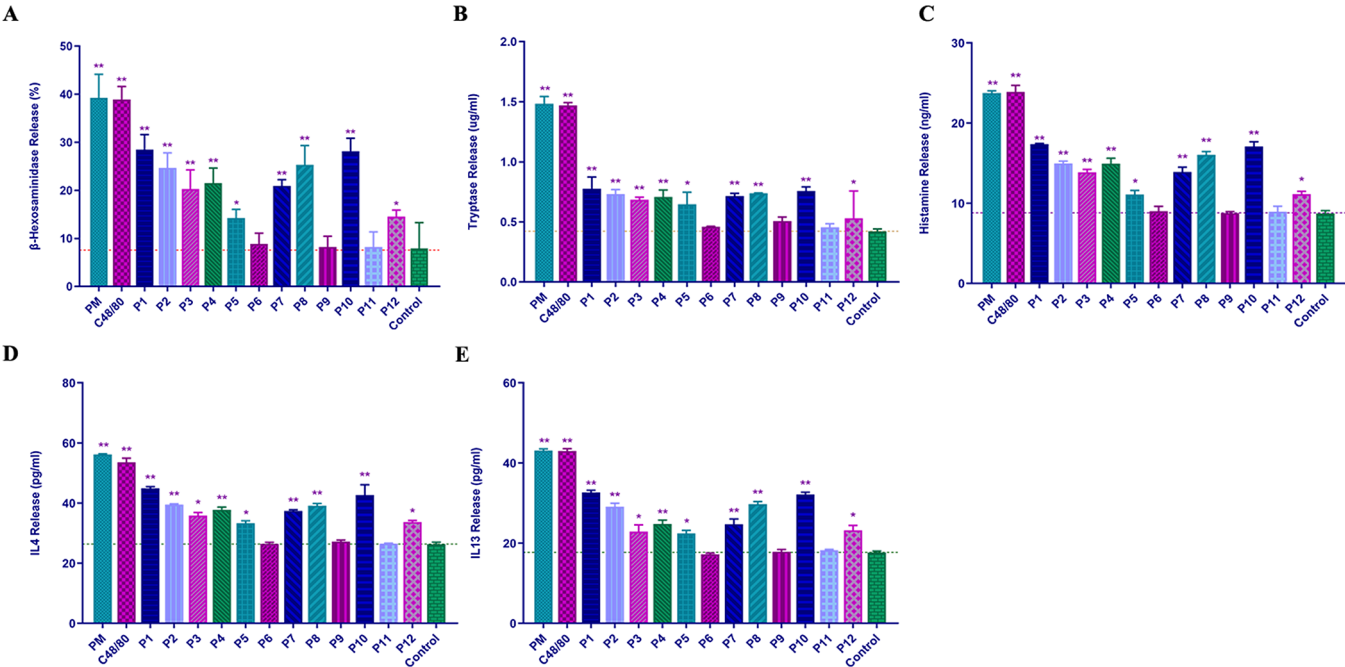


Figure 3. Verification of allergenic epitopes by IgE-mediated KU812 cells sensitized with sera obtained from sea-snail-allergic patients. (A) Release of β -hexosaminidase, (B) histamine, (C) tryptase, (D) IL-4, and (E) IL-13. Tyrode buffer was used as a negative control. C48/80 was used as a positive control. Data represent the mean $\pm$ SD ($n = 3$). * $p < 0.05$ and ** $p < 0.01$ compared with the negative control.

Validation of Allergenic Epitopes by Dot-Blot Inhibition Assay. Dot-blot inhibition was performed with sera from sea-snail-allergic patients to determine the IgE-binding capacity and frequency of the potential epitopes

predicted by immunoinformatics tools. Figure 2 illustrates the IgE binding of healthy and patient individuals' serum with all the predicted peptides. The negative control serum (Controls 1 and 2) had no IgE reactivity with the predicted peptides; P1,

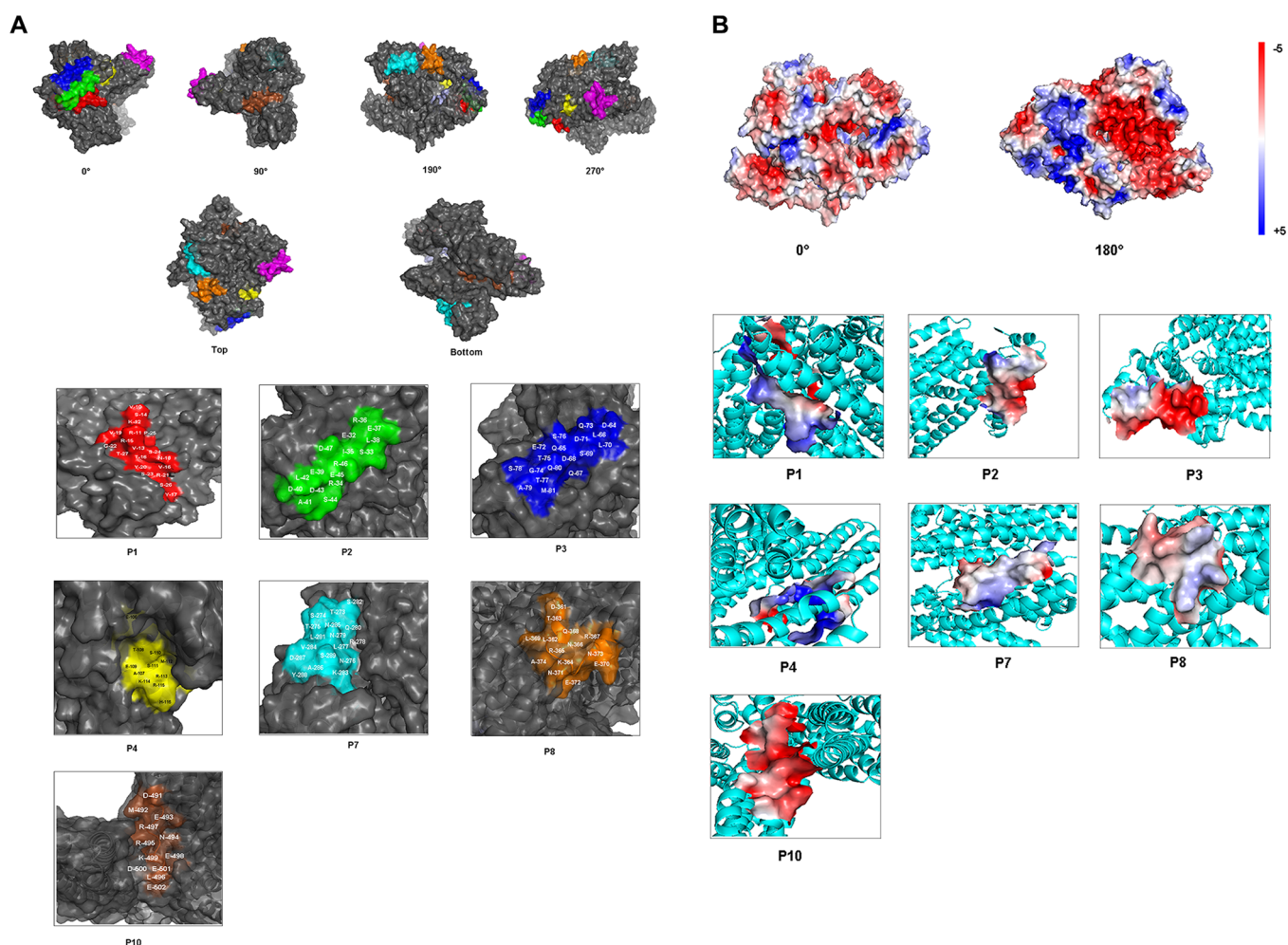


Figure 4. Distribution and electrostatic potential of Rap v 2 allergenic epitopes. (A) Position of allergenic epitopes determined in the present study on the 3D structure model and amino acid composition of Rap v 2. The validated pre-epitopes labeled with different colors. (B) Mapping of the electrostatic potentials representation and location of the allergenic epitopes on the molecular surface of Rap v 2. Electropositive and electronegative regions are colored blue and red, respectively. Neutral regions are white.

P2, and P10 of predicted peptides of Rap v 2 could be recognized with two molluscan control sera (Controls 3 and 4), while most of the predicted peptides were recognized by serum IgE of the sea-snail-allergic, Rap v 2-reactive sera. An intensity score (0–3) for the predicted epitopes was figured by adding the individual scores obtained with each individual serum using dot-blot as previously described.²⁸ The mean intensity score of all predicted epitopes was obtained by adding all the epitope scores and dividing by 12 predicted epitopes (mean epitope score). A major allergenic epitope was defined as a region recognized by serum IgE from more than three out of six subjects (50%) and/or with an intensity score of IgE binding larger than the mean epitope score (6.5 ± 3.5) plus 1 standard deviation.²⁸ Based on the frequency and intensity of IgE reactivities, seven major predicted allergenic epitopes were identified as follows: P1: Rap v 2 (10–27), P2: Rap v 2 (32–47), P3: Rap v 2 (64–81), P4: Rap v 2 (106–116), P7: Rap v 2 (273–289), P8: Rap v 2 (361–374), and P10: Rap v 2 (491–502). The scores for the intensity of each predicted epitope IgE recognition were 10, 8, 8, 6, 8, 7, and 12, respectively.

Validation of Allergenic Epitopes by the KU812 Mediator and Cytokine Release Assays. Currently, human basophil and mast cells can be used as an effective in

vitro model to evaluate the allergenicity of potential allergenic epitopes. The potential allergenic peptides of Rap v 2 were evaluated by basophil degranulation assay and we determined five biomarkers (β -hexosaminidase enzyme, histamine, tryptase, IL4, and IL13) of KU812 cells to further validate the allergenicity of the predicted epitopes as previously described.²¹ C48/80, an effective activator that can increase the intracellular Ca^{2+} concentration and lead to the degranulation of basophils, was used as a positive control in this study. Compared with the negative control, predicted peptides that show a significant difference ($p < 0.01$) were considered as potential allergenic epitopes. The overall trends of the release of β -hexosaminidase (Figure 3A), histamine (Figure 3B), and tryptase (Figure 3C) contents were relatively consistent. There was no significant difference between P6, P9, and P11 peptides and the negative control group ($p > 0.05$), while PM (Rap v 2) and predicted peptides including P1, P2, P3, P4, P7, P8, and P10 had significant degranulation on basophils ($p < 0.01$). In addition, the results of IL-4 (Figure 3D) and IL-5 (Figure 3E) cytokine contents were similar; the predicted peptides P1, P2, P4, P7, P8, and P10 displayed significant degranulation efficiency ($p < 0.01$). In total, based on the results of the five biomarkers, P1, P2, P3, P4, P7, P8, and P10 of Rap v 2 induced significant degranulation, which

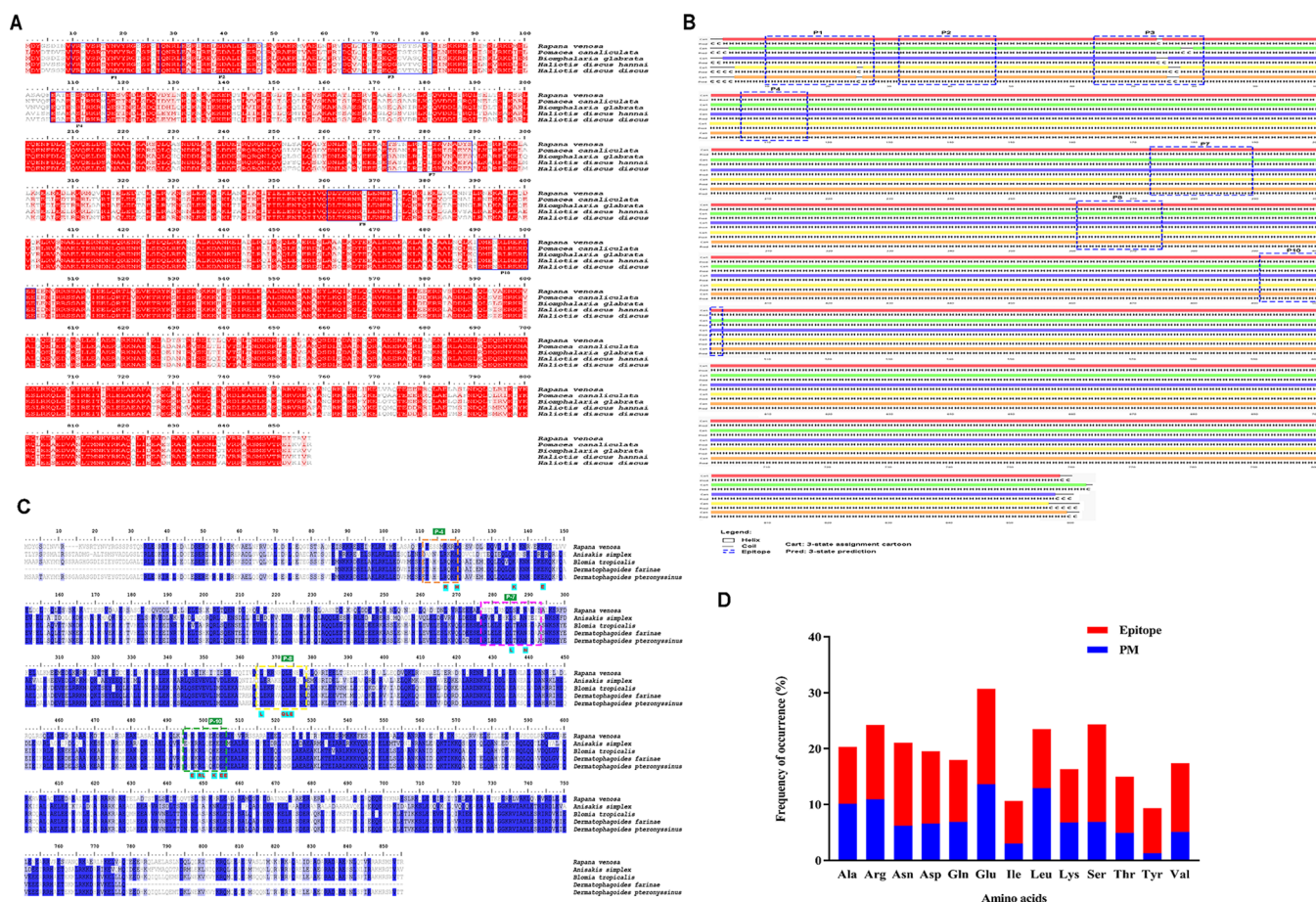


Figure 5. Analysis of allergenic epitopes and amino acid of paramyosin allergens. (A) Analysis of sequence conservation of paramyosins in different gastropod species. Five gastropods' paramyosins were analyzed in the alignment using Rap v 2 as the reference, which contained *Pomacea canaliculata* (XM_013222942.1), *Biomphalaria glabrata* (AB571843.1), *Haliotis discus hannai* (ASU87569.1), and *Haliotis discus discus* (BAJ61596.1). Sequences of the 12 paramyosins that correspond to the predicted epitope regions of *R. venosa* paramyosins are displayed in blue boxes. (B) Location of PM predicted epitopes on secondary structure. (C) Epitope alignment of Rap v 2 and with other species in IUIS. (D) The distribution frequency of amino acids present in the allergen of Rap v 2 and epitopes.

suggested that they were the allergenic epitope regions. The results were also consistent with dot-blot inhibition assays.

Allergenic Epitopes Mapping and Electrostatic Potential in 3D Model Structures of Rap v 2. Seven allergenic epitopes on the surface of Rap v 2 were mapped as indicated in Figure 4A. In addition, we can see from the spatial distribution of Rap v 2 allergenic epitopes that seven epitopes were all linear, and the epitopes mainly showed an obvious α -helix structure. Electrostatic interaction can determine the orientation of the molecule and the stability of the antigen–antibody complex, such as distinguishing the differences in their own and IgE binding capabilities between different allergenic proteins. The surface electrostatic potential result indicated that both faces of the allergenic protein tend to exhibit different electrically charged properties due to their charge; most of the surfaces exhibit an essentially negative electrostatic potential as a whole. Interestingly, most of the allergenic epitopes of Rap v 2 were distributed in the blue region and showed a predominantly positive potential (Figure 4B).

Analysis of Sequences Corresponding to Those in Other Gastropods and the Amino Acid Composition of the Allergenic Epitopes. As shown in Figure 5A, the multiple sequence alignment with other gastropods' para-

myosin was performed using Rap v 2 aligned against the reference. Sequence similarity analysis showed that Rap v 2 had a high degree of sequence identity with paramyosin from different gastropods: *Pomacea canaliculata* (82.28%), *Biomphalaria glabrata* (78.00%), *Haliotis discus hannai* (71.91%), and *Haliotis discus discus* (71.56%). Furthermore, most of the allergenic epitopes were distributed in the conserved regions with >70% identity, and some of them shared common sequences. Afterward, PM in these species was compared with the secondary structure, and the results showed that all species of PM in the α -helical region had allergenic epitope distribution (Figure 5B). Compared with the known allergenic sequences in the WHO/IUIS database, there is a large difference in the genetic relationship between the known allergen species and *R. venosa*, and it only shares 34.46 to 39.43% identity with the paramyosin of *Blomia tropicalis*, *Anisakis simplex*, *Dermatophagoides farinae*, and *Dermatophagoides pteronyssinus*. However, four allergenic epitopes were distributed in the relatively conserved regions among known allergens. We then analyzed the key amino acids of these epitope regions, and the results showed that the amino acids Glu and Leu have a high propensity to occur in IgE binding sites (Figure 5C). The frequencies of each amino acid in Rap v 2 and seven allergenic epitopes were analyzed as shown in

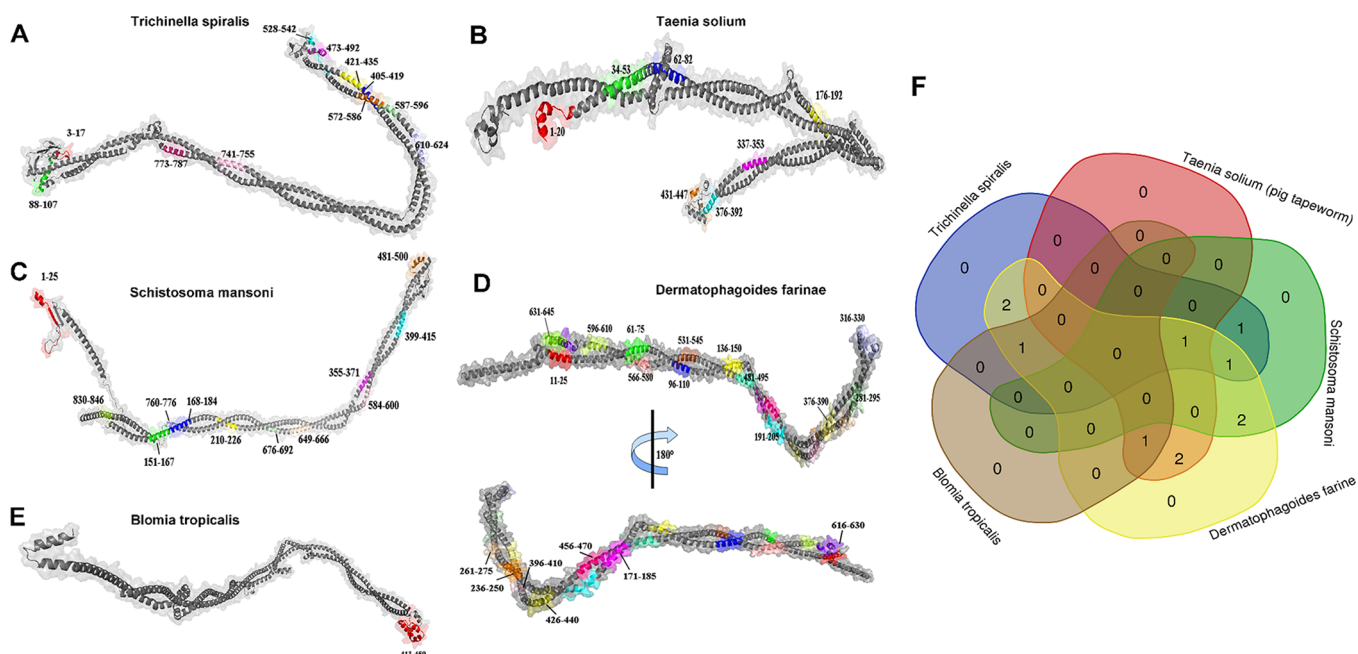


Figure 6. Distribution of paramyosin allergenic epitopes in the Immune Epitope Database and epitope alignment of Rap v 2. (A–E) The molecular modeling and distribution of known allergens' PM allergenic epitopes in the Immune Epitope Database (IEDB). The protein structure of PM was modeled using the Robetta server, and the visualization of allergenic epitopes was accomplished by the PyMOL software. (A) Position of allergenic epitopes on the *Trichinella spiralis* PM protein 3D structure model. (B) Position of allergenic epitopes on the *Taenia solium* PM protein 3D structure model. (C): Position of allergenic epitopes on the *Schistosoma mansoni* PM protein 3D structure model. (D) Position of major allergenic epitopes of *Dermatophagoides farinae* PM Der f 11. (E) Position of major allergenic epitopes of *Blomia tropicalis* PM Blo t 11. (F) Venn diagram showing the similarities among the conserved PM allergenic epitopes across different species in the IEDB.

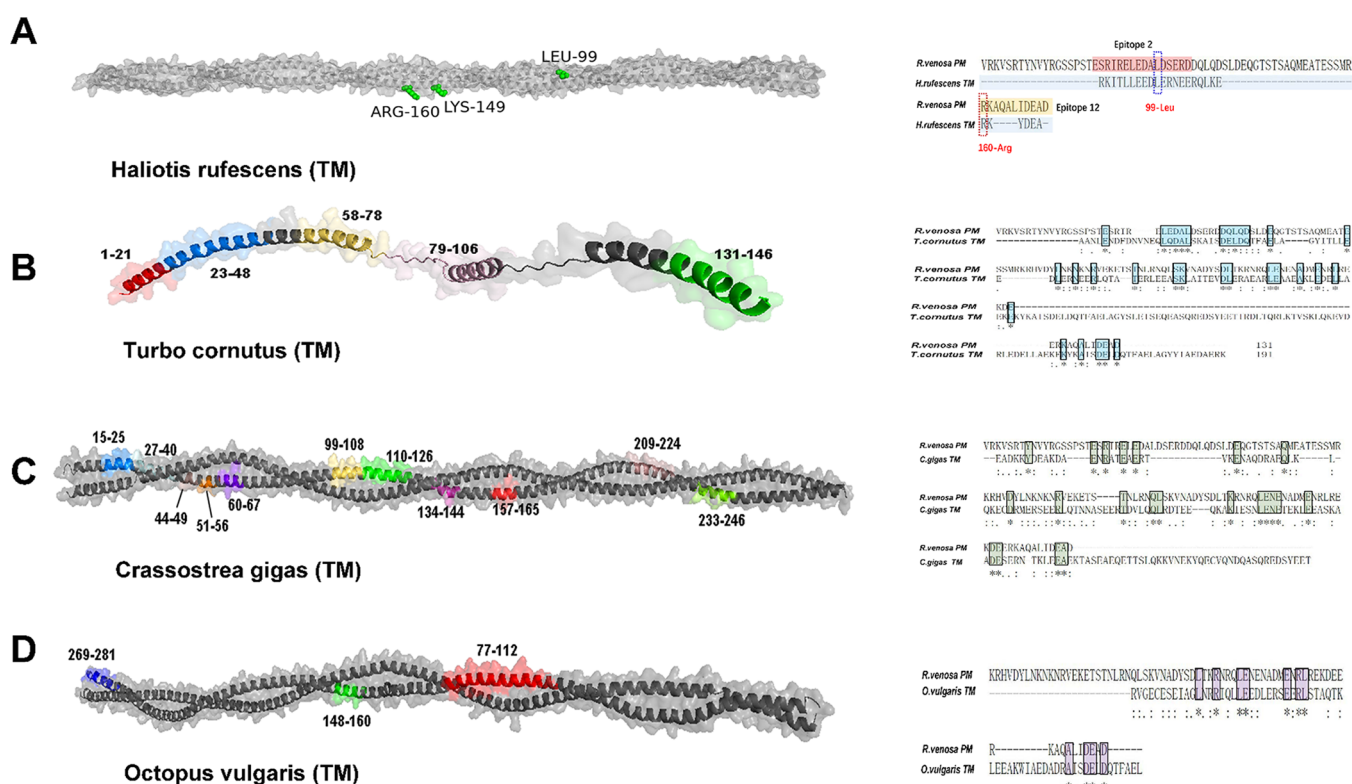


Figure 7. Distribution of tropomyosin allergenic epitopes in molluscan species and epitope alignment of Rap v 2. The protein 3D structures of TM were homology modeled using the SWISS-MODEL server (<http://swissmodel.expasy.org/>), and the visualization of allergenic epitopes was performed using the PyMOL software. Four molluscan tropomyosins were analyzed in the alignment using Rap v 2 allergenic epitopes as the reference, which contained *Haliotis rufescens* (Q25145) (A), *Turbo cornutus* (Q7M3Y8) (B), *Crassostrea gigas* (ARX70262.1) (C), and *Octopus vulgaris* (BAE54433.1) (D). The allergenic epitope regions of PM and different TMs are displayed with different colored boxes.

Figure 5D. In the allergen sequence, Glu has the highest content followed by Leu and Arg. In the epitope sequence, the frequency of a total of 13 amino acids was significantly higher than that in the allergen sequence, and the contents of Glu, Arg, Ser, and Asn were the highest, indicating that these amino acids have potential as key amino acids.

Analysis of Structural Epitopes of the Identified Paramyosin and Tropomyosin Proteins. The allergenic epitopes of the identified paramyosin in the IEDB database were mapped on three-dimensional models of the proteins built up by the Robetta modeling method. As shown in **Figure 6A–E**, the structure of paramyosin is mainly composed of α -helix, which is interwoven by amino acid residues of α -helix. Different from the paramyosin of these species, the spiral structure of other gastropod paramyosins including Rap v 2 is more complex and changeable in space. To verify the specificity of the Rap v 2 allergenic epitope identification results, we further analyzed the antigenic epitopes previously found in paramyosin to observe their sequence coverage. The overlapping regions of five groups of paramyosin epitopes were shown by the Venn chart (**Figure 6F**), and two groups (Epitope: 1–20; 61–80) of highly overlapping epitope regions with Rap v 2 allergenic epitopes were found in all the epitope sequences. The reason for the structural difference and low epitope coverage may be related to a large difference in genetic and evolutionary relationships between them. On the other hand, the allergenic epitopes of the identified tropomyosin in the IEDB database were mapped on three-dimensional models of the proteins built up by the Swiss modeling method. As indicated in **Figure 7**, both TM and PM are proteins associated with muscles, composed of coiled-coil α -helices, but the α -helix structure of PM is more irregular and complicated than that of TM. Moreover, in terms of primary structure, the homology between TM and PM in four mollusc species was more than 30% except for abalone. Amino acids in the two antigenic epitopes were analyzed, and it was found that Leu, Glu, and Arg were the critical amino acids in the two helical structure proteins.

DISCUSSION

Allergen epitopes are the key factors to determine the allergenicity and specificity of allergens. The systematic study of antigen epitopes is of fundamental importance for further understanding the mechanism of food allergens in allergic reactions.¹⁷ Earlier reports provided the first hints on the potential allergenicity of PM from abalone and sea snail. This study further analyzed and screened IgE epitopes of molluscan allergen PM (Rap v 2) that used well-characterized sera from allergic patients.^{9,14} At present, the immunoinformatics predicting method has become a common and useful tool and widely used in the analysis and prediction of food allergenic epitopes, which can effectively reduce the screening range of allergenic epitopes, significantly improve the working efficiency, and further reduce the synthesis and verification of candidate epitopes.²⁰ Since solid-phase carriers such as ELISA plate and NC membrane cannot bind epitope peptides well, an immune array experiment based on inhibitory dot-blot was designed to further verify the IgE reactivity of predicted epitopes by using the properties of epitope peptides that can compete with Rap v 2 to bind serum IgE antibodies after direct binding with the serum. Furthermore, human KU812 basophil was used to stimulate the degranulation of basophil cells mediated by epitope peptides to bind specific serum IgE

antibodies to validate the results of the inhibitory dot-blot assay, which is a more straightforward method to reveal the interaction between specific IgE and allergenic epitope and has greater biological relevance. In the present study, 12 predicted epitopes of Rap v 2 were developed and selected based on 5 immunoinformatics tools that fulfilled all the criteria of antigenicity, surface accessibility, hydrophilicity, and flexibility. Subsequently, 7 of the 12 Rap v 2 predicted epitopes were identified as the allergenic epitopes via inhibitory dot-blot, and the final human KU812 basophil degranulation assay also verified this result, which also indicates the reliability of our results. It is worth mentioning that three identified allergenic epitopes of Rap v 2 (P1, P2, and P10) could be recognized by the sera from clam-allergic individuals, suggesting that there may be cross-reactivity between paramyosins in molluscan shellfish. Moreover, compared with allergen Rap v 2, seven single identified allergenic epitopes could trigger weaker degranulation and cytokine release, and this may be due to the fact that Rap v 2 has multiple epitopes to get higher accessibility to improve the IgE recognition, resulting in a higher basophil allergy response. Recent researches have also demonstrated that using bioinformatics methods combined with dot-blot and cell degranulation assays can accurately and efficiently screen allergen epitopes.^{20,21,35}

In addition, the biochemical properties of the epitope regions such as hydrophobicity, hydrophilicity, solvent exposure, and electrostatic character affect the IgE-binding potential. The study of these properties will help further to reveal the specific binding with serum and cross-reactivity of allergenic epitopes of allergens.^{36,37} It was proposed that high-affinity antibody–antigen complexes have stronger electrostatic interactions. The surface electrostatic potential of the screened Rap v 2 allergenic epitopes was analyzed through the PQR server, and it was found that these epitope regions essentially showed a positive potential. Roug et al. identified eight allergenic epitopes of allergen Ara h 3 from peanut using the Spot technology. They correspond to the electro positively charged areas exposed on the surface of protein molecules and have the ability to bind to IgE antibodies.³⁸ This conclusion is consistent with the result of our molecular epitope electrostatic potential. Barre et al. found that consensual molecular surface-exposed characteristics of IgE-binding epitopes exhibited some structural homology that possibly accounts in part for the cross-reactivity reported among peanut and tree nut allergens.³⁹ Therefore, this suggests that the charged properties exposed on the surface of protein molecules affect the binding of the IgE epitope region, which in turn affects the conservation of IgE-binding epitopes of allergens, which is also an essential link that leads to food allergen cross-reactivity. Furthermore, the hydrophilic–hydrophobic property of amino acids can affect IgE binding of the allergenic epitopes. It was reported that most IgE-binding epitopes are composed of a high proportion of hydrophobic amino acids.⁴⁰ Brinda et al. showed that hydrophobic residues can function as weak interface hubs to facilitate IgE-binding capacity.⁴¹ In the current study, it was found that highly hydrophobic amino acids, particularly Glu, Arg, Ser, and Asn, play a key role in the Rap v 2 allergenic epitope regions. The previous study also demonstrated that the presence of these charged residues would raise IgE-binding capacity because of contribution with high solvent accessibility.⁴² For example, the solvent-accessible arginine plays a major role as a central hub through interacting with several amino acid residues to promote subunit

interaction.⁴³ Meanwhile, PM itself contains more hydrophobic residues, which may play an important role in the structural folding of PM. The molecular phylogenetic comparison of paramyosin amino acid sequences, among different invertebrate and vertebrate species, indicated that the paramyosin of molluscs, trematodes, cestodes, and mites is closely related in the present study. This fact may suggest that PM might be the second invertebrate pan-allergen, implicating allergy cross-reactivity to molluscs, trematodes, cestodes, and mites. In addition, early research found that PM is a cross-reactive allergen with molluscan TM. The characteristic α -helical coiled-coil structure and certain similar residues on the epitopes of TM and PM may be the potential factors of their cross-reactivity.⁹

In conclusion, as a novel allergen of molluscan shellfish, more attention should be given to PM, which will be particularly crucial to supplement information on PM epitopes. There have been still no reports on the food allergen PM epitopes, and the identification of PM-specific allergenic epitopes allows the prediction of allergic reactions to ingested molluscs. The identification and analysis of allergenic epitopes and amino acids of Rap v 2 in this study for the first time will not only give a better understanding of the molecular mechanism of interaction between the novel allergen Rap v 2 and sea-snail-specific IgE but also offer new information regarding allergenic epitopes of food allergen PM for the clinical diagnosis and immunotherapy of mollusc allergy.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.1c00775>.

The studied patient information ($n = 11$), clinical history, symptoms, and purified protein specific IgE immunoglobulin in sea-snail-allergic patients (Table S1) and the bioinformatics tools, websites, and references used in the methods section (Table S2) (PDF)

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Notes

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■ ABBREVIATIONS USED

PM, paramyosin; TM, tropomyosin; AK, arginine kinase; SCP, sarcoplasmic-calcium-binding protein; Rap v 2, *Rapana venosa* paramyosin; IgE, immunoglobulin E; ECL, enhanced chemiluminescent kit; KU812, human basophil cell; WHO/IUIS, World Health Organization and International Union of Immunological Societies.

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Supporting Table 1. The studied patient information (n = 11), clinical history, symptoms and purified protein specific IgE immunoglobulin in sea snail allergic patients.

No.	Serum ID	Age (years)	Sex ^a	Clinical history ^b	Symptoms ^c	Rap v 2 protein specific IgE (OD) ^d
1	399	50	F	SSA, WA, EA	As, Ar, Dz, Uc,	1.036
2	294	46	M	SSA, SA, CRA	As, Fe, Dz, Uc	1.065
3	647	30	F	SSA, PA, CRA, DMA	U, A	0.741
4	683	16	M	SSA, SA, FA, CRA	Ar, U, A	1.124
5	776	24	M	SSA, SA, OA	U, V&D, A	0.728
6	574	49	M	SSA	U, A	0.640
7	628	36	F	CLA, OA	U, Ar	0.423
8	259	69	M	CLA, CRA, SA	U, AD	0.407
9	341	21	M	SA	U, Ar	0.310
10	332	36	F	ND	--	0.168
11	357	26	M	ND	--	0.219

^a M, male; F, female.

^b SSA: sea snail allergy; SA: shrimp allergy; FA: fish allergy; CRA: crab allergy; WA: wheat allergy; EA: egg allergy; PA: pollen allergy; DMA: dust mite allergy; OA: oyster allergy; CLA: clam allergy; ND: not determined/unknown

^c Ar: allergic rhinitis; U: urticaria; Dz: dizziness; Uc: unconsciousness; As: anaphylactic shock; A: asthma; Fe: facial edema; AD: atopic dermatitis; V&D: vomiting and diarrhea; --: means no symptoms.

^d Negative control for specific IgE (No. 9-11 sera from shrimp allergic patient and healthy individuals): OD 0.16–0.31. The levels of IgE immunoglobulin against Rap v 2 protein was significantly increased in sea snail-allergic patients compared to negative control.

Supporting Table 2. The bioinformatics tools, websites and references used in the methods section.

Name	Web addresses	References
Uniprot Swiss-Prot/TrEMBL	http://www.uniprot.org/	22
NCBI tBLASTn	http://blast.ncbi.nlm.nih.gov/Blast.cgi	22
Clustal Omega	https://www.ebi.ac.uk/Tools/msa/clustalo/	23
ABCpred server	https://webs.iiitd.edu.in/raghava/abcpred/dataset.html	21, 27
BepiPred 2.0 server	http://www.cbs.dtu.dk/services/BepiPred/	21, 27
Immunomedicine Group Server	http://imed.med.ucm.es/Tools/antigenic.pl	21, 27
PSIPRED server	http://bioinf.cs.ucl.ac.uk/psipred/	30
SWISS-MODEL server	https://swissmodel.expasy.org/	31
ROBETTA server	http://new.rosetta.org/	32
SAVES server	https://saves.mbi.ucla.edu/	33
PDB2PQR server	http://nbcr-222.ucsd.edu/pdb2pqr_1.8/	34