

Purification, Characterization, and Three-Dimensional Structure Prediction of Paramyosin, a Novel Allergen of *Rapana venosa*

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Cite This: *J. Agric. Food Chem.* 2020, 68, 14632–14642



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ABSTRACT: Paramyosin (PM) is an important structural protein in molluscan muscles. However, as an important allergen, there is a little information on PM in the molluscs. In this study, a 99 kDa molecular weight allergen protein was purified from *Rapana venosa* and confirmed as PM by mass spectrometry. The results of immunoglobulin E (IgE)-binding activity and physicochemical characterization showed that *R. venosa* PM could react with a specific IgE of the sera from sea snail-allergic patients, and the IgE-binding activity could be reduced by thermal treatment. The full-length cDNA of *R. venosa* PM was cloned, which encodes 859 amino acid residues, and it has a higher homology among molluscan species. According to the circular dichroism results, Fourier transform infrared, and 2D and 3D structure analysis, both PM and tropomyosin are conserved proteins, which are mainly composed of the α -helix structure. These results are significant for better understanding the anaphylactic reactions in sea snail-allergic patients and allergy diagnosis.

KEYWORDS: paramyosin, allergen, purification, *Rapana venosa*

INTRODUCTION

Seafood and its products have become an integral part of human diet and nutrition as an important source of high-quality animal protein. The increasing production and consumption of seafood have been accompanied by the growth in the prevalence of seafood allergies, which is severely affecting people's quality of life and increasing the expenses on health care all over the world. Similar to fish and crustacean allergies, molluscs are also prone to cause allergic reactions in some sensitive individuals.^{1,2} However, there is only a few research focus on mollusc-induced allergic reactions, although a recent study of mollusc allergy based on self-reporting data showed that the prevalence of molluscan allergy in the pediatric population of the United States was as high as 0.15%.³ It has also been well demonstrated that the allergic reactions to molluscs are often associated with the oral allergy syndrome, urticaria, and severe life-threatening anaphylaxis including anaphylactic shock and severe asthma attacks.^{4,5}

Tropomyosin (TM) is a salt-soluble and heat-stable 34–36 kDa myofibrillar protein and has been identified as a major allergen in a number of mollusc species.^{6,7} Arginine kinase (AK) and triose phosphate isomerase (TIM) have also been discovered to be allergenic proteins in Octopus, and the cross-reactivity of these allergens has only been investigated in other crustaceans.^{8,9} At present, there are still many unknown proteins in other molluscs, which need to be identified and studied. Lopata et al.¹⁰ isolated and identified a 49 kDa heat-stable allergen protein from fresh, cooked, and dried abalone, which was recognized by immunoglobulin E (IgE) in the serum of abalone-allergic patients, but there is no further data available on the biochemical properties. Lee and Park¹¹ pointed out that three proteins with molecular weights

(MWs) of 40, 71, and 82 kDa can be recognized by IgE in the sera of allergic patients with *Buccinum undatum*. Therefore, extensive research on the impact of novel allergens and their identification in molluscan species is highly essential.

Paramyosin (PM) is a major structural component of the molluscan muscle thick filaments and plays an important role in the “catch” mechanism, which enables sustained contraction of the muscles in invertebrates.^{12,13} It is reported that this protein also forms a significant component of the bivalve myofibril and the mantle muscle of squid and the foot muscle of turban shell comprising 9–14% of PM in their myofibril.¹⁴ PM is not only an essential structural protein for the growth of certain invertebrates but also an allergenic protein capable of causing an immune response in the body.^{15,16} Apart from TM, PM has also been investigated as a potential allergen in molluscan shellfish. Suzuki et al.¹⁷ found that the PM from the disc abalone could react to a protein with 100 kDa MW in 16 out of 18 patient's sera, as observed by western blot analysis. Furthermore, PMs have been confirmed as a major allergen in *Anisakis simplex* (Ani s 2), *Blomia tropicalis* (Blo t 11), *Dermatophagoides farinae* (Der f 11), and *Dermatophagoides pteronyssinus* (Der p 11) (<http://www.allergen.org/>). To be mentioned, the World Health Organization (WHO) has listed PM as one of the candidate vaccine antigens for schistosomiasis and *Dermanyssus gallinae*.^{18,19} At present, studies

Received: July 12, 2020

Revised: October 8, 2020

Accepted: October 29, 2020

Published: November 11, 2020



focusing on mollusc allergy and PM is not up to the mark; therefore, an extensive research should be explored to further understand this particular allergen.

Rapana venosa is a mollusc widely found in the Bohai and Yellow coastal areas of China, which is a kind of popular seafood and commercially important gastropod in Asia.^{20,21} Thus, these allergenic protein components are of great concern in *R. venosa* and attract more attention because their high consumption. Generally, an allergic reaction to certain proteins can be attributed to a variety of factors, including the amino acid sequence of the allergens, their structures, and thermal and pH stabilities in certain circumstances.^{22,23} In our study, the 99 kDa protein was purified from *R. venosa* for the first time, and the purified protein was identified by mass spectrometry (MS) and cloned by genetic engineering. The sequence homology and alignment of PM were then analyzed in different species, and the three-dimensional model of the protein structure was established and used to predict its epitope conservation. In addition, its physicochemical characteristics and protein structure including the secondary structure and IgE-binding capacity were determined and characterized. These findings will enhance our understanding of sea snail allergens and provide valuable references for more molecular diagnostic studies on molluscan allergens.

MATERIALS AND METHODS

Chemicals. Ceramic hydroxyapatite type I was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Total RNA Extraction kit and RACE 5'/3' kit were purchased from TaKaRa (Takara Bio, Mountain View, CA, USA). Bovine serum albumin (BSA) and β -mercaptoethanol (β -Me) were procured from Sigma Chemical Co. (St. Louis, MO, USA). Rabbit serum against PM was commissioned by BGI Genomics Co., Ltd, and anti-rabbit IgG and horseradish peroxidase (HRP)-conjugated goat anti-human IgE antibodies were obtained from Zhongshan Jinqiao Co. (Beijing, China). The enhanced chemiluminescent (ECL) substrate used in western blot analysis was procured from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The protein standard for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was procured from Solarbio Co. (Beijing, China). All other chemical reagents used were of analytical grade and pure.

Human Sera. Sera from sea snail-allergic patients were provided by the Affiliated Hospital of Qingdao University (Qingdao, China). Five serum samples contained a specific IgE of *R. venosa* PM (no. 1–5). Three sera from shrimp-allergic patients and healthy individuals (no. 6–8) were used as a negative control. Table S1 shows the individual sensitive profiles and clinical symptoms, which were used in the IgE western blot and dot blot tests. All the patient sera were kept at $-80\text{ }^{\circ}\text{C}$ until used. Written consents were obtained from each patient and healthy individual before collecting the sera, who were aware of the usage of their sera for experimental purposes. The medical ethics committee approved the sera collection protocol (file no. QD-20140314551).

Immunoassay of Myofibrillar Protein. Sea snails (*R. venosa*) were purchased live at Liqun Supermarket (Qingdao, Shandong, China). The muscle collections were used or processed immediately by freezing at $-80\text{ }^{\circ}\text{C}$ before being used to extract and purify PM. The *R. venosa* muscle was mashed and homogenized with 10 volumes of precooled 20 mM Tris-HCl (pH 6.8) at $4\text{ }^{\circ}\text{C}$, and then the homogenate was centrifuged at 8000g for 30 min at $4\text{ }^{\circ}\text{C}$. The supernatant obtained was regarded as a sarcoplasmic protein fraction. The precipitate was resuspended in 10 volumes of 20 mM Tris-HCl, and the step was repeated three times. Finally, the precipitate was dissolved in seven volumes of 20 mM Tris-HCl including 1 mol/L KCL and 0.1 M DTT (pH 6.8), and the solution was termed myofibrillar protein. SDS-PAGE and western blot analysis were performed using the human serum.

Purification and Identification of a Target Allergen. The extraction and purification methods of PM mainly refer to the method described by Suzuki et al.¹⁷ with slight modifications. The muscle (50.0 g) of *R. venosa* was homogenized with 400 mL of precooled buffer A (phosphate buffer containing 0.15 M KCl and 0.5 mM L-cysteine, pH 6.8), followed by centrifugation at 8000g for 30 min, followed by removing the supernatant. The remaining precipitate was washed repeatedly with buffer A four times, and the last precipitation was extracted with 500 mL of ice-cold buffer B (10 mM phosphate buffer with 0.5 M NaCl and 0.1% β -Me, pH 6.8) overnight after centrifugation at 12,000g for 30 min. Then, MgCl_2 and ATPN_2 were added to the supernatant solution until a final concentration of 1 mM was reached and shaken again for 1 h. Followed by centrifugation at 12,000g for 30 min, the supernatant obtained was regarded as a myofibrillar protein solution. The salting-out condition of 10–30% saturation of ammonium sulfate was explored for the myofibrillar protein solution, and then the gradient with the most target protein and the least impurity protein (10–20%) was selected as the final ammonium sulfate salting-out gradient. Further, the obtained precipitate was dissolved from 10 to 20% saturation of ammonium sulfate with buffer B and dialyzed thoroughly with the same buffer. The fully dialyzed sample was then filtered with a $0.22\text{ }\mu\text{m}$ membrane filter and loaded on a ceramic hydroxyapatite type I column ($1.0 \times 10.0\text{ cm}$; Bio-Rad Laboratories, Hercules, CA, USA). The unadsorbed protein was washed with buffer B, and the adsorbed part of the protein was linearly eluted with an equal amount of buffer B and buffer C (200 mM phosphate buffer with 0.5 M NaCl and 0.1% β -Me, pH 6.8) at a flow rate of 0.8 mL/min to finally obtain the purified protein. The proteins were monitored at 220 nm with a UV spectrophotometer and the eluate containing the 99 kDa allergen was collected. The target protein band cut from the gel was sent to Beijing Protein Institute (Beijing, China) and analyzed by matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF-MS), which was based on the method as described by Zhang et al.²⁴ The peptide mass fingerprint was compared with the sequences of *R. venosa* 99 kDa allergen in the NCBI database.

IgE Immunoblotting and Specific IgE Sea Snail-Allergic Patient's Sera. For the confirmation of IgE binding potential of 99 kDa purified protein, the levels of specific IgE were estimated, and IgE immunoblotting was performed on sea snail severe allergic patient's sera with reference to the methods as described by Misra and Xia et al.^{25,26}

Glycoprotein Characterization of the Target Protein. β -elimination was used to characterize the carbohydrate–peptide linkage of protamine. The protocol was carried out according to the method described by Zhang et al.²⁴ with some slight modifications. The target protein solution (90 μL , 0.2 mg/mL) was mixed with 10 μL of 0.5 mol/L NaOH and incubated at $37\text{ }^{\circ}\text{C}$ for 16 h. The pH of the reaction system was adjusted to neutral using HCl (1 mol/L) to stop the reaction. The ultraviolet absorption values of the reaction system were then scanned in the wavelength range of 210–300 nm using a UV-1101 system (Techcomp Co. Shanghai, China).

Thermal and pH Stabilities of PM. To evaluate the thermal and pH stability of the main allergen, the purified target protein was adjusted to a concentration of 0.2 mg/mL and incubated at different temperatures (4, 35, 45, 65, 70, 80, 90, and $100\text{ }^{\circ}\text{C}$) for half an hour. In the study of pH stability, the purified PM protein was mixed with buffer solutions of different pH values (1.0, 3.0, 5.0, 6.0, 7.0, 8.0, 9.0, 11.0, and 13.0) at a ratio of 1:4 (v/v) and incubated at room temperature for 2 h. The proteins (20 μg /mL purified protein) were directly blotted on the nitrocellulose membrane (Merck Millipore Inc., Burlington, MA, USA) and incubated with human sera (1:4 dilution) after blocking. Following incubation with HRP-conjugated goat anti-human IgE (1:3000 dilution), the immunoassay was carried out using ECL.

Analysis of Changes in the Protein Structure of Different Temperatures and pH Values by CD Spectroscopy. The method for obtaining samples with different temperatures (0 and $100\text{ }^{\circ}\text{C}$) and pH values (pH 1, 6.8, and 11) was the same as that for the determination of thermal stability and pH stability of PM.

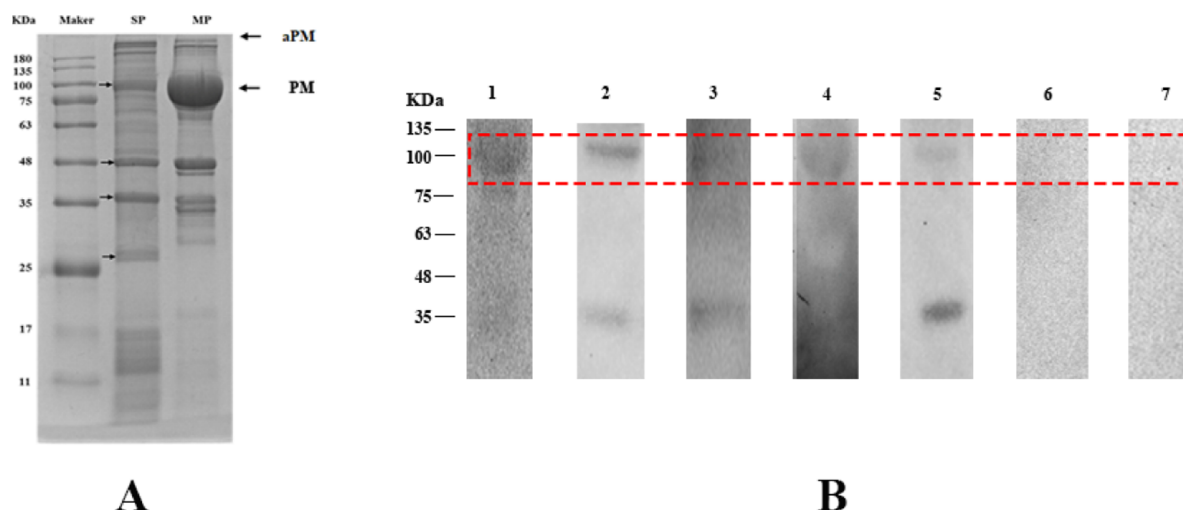


Figure 1. SDS-PAGE and IgE immunoassay analysis of myofibrillar protein from *R. venosa*. (A) SDS-PAGE analysis. Lane M, protein marker; lane SP, sarcoplasmic protein; and lane MP, myofibrillar protein. (B) IgE immunoassay analysis. Human sera (1:4 dilution) and HRP-conjugated goat anti-human IgE (1:3000 dilution). Strips 1–5 were five different sensitive individual sera, and the profiles are shown in Table S1. Strips 6 and 7 were shrimp-allergic patient and pooled sera of two healthy individuals (negative control), respectively.

According to the methods described previously by Ahmed et al., the parameters of CD analysis were a scanning wavelength range of 190–280 nm and a scanning speed of 100 nm/min using a 1 nm bandwidth at an interval of 2 s via a JASCO J-815 CD spectrometer (Japan 218 Spectroscopic Co. Inc., Japan).²⁷

Fourier Transform Infrared Analysis. The powdered sample (~1–2 mg) was mixed with spectroscopy KBr (100–200 mg) and formed a thin film with a pressure of 20 MPa. Fourier transform infrared (FTIR) spectra were scanned from 400 to 4000 nm in the absorbance mode. Interferograms were averaged for 32 scans at a 4 cm^{−1} resolution. Each analysis was carried at least three times to obtain reproducible results.

Differential Scanning Calorimetry Analysis. DSC measurement was performed using a TA Instruments DSC 200PC. All tests were conducted in the temperature range of 20–150 °C at a heating rate of 8 K/min. Netzsch proteus analysis software was used to analyze the glass-transition inflection point and the temperature at the start and end of the glass transition. Each experiment was carried out for at least three times to ensure the reliability of the results.²⁸

Cloning and Sequencing the PM Genes. Total RNA was extracted from the muscle of *R. venosa* with a TRIzol Reagent (Invitrogen, Tokyo), as described in the manufacturer's protocol. Total RNA was treated with RQ1 RNase-FreeDNase (Promega, USA) to remove contaminated DNA. The synthesis of cDNA was carried out by using a Full Gold Reverse Transcription Kit (TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix) according to the manufacturer's procedure. On the basis of the DNA sequences of PM (XM_025230583.1, *Pomacea canaliculata*; XM_013222942.1, *Biomphalaria glabrata*; AB571843.1) in the NCBI database, two degenerate oligonucleotide primers were designed as sense primers for PCR. We designed a pair of primers including a forward primer (FPM, 5'-CGACTAYGACAACYT-SAACG3') and a reverse primer (RPM, 5'-GTTGAKGGCVTCBTC-CAGGT3') based on the above gene sequence of homologous species. PCR amplification was performed using the cDNA template from hemocytes (1 cycle of 98 °C for 2 min; 30 cycles included denaturation at 98 °C for 10 s, annealing at 53 °C for 15 s, and extension at 72 °C for 5 min, followed by 1 cycle of 72 °C for 5 min). PCR products were analyzed on 1% agarose gels, extracted with a OMEGA quick gel extraction kit and sequenced. Based on the target gene information obtained previously, 5'RACE and 3'RACE gene-specific primers (sea snail 5'R: 5'-CCAGCAGGGTCTC-CAGCTCCTTGACGCG3' and sea snail 3'R: 5'-GACCT-CAAGCGCCGCGCATGCAGGTGCGGA3') were designed, respectively. RACE and RACE-PCR were conducted with the

SMARTer RACE 5'/3' Kit (TAKARA) according to the manufacturer's instruction. DNA sequencing was acquired from Paisenno Biotechnology Co., Ltd. (Shanghai, China). The amino acid sequence of PM was deduced from the full-length gene sequence by DNAMAN software.

Sequence Retrieval, Alignment, and Analysis of PM. The amino acid composition, protein MW, hydrophobicity, and calcium binding site of the PM gene were analyzed and predicted by ExPASy. The multiple sequence alignment was performed with *R. venosa* PM in different species using BioEdit, which is highly integrated software for molecular biology. The analysis involved eight amino acid sequences, including three different species category, namely, mollusc, arthropod, and platyhelminthes. A phylogenetic tree analysis was executed in MEGA 5.0, which is an integrated tool for molecular biology, using the neighbor-joining method.

Three-Dimensional Structure Analysis and Validity Evaluation of PM. The PSIPRED online server (<http://bioinf.cs.ucl.ac.uk/psipred/>) was used to predict the secondary structure of PM. Information on the simulated three-dimensional structure of PM (PM1 and PM2) could be obtained when amino acid sequences of PM were predicted by prediction programs, iterative threading assembly refinement (I-TASSER) and Rosetta.^{29,30} The stereochemical quality of the selected model was evaluated by using SAVES (http://nihserver.mbi.ucla.edu/SAVES_3/saves.php) server, which includes ERRAT, PROCHECK, and Verify 3D online tools. Moreover, the amino acid sequences of *R. venosa* PM were input into the ConSurf online server (<https://consurf.tau.ac.il/>), which matched the PM of 30 correlated species through automatic search, and compared and analyzed the obtained amino acid sequences to complete the sequence conservatism analysis of PM. The three-dimensional structure of PM was analyzed using pyMOL software.

RESULTS

Purification of the 99 kDa Protein. Several bands appeared in both sarcoplasmic and myofibrillar proteins, such as PM, aggregated paramyosin (aPM), actin, and TM in *R. venosa* (Figure 1A). Meanwhile, the 99 kDa bands in the myofibrillar protein reacted with sea snail-allergic serum samples (no. 1, 2, 4, and 5), while the 35 kDa (TM) bands reacted with serum samples (no. 2, 3, and 5), which led us to speculate that the 99 kDa protein was a novel allergen in *R. venosa* (Figure 1B).

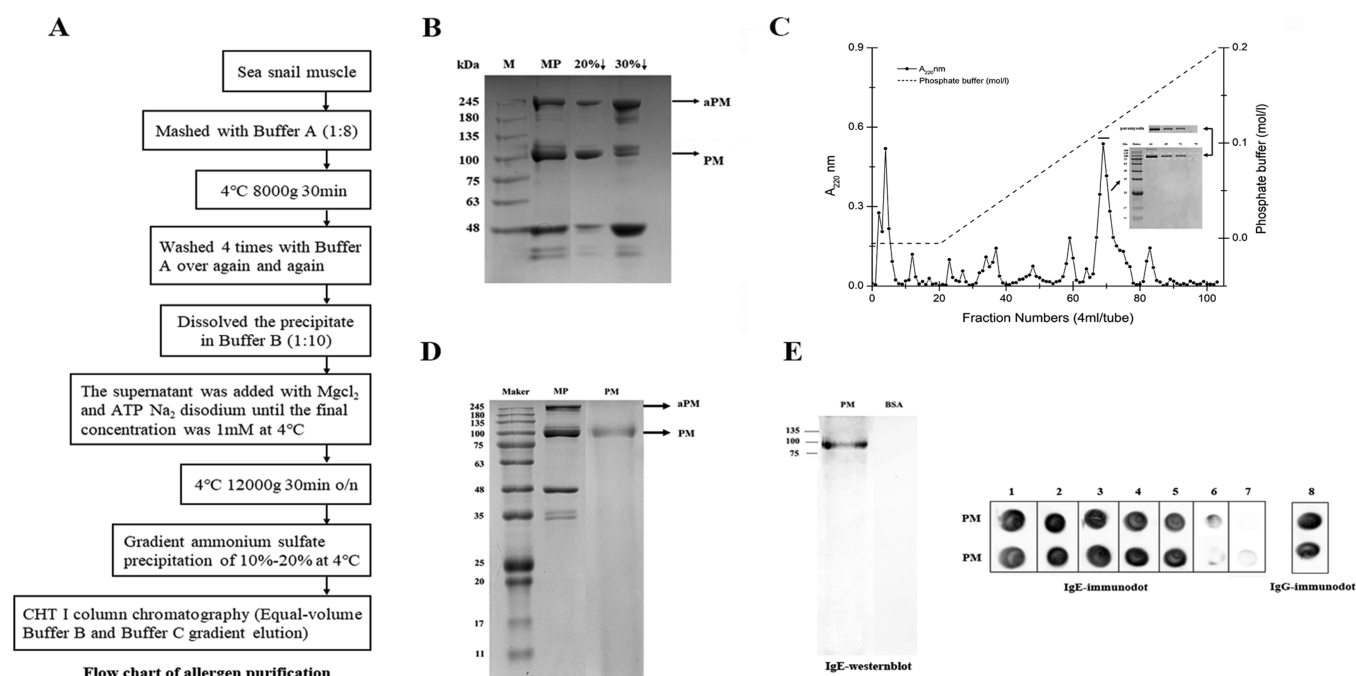


Figure 2. Extraction, purification, and immune activity analysis of the 99 kDa protein in *R. venosa*. (A) Flow sheet for the purification of 99 kDa allergen protein. (B) SDS-PAGE analysis of ammonium sulfate precipitation. Lane M, protein marker; lane MP, myofibrillar protein; and lane 20%↓, 10–20% ammonium sulfate precipitation. Lane 30%↓, 20–30% ammonium sulfate precipitation. (C) Hydroxyapatite chromatogram of the protein extract of 10–20% ammonium sulfate precipitation used for the purification of 99 kDa protein. The numbers on top of the lanes correspond to the fraction number. (D) SDS-PAGE analysis of the 99 kDa purified protein from *R. venosa*. Lane MP, myofibrillar protein; lane PM, purified protein. (E) Immunoassay analysis of the purified protein including western blot assay of IgE capacity to PM from *R. venosa* and dot blot assay of IgE and IgG capacity to the 99 kDa purified protein. Human sera (1:4 dilution) and HRP-conjugated goat anti-human IgE (1:3000 dilution). Sea snail sensitive patient's serum pool was used to determine the IgE capacity of PM and BSA (as the negative control) in western blot assay; for the dot blot assay, strips 1–5: sera of five different sea snail-sensitive patients; strip 6: shrimp allergic patient; strip 7: the pooled sera from nonallergic individuals as the negative control; strip 8: rabbit anti-sea snail PM antiserum (1:10⁵ dilution).

Purification of the 99 kDa protein was achieved by the myofibrillar protein from the raw *R. venosa* muscle, followed by stepwise ammonium sulfate precipitation and CHT-I hydroxyapatite chromatography. A flow sheet for the purification process of the 99 kDa protein is shown in Figure 2A. As shown in Figure 2B, upon stepwise addition of ammonium sulfate to the myofibrillar protein, aPM with a MW greater than 200, 99 kDa PM, and 48 kDa actin can be clearly observed in myofibrillar protein (lane MP). After exploring the conditions of 10–30% ammonium sulfate salting-out, generally the target protein was mostly distributed in the range of 10–20% ammonium sulfate precipitation (lane 20%↓), while aPM and actin were mainly distributed in 20–30% ammonium sulfate precipitation (lane 30%↓). Subsequently, 10–20% ammonium sulfate salted-out samples were loaded into hydroxyapatite chromatography and the target protein was eluted when the phosphate concentration was about 0.16 mol/L (Figure 2C). The highly purified protein migrated on SDS-PAGE as a single band with a MW of about 99 kDa (Figure 2D). Moreover, the IgE-binding capacity of the 99 kDa purified protein was evaluated by western blot, which revealed a positive binding capacity to the allergic patients' sera pool while showed a negative result to the BSA control. In the dot blot analysis, five serum samples from sea snail-allergic patients all reacted with the target protein, but no IgE-binding capacity was detected in the negative control sera. Meanwhile, the rabbit anti-sea snail PM polyclonal antibody was confirmed to have a strong IgG-binding activity (Figure 2E).

Identification by MALDI-TOF-MS. Peptide mass fingerprinting of the purified 99 kDa proteins showed multiple peaks ranging from 800 to 4000 Da (Figure 3A). The effective peaks were further analyzed by MS/MS (MS/MS); the matched effective peptides had 100% identity with aligned full-length sequence data of the PM from *R. venosa* (Table 1), and then the effective peptides were compared with the NCBI database. The only one protein listed by the Mascot score standard ($p < 0.05$) with high score was *P. canaliculata* PM (XP_025086368.1), indicating that 11 peptides with amino acid residues in total were obtained, and these were 100% identical to 11 peptide fragments of PM from *P. canaliculata* (Figure 3B). Thus, we inferred that the 99 kDa MW purified protein was PM in the *R. venosa*.

Characterization of Purified PM. As shown in Figure 4A, the UV absorbance at 214 and 242 nm of the alkali-treated PM sample was significantly increased and shifted compared with the sample without alkali treatment, which showed that one or more polysaccharide components exist in the PM structure.³¹ In the thermal and pH stability assay, when the reaction temperature increased to 70 °C, the protein's IgE-binding capacity gradually weakened, and until the reaction temperature reached 100 °C, almost no IgE-binding capacity was detected (Figure 4B). Moreover, compared with the control sample (pH 7.0), no obvious change was detected in the IgE-binding capacity of *R. venosa* PM in different pH values (Figure 4C).

The circular dichroism (CD) results showed that PM has a typical α -helix structure, and when *R. venosa* PM was heated at

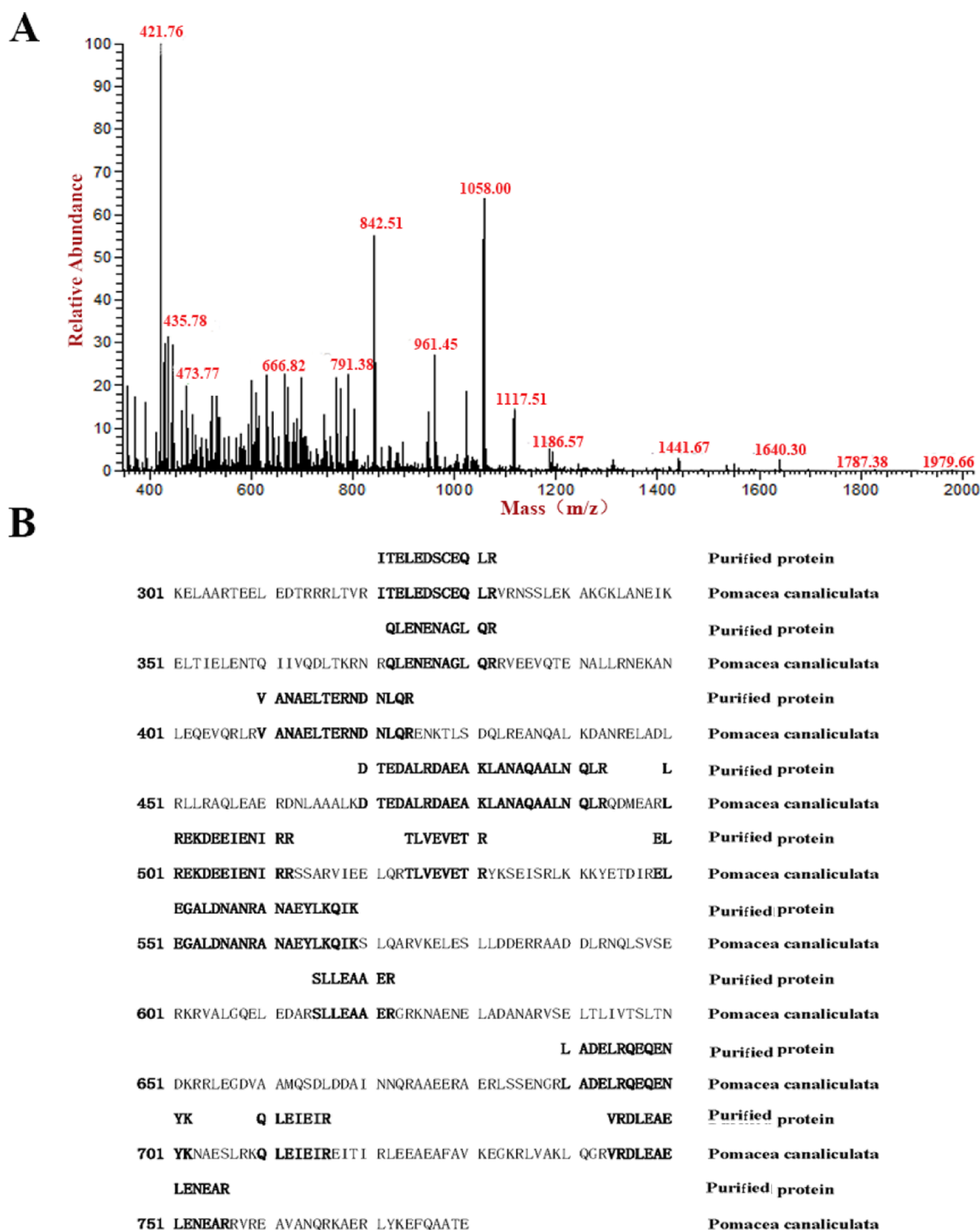


Figure 3. Mass spectrometric results of the 99 kDa purified protein. (A) Peptide mass fingerprinting of purified protein from *R. venosa* and (B) protein sequence alignment of the MS peptides from *P. canaliculata*.

100 °C, the α -helical structure was significantly decreased, its positive and negative absorption peaks were also significantly reduced, and the extension chains and random structures in the structure were heightened (Figure 4D). However, the pH after treatment with strong alkali did not change distinctly (Figure 4E). IR spectroscopy showed that the peak position of *R. venosa* PM amide A band was 3411.06 cm^{-1} , which indicated the existence of N–H stretching vibration, and the

hydrogen bonds formed by these N–H groups play an important role in maintaining the α -helix structure. The peak frequencies of PM amide II and amide III bands were 1553.00 and 1221.66 cm^{-1} , respectively. The presence of amides II and III also proved the complete existence of the helical structure in the PM (Figure 4F). Furthermore, the DSC thermograms showed an endothermic peak at 82.30 °C, indicating that the

Table 1. Effective Purified PM Peptides from MS Compared with Nucleotide Sequence Data from *R. venosa* PM

start–end	calc. mass	obsrv. mass	±da	peptide sequence
35–49	1772.8653	1772.8722	0.0069	IRELEDALDSEDRGR
55–62	978.4957	978.5011	0.0054	MVAELNFR
63–85	2525.1439	2525.1566	0.0127	VDQLQDSLDEQGTSTSAQ MEISK
96–113	2061.8983	2061.9052	0.0069	KDMELASAQFEATES SMR
115–132	2002.9232	2002.9317	0.0085	RHQESVNDLSQVDYL NK
189–197	1000.5503	1000.5441	−0.0062	QLSELTSLK
200–223	2711.3151	2711.3243	0.0092	LTQENFDLQHQVQELDSNNAA LGK
267–278	1390.6599	1390.6576	−0.0023	LEEEAETSTNLR
284–292	979.4974	979.4996	0.0022	VNADYSA LK
295–302	962.5437	962.5456	0.0019	FDKELA LK
302–311	1023.4252	1023.4300	0.0048	MEEMEDLK
317–328	1505.7099	1505.7032	−0.0067	ITELEDTCQQLR
368–378	1253.6000	1253.6025	0.0025	QLENENAGLQR
380–391	1387.6943	1387.6972	0.0029	I EELTGENNTLR
395–403	1042.5659	1042.5683	0.0024	ALLEQDVQK
451–465	1600.8420	1600.8503	0.0083	SQLEAERDSLAAALK
466–477	1346.6313	1346.6323	0.001	DTEEARLDAEAK
478–489	1226.6982	1226.7053	0.0071	LASAQAALNQLK
520–527	945.5131	945.5149	0.0018	TLVEVETR
545–555	1214.5891	1214.5927	0.0036	ELEAALDNANR
571–582	1486.7878	1486.7970	0.0092	VKELETLLEER
600–610	1213.6302	1213.6363	0.0061	VALAQELEDAR
622–633	1306.6001	1306.5997	−0.0004	NAETELADTSTR
634–649	1816.9894	1816.9969	0.0075	LSEITLQVTSLTNDKR
686–698	1634.7900	1634.7860	−0.004	LADELRLQEQENYK
706–712	868.4654	868.4699	0.0045	QLEVEIR
718–727	1107.5448	1107.5490	0.0042	LEEAEAFATK
740–752	1542.7637	1542.7704	0.0067	V RDLEAELENEAR
770–779	1202.5779	1202.5811	0.0032	ELVAQTEER
781–795	1710.9264	1710.9336	0.0072	QLAELASLNDQLQLR
801–816	1832.8938	1832.9027	0.0089	RQLEEAEDVASLTMNK
820–830	1171.5833	1171.5882	0.0049	AQALIDEADAR

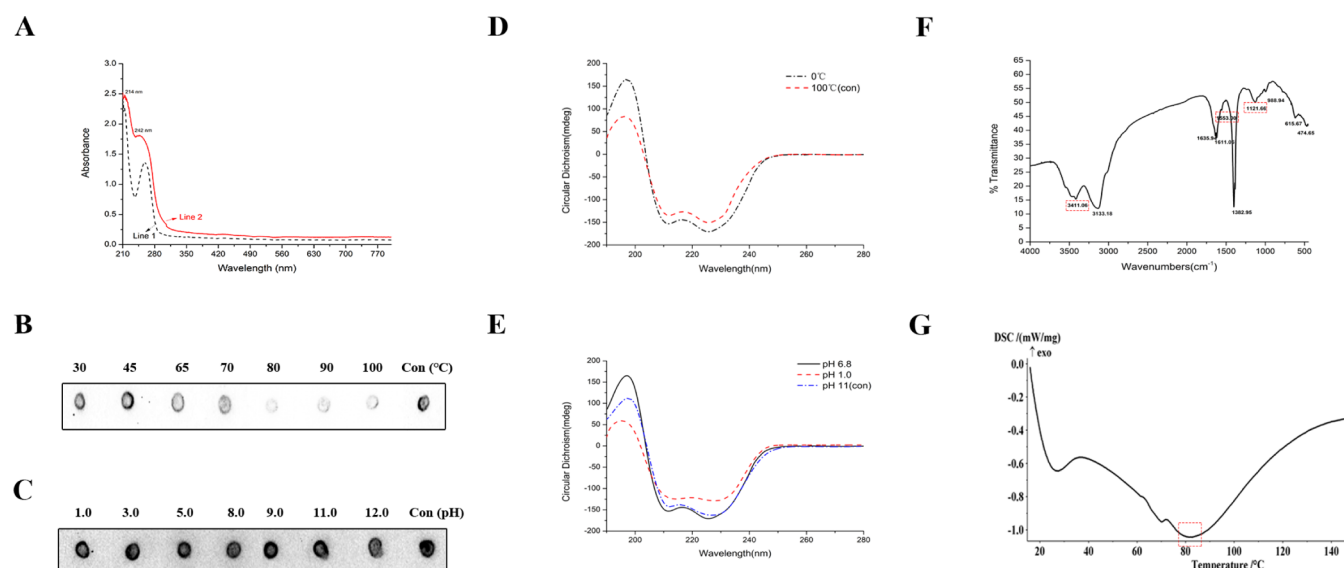


Figure 4. Physicochemical and structural characteristics of PM. (A) UV wavelength scanning atlas of PM of *R. venosa* (line 1, PM treated with low-concentration alkali; line 2, untreated PM; the change at 214 and 275 nm was marked by an arrow); (B) dot blotting analysis of the IgE-binding capacity of *R. venosa* PM treated at different temperatures (PM treated at 4 °C as the control); (C) dot blotting analysis of the IgE-binding capacity of *R. venosa* PM treated with buffers at different pH values [PM treated with buffer (pH 7.0 as the control)]; (D) CD spectral analysis of PM subjected to high temperature (100 °C, - - -) or 0 °C (—) as the control. (E) CD spectral analysis of PM subjected to strong acid (pH 1.0, - - -), strong alkali (pH 11.0, - - -), or pH 6.8 (—) as the control. (F) Secondary structural analysis of PM by IR spectroscopy. (G) DSC heating curves (8 K/min) of *R. venosa* PM. Peak: 29.6 and 82.30 °C, onset: 19.96 and 42.31 °C; end: 104.3 °C.

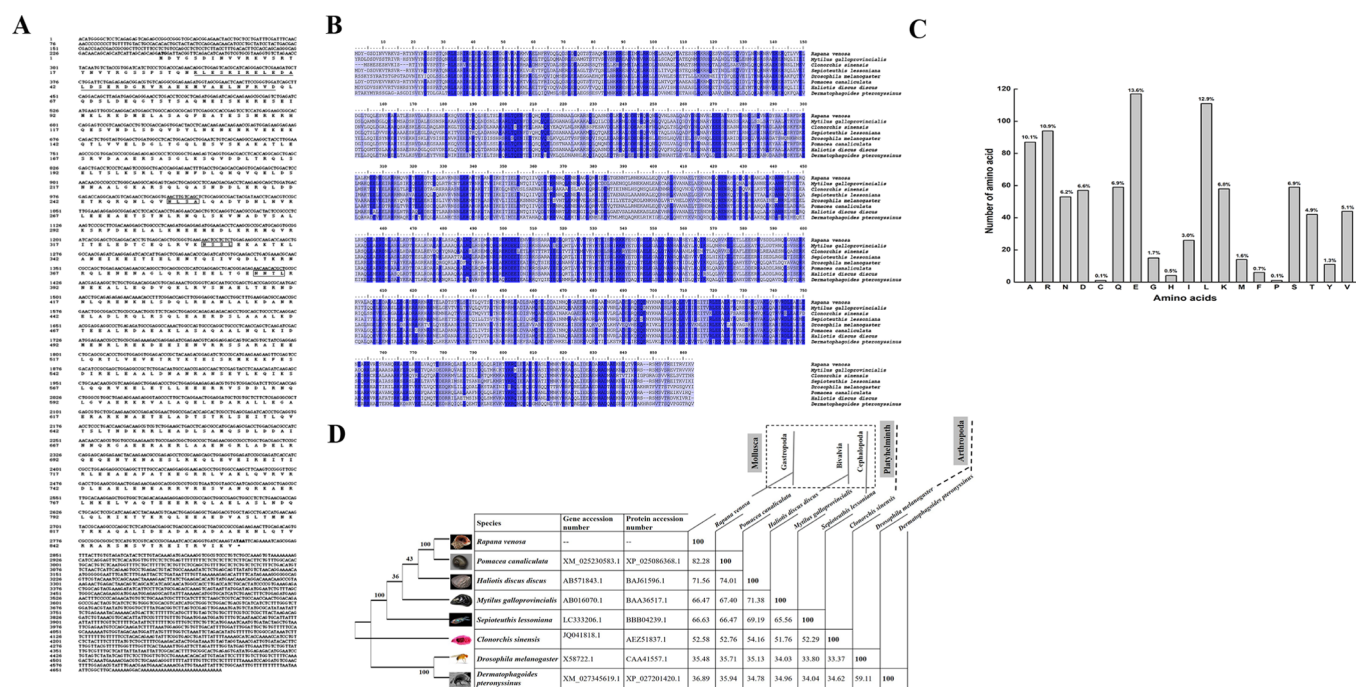


Figure 5. Invertebrate and vertebrate PMs investigated in this study. (A) The complete nucleotide and deduced amino acid sequences of *R. venosa* PM, the residue numbers for both nucleotides and amino acids are shown on the left, the letters in boldface indicate the start codon (ATG) and stop codon (TGA). The N-glycosylation site is marked by the box; the horizontal line represents the calcium-binding domain profile. (B) Comparison of invertebrate and vertebrate PM amino acid sequence information. Multiple alignment of the amino acid sequences of the PM from *R. venosa* with the representative amino acid sequences of *P. canaliculatus* (XP_025086368.1), *Haliotis discus discus* (BAJ61596.1), *Mytilus galliprovincialis* (BAA36517.1), *S. lessoniana* (BBB04239.1), *Clonorchis sinensis* (AEE51837.1), *Drosophila melanogaster* (CAA41557.1), and *D. pteromyximus* (XP_027201420.1) for visual comparison of primary structure similarity of invertebrate and vertebrate PMs. (C) Compositions of amino acids of *R. venosa* PM. (D) PM amino acid sequence identity table calculated using Clustal Omega and phylogenetic tree of different species PM inferred using the minimum evolution method.

structure and thermal stability of the purified protein were likely to change at 82.30 °C (Figure 4G).

Amino Acid Sequence Analysis of PM. As shown in Figure 5A, the full-length cDNA of *R. venosa* PM was obtained, which was 4699 bp with an open reading frame encoding 859 amino acid residues. The nucleotide sequence data was submitted to the GenBank/EBI databases (MN784956). There are three potential N-glycosylation sites at Asn₂₅₂–Leu₂₅₃–Ser₂₅₄–Ala₂₅₅, Asn₃₃₁–Ser₃₃₂–Ser₃₃₃–Leu₃₃₄, and Asn₃₈₇–Asn₃₈₈–Thr₃₈₉–Leu₃₉₀ residues of PM, which was marked with a solid black box. In addition, the residues of amino acids were represented by the calcium binding domain located on the horizontal line. The ProtParam online tool analyzed the amino acid composition of *R. venosa* PM, of which the glutamic acid (Glu) content was the highest, accounting for 13.6%, followed by the leucine (Leu) content accounting for 12.9% (Figure 5C). As shown in Figure 5B,D, the sequence homology of *R. venosa* PM ranges between 66.47 and 82.28% with several PM proteins of molluscan species. However, it is worth mentioning that when compared to arthropoda PM, the sequence homology range was only 35.48–36.89%.

Secondary Structure Prediction and Three-Dimensional Modeling of PM. The secondary structure prediction showed that *R. venosa* PM is mainly composed of α -helices (98.02%) and random coils (1.51%) with a few extended strands (0.23%) analyzed by the PSIPRED server (Figure 6A). The three-dimensional structure of *R. venosa* PM1 and PM2 was constructed based on its amino acid sequence by Robetta and I-TASSER methods, respectively (Figure 6B,E). ERRAT is

a quality assessment tool; the overall quality factor of PM2 was 94.36, while PM1 came out to be 99.76% (Figure 6G,D). Procheck is an online tool for stereochemical quality assessment of the model; the main chain conformations of modeled protein were located in the acceptable regions of the Ramachandran plot. Majority of residues (PM1 92.4 vs PM2 84.1%) were in most favored regions; the PM1 (0.7%) and PM2 (2.5%) of the residues were in disallowed regions of the plot (Figure 6C,F). Thus, we choose PM1 as the established three-dimensional model for subsequent analysis.

Molecular Evolutionary and Conservation Analysis of PM. A consensus tree generated for all protein groups showed similar topologies with a well-branched support for PM (Figure 7A). The phylogenetic tree for PM demonstrated that the mollusc clustered closer with platyhelminthes, while the arthropoda formed a distinct cluster. The conservation grades identified with ConSurf were mapped to the structure using the ConSurf color code, with cyan-through-purple corresponding to a variable (grade 1)-through-conserved (grade 9) positions. As shown in Figure 7B, it can be seen that the amino acid residues of PM from different species are highly conserved, and the regions with large differences are mainly distributed in AA_{151–159}, AA_{188–197}, AA_{214–222}, AA_{411–419}, and AA_{625–632}.

DISCUSSION

With the large-scale capture and the high frequency of consumption around the world, mollusc-induced allergic reactions are prevalently increasing. However, there are few studies on the allergenic proteins in molluscan species, which

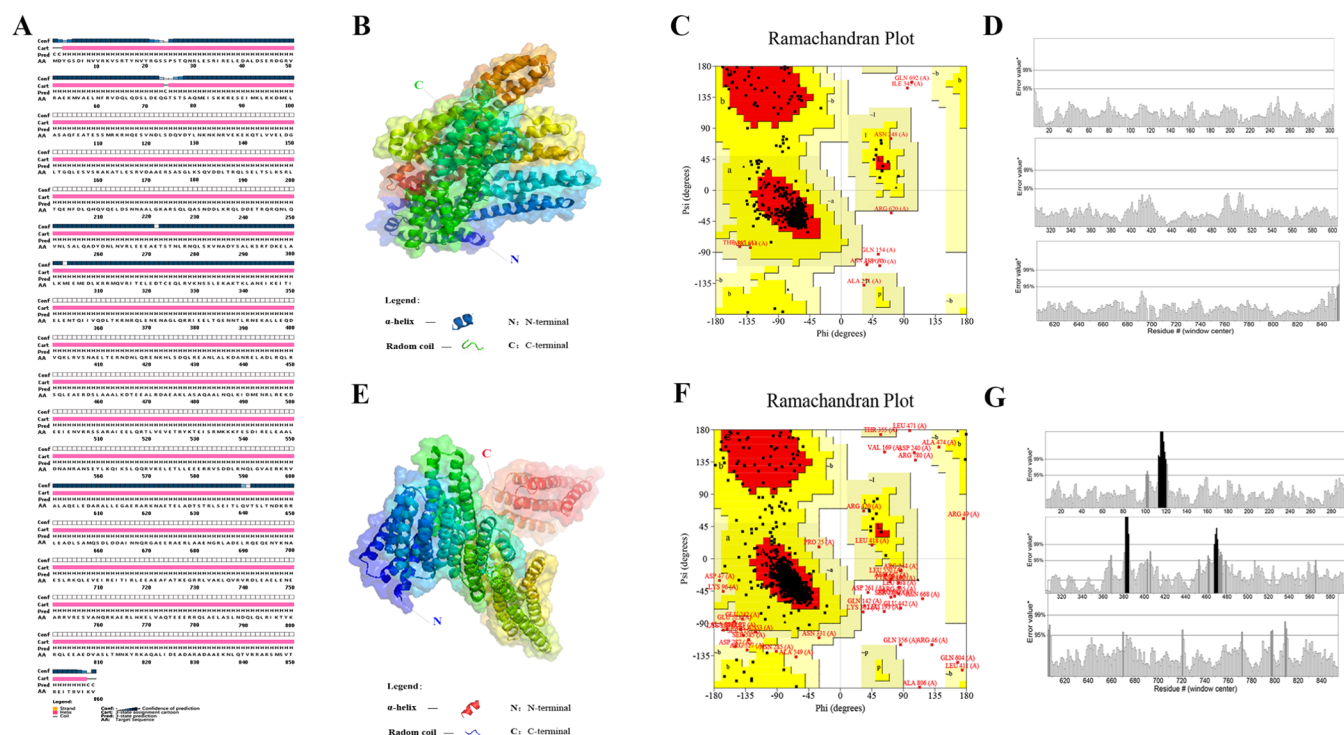


Figure 6. Secondary structure prediction and three-dimensional structure modeling of *R. venosa* PM. (A) Secondary structure prediction for PM. (B) Three-dimensional structure model of PM1 by Robetta server using the comparative modeling method. (C) Ramachandran plot for the PM1 model. (D) Stereochemical quality assessment of the PM1 model by ERRAT plots. (E) Three-dimensional structure model of PM2 by I-TASSER server using the reassembling fragments excised from threading templates. (F) Ramachandran plot for the PM2 model. (G) Stereochemical quality assessment of the PM2 model by ERRAT plots.

mainly focused on TM, the major allergenic protein. Apart from TM, no other allergens have been intensively studied in molluscs. In some recent studies, TIM and sarcoplasmic calcium binding protein have been shown to be a novel allergen in Octopus and Oyster, respectively.^{9,32} Moreover, PM from disc abalone and pacific oyster have also been identified as a novel mollusc allergen, but no biochemical characterization has yet been reported.^{17,33} *R. venosa* is one of the most widely distributed and a commonly consumed sea snail in China, South Korea, and Japan.^{20,21} Previous studies have shown that the ingestion of sea snail could cause a severe anaphylaxis in sensitized patients, but there have been no reports of the associated allergen and the level of molecular immunity studies in sea snail.^{34,35} In the present study, the IgE-binding 99 kDa protein was purified by a combination of ammonium sulfate precipitation and hydroxyapatite chromatography from *R. venosa* for the first time and then identified as PM with MALDI-TOF-MS and immunological reactivity, which is clearly similar to that of disc abalone (100 kDa) and *D. farinae* PM (98 kDa).^{17,36}

In addition, the IgE-binding activity of *R. venosa* PM and TM was simultaneously detected in a serological assay, suggesting that PM may be an important allergen besides TM in sea snail. Similar to TM, PM is also a salt-soluble protein in the myofibril, but it is only soluble in high-salt buffers and tends to aggregate in low-salt environments. In addition, PM is also an unstable protein in molecular properties and its common characteristic is its susceptibility to degrade;^{37,38} thus, these factors lead to certain difficulty in its extraction and purification. In terms of the unstable characteristics of PM protein, this view has also been confirmed by our study, that *R. venosa* PM is relatively

unstable under high-temperature conditions, and when the reaction temperature increased to 70 °C, the IgE-binding capacity of PM decreased significantly. The thermal stability characteristics of *R. venosa* PM are similar to that of disc abalone PM.¹⁷ Moreover, the IgE-binding activity of purified PM was resistant to acid-alkali treatments, which maintained the protein IgE-binding capacity at extreme conditions at a pH 1.0–12.0. Although PM is heat-labile and suffers conformational changes with extremely high temperature, it can still cause allergic reactions, which may be due to the retention of some IgE binding epitopes that are not affected by elevated temperature. Thus, the thermal instability of PM may be a major reason that seriously affects the research progress of the molluscan allergen PM.

In this research, the recently identified PM clone encodes a major molluscan allergen containing 859 amino acid residues and up to 99 kDa of MW. The β -elimination indicated the existence of O-linked glycan in the PM structures. Ser or Thr amino acid on O-glycans is unstable under low temperature and alkaline conditions. After NaOH treatment, Ser and Thr are transformed to α -amino acids and exhibits characteristic UV absorption at a wavelength of 240 nm.^{22,39} There are three putative N-glycosylation sites in the sequence; this particular result further strengthened the results of β -elimination experiment. Moreover, many Ca^{2+} binding regions also exist in the sequence, which are probably functional. It was reported that Ca^{2+} binding was essential for the formation of the conformation structure of calcium-binding proteins, and the consumption of Ca^{2+} may lead to the decrement of IgE-binding capacity.^{40,41} The influences of Ca^{2+} and N-glycosylation sites on the structure of PM and their allergenicity need further research. Sequence alignment

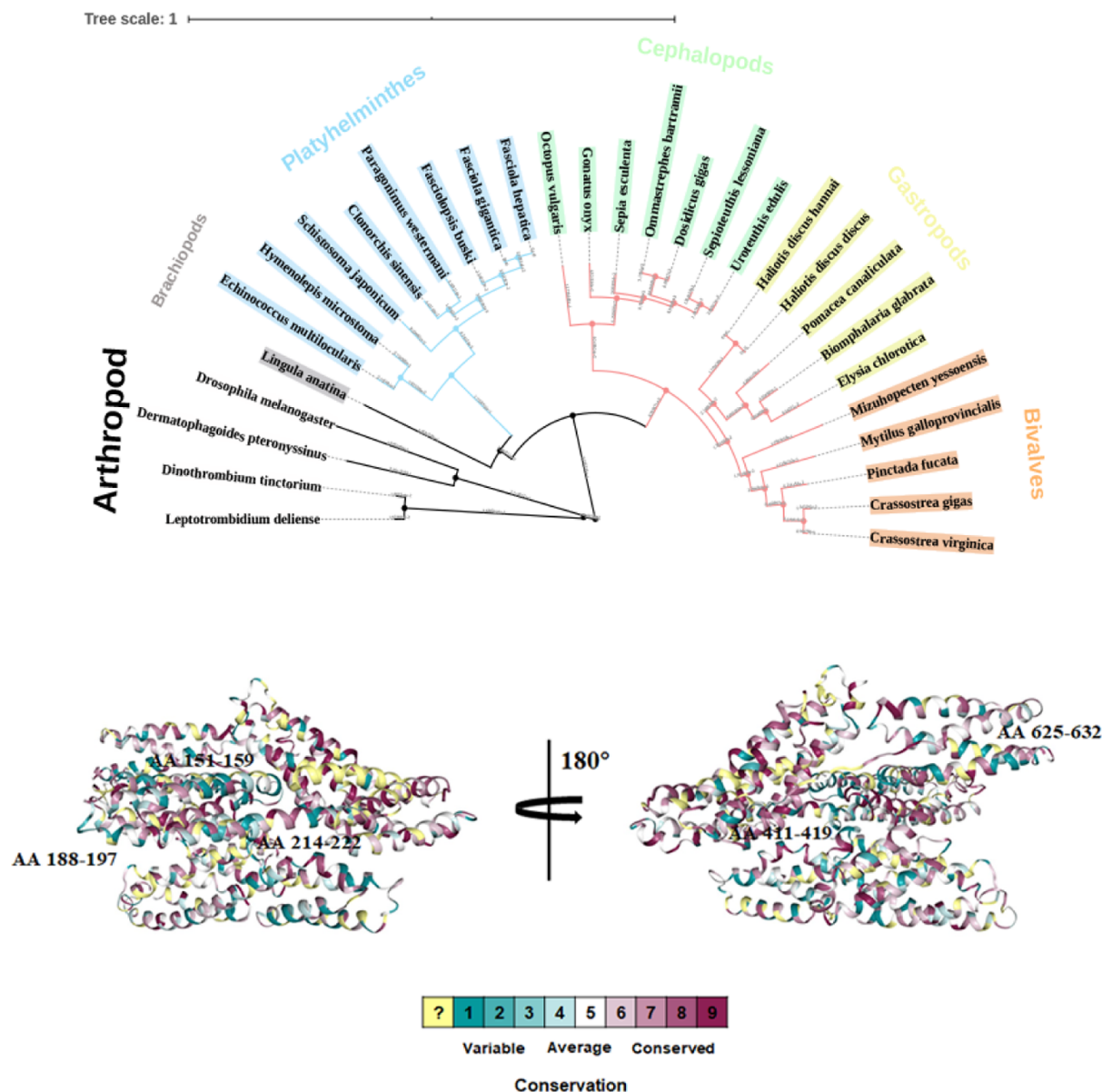


Figure 7. Molecular evolutionary and conservation analysis of *R. venosa* PM by comparing amino acid residues of PM in 30 different species. (A) The phylogenetic tree was drawn using Bayesian and maximum likelihood approach for PM. (B) Conservation analysis of individual amino acids in PM determined using the Consurf server color code, with blue-through-red corresponding to a variable (grade 1)-through-conserved (grade 9) positions.

indicated that the *R. venosa* PM was more closely related to *P. canaliculata*, which belonged to the Gastropoda branch. The phylogenetic tree of PM showed evolutionarily high similarity between PM from other gastropods, which may be evolved originally from the same ancestor gene. These studies revealed that the *R. venosa* PM had a high degree of sequence identity with PM from invertebrates, which also indicated that PM may cross-react with the PM of other molluscs. This supports the conclusion of the previous study where PM is a cross-reactive allergen in some molluscs.⁴² The 2D and 3D PM structures in the *R. venosa* were analyzed in order to enhance the knowledge of the structure function relationship and the conservation status of PM. In this work, 3D structure of PM was modeled based on the Robetta server to build model for domains with sequence homology to proteins of known structure using comparative modeling. The stereochemical evaluation by Ramachandran plots and ERRAT demonstrated that the

homology-based model of PM 3D structure was more reasonable than the I-TASSER method. In the previous study, the model structures of Ara h 2 were generated using ROBETTA and I-TASSER to obtain the disordered loop region, and the comparison between the simulated structures of Ara h 2 and the X-ray structures showed that these structures shared similar protein folding.⁴³ The predicted results of 2D and 3D PM structures demonstrated that PM and TM were both conserved proteins and mainly composed of α -helixes, but unlike TM, it is not a long chain structure. At present, because of the lack of more information on the three-dimensional structure and epitopes of PM, the influence of the structural similarity between TM and PM on allergenicity should be further explored. To be mentioned, it has been reported that the conserved structure of PM across invertebrate species was evidenced by its cross-reactive antigenicity.⁴⁴ Therefore, the analysis-conserved regions of

PM in this study provide a reference for the subsequent research of IgE-binding epitopic regions and cross-reactivity of PM.

In summary, this study reported for the first time a potential novel allergen of 99 kDa from *R. venosa*, obtained the genetic information, and characterized its physicochemical properties and structure. Moreover, the three-dimensional structure based on the cloned sequence and the constructed model was used to further analyze the conservatism in the end. These studies are essential to further understand the *R. venosa* PM IgE-binding epitopes and clarify the relationship between structure properties and allergenicity. As an extremely important functional protein and the potential allergen of mollusc, more attention should be paid that will be particularly crucial to [Supporting Information](#) on PM biochemistry. This research will thus enhance our understanding of PM derived from *R. venosa* and will provide guidelines for assessing the risk of mollusc allergies and further enhancing the diagnostic steps.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.0c04418>.

Chinese patient information ($n = 8$), clinical history, symptoms, and purified protein-specific IgE immunoglobulin in sea snail-allergic patients; location and description of characteristic absorption peaks of the FTIR spectra of *R. venosa* PM; and general peak assignment of the DSC heating curves of *R. venosa* PM (PDF)

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Funding

The authors would like to express their sincere thanks to the National Natural Science Foundation of China (31771892) and the CAMS Innovation Fund for Medical Science (CIFMS 2019-I2M-5-024) for supporting the research study.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

PM, paramyosin; TM, tropomyosin; aggregated paramyosin aPM; MW, molecular weight; 2D and 3D structures, secondary and three-dimensional structures; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ORF, open reading frame; BSA, bovine serum albumin; bp, base pair; IgE, immunoglobulin E; UV, ultraviolet; FTIR, Fourier transform infrared; CD, circular dichroism; DSC, digital signal controller

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