

Immunological Cross-Reactivity Involving Mollusc Species and Mite–Mollusc and Cross-Reactive Allergen PM Are Risk Factors of Mollusc Allergy

Chuang Yu, Xue Ding, Xiang Gao,* Hong Lin, Mati Ullah Khan, Hang Lin, Xuwen Dang, and Zhenxing Li*



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ABSTRACT: Marine molluscs are seafood consumed worldwide and could cause food allergies, while investigation on their sensitizing components and cross-reactivity seems to be rare. Furthermore, allergy to mites may result in anaphylaxis in mollusc-allergic individuals owing to their cross-reactivity. The aim of the study was to identify cross-reactive allergens and investigate the cross-reactivity between different mollusc groups and mite–mollusc. The extracted mollusc and dust mite proteins were separated by SDS-PAGE, and IgE-binding components were recognized by immunoblotting with sera from patients sensitized to mollusc and mite. Cross-reactivity of different mollusc groups and mite–mollusc was assessed using ELISA and inhibition ELISA. The results of the immune detection, ELISA, and inhibition ELISA indicated that different mollusc groups and mite–mollusc showed varying degrees of cross-reactivity. The most frequently recognized cross-reactive protein was paramyosin from different mollusc groups and dust mite, while cross-reactive allergen paramyosin in the mite extract was identified and evaluated by MS and Allermatch, respectively. Inhibition ELISA studies also revealed that paramyosin played an important role in molluscan and mite–molluscan cross-reactivity. These findings contribute to a better understanding of the cross-reactivity involving mollusc species and mite–mollusc, which can be used to assist in the diagnosis and treatment of mite- and mollusc-allergic disorders.

KEYWORDS: mollusc allergy, mite–mollusc, cross-reactivity, paramyosin

INTRODUCTION

Mollusc allergy is becoming an increasingly important public health issue in both children and adults because of the fact that molluscan shellfish have been currently designated as a commonly allergenic food in the European Union, Canada, and some coastal Asian countries,^{1,2} while molluscs are also often eaten as an added ingredient in some processed foods like sauces, garnishes, and sushi products.³ Marine molluscs are a major group of shellfish and, together with crustaceans, are the leading cause of shellfish allergy, with symptoms ranging from mild rashes and hives to life-threatening anaphylactic shock.⁴ Unfortunately, although numerous clinical cases of sensitized patients to mollusc are often reported, there are still no reliable clinical diagnostic approaches and specific immunotherapy of mollusc allergy due to undefined allergens and extensive immunological cross-reactivity with other allergen sources containing similar proteins in different species.^{5,6} Therefore, it is essential to further understand the allergic risks associated with the consumption of molluscan shellfish in order to inform at-risk populations in advance.

Cross-reactivity is an immune-mediated phenomenon in which the specific IgE antibody reacts not only to the original allergen but also to the homologous allergenic molecules. When one food allergen shares sufficient structural or sequence similarity with another food allergen or aeroallergen, epitopes on the second allergen are bound by cross-reactive antibodies, resulting in adverse reactions similar to those caused by

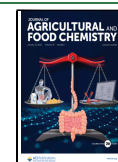
original food allergens.⁷ Cross-reactivity between the classes of mollusc shellfish, especially within the same group, is quite common, and patients with allergies to certain mollusc frequently show similar clinical allergic symptoms to other mollusc species,^{8–10} therefore suggesting that individuals with an allergic reaction to one mollusc species or evidence of IgE reactivity against that species should be counseled to avoid eating other molluscan shellfish.¹¹ Besides, cross-reactivity between dust mite aeroallergens and foods may also cause food allergies in some respiratory allergic patients. In particular, the allergic symptoms with chronic respiratory symptoms and severe asthmatic attacks triggered by snails invariably occur in patients with allergies to house dust mites (HDM, referring to the species *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), which has been called “mite-shellfish-mollusc-syndrome”.¹² Several studies have reported a strong correlation between dust mite sensitization and gastropod hypersensitivity (snails and limpets).^{13,14} However, cross-reactivity between molluscs has been studied less frequently than that of crustaceans, or even very little is known about cross-reactive

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allergens between mollusc species as well as mite–molluscs, despite the fact that molluscs are commonly consumed in Chinese coastal parts.

Cross-reactivity involves cross-reactive allergens that can trigger IgE antibody binding, while cross-reactive allergens are ubiquitous proteins that cause IgE cross-reactivity with different related and unrelated allergenic sources.¹⁵ Previous studies have indicated that tropomyosin (TM) was a major molluscan shellfish cross-reactive allergen responsible for clinical cross-reactivity not only within the mollusc groups but also with other invertebrates such as HDM and fish parasites (*Anisakis*).^{16,17} Some allergens found in shellfish, such as paramyosin (PM), arginine kinase (AK), hemocyanin, myosin heavy chain (MHC), amylase, and triosephosphate isomerase (TIM), may also play an essential part in cross-reactivity to seafood and dust mites as they share similar IgE-binding regions on these proteins, regardless of the fact that their clinical relevance and role in cross-reactivity are unspecified.¹⁸ PM is a 99 kDa allergen protein found in diverse invertebrates, such as abalone, sea snail, and dust mite.^{19–21} In fact, based on the results of molecular characteristics and structural homology, our recent findings suggested that the allergen PM was potentially responsible for cross-reactivity in mollusc and dust mite species.²² Furthermore, several non-TM allergens, including α -amylase and hemocyanin, were also found in molluscs and are possibly involved in cross-reactivity with HDM aeroallergens. α -Amylase (Der p 4) has also been described as an allergen that might be responsible for cross-reactivity between *D. pteronyssinus* and gastropods.^{3,14,23}

To date, no immunologic studies on the Chinese population of mollusc and associated dust mite allergic patients have been conducted. Immunological cross-reactivities within mollusc species are often observed in allergic individuals. Moreover, it is worth noting that HDM allergies can sometimes cause allergic reactions to molluscan-sensitized individuals, while HDM hypersensitivity may also occur after sensitization to molluscs.^{24,25} Furthermore, cross-reactivity with similar taxonomically related food allergens, such as gastropods, bivalves, and cephalopods, as well as inhalant allergens, such as HDM, might be accountable for the adverse reactions described after the ingestion of molluscs. The objective of this research was to identify the cross-reactive proteins responsible for mollusc allergy and to examine the extent of cross-reactivity among the mollusc species and mite–mollusc, in order to indicate whether allergic reactions in some mollusc-allergic patients might be induced by cross-reactivity among other molluscs and mite–mollusc and also to assess the potential risk of cross-reactivity with mollusc-allergic individuals.

MATERIALS AND METHODS

Subjects and Clinical Information. Sera were obtained from 17 individuals that attended an outpatient allergy clinic at the Affiliated Hospital of Qingdao University in Qingdao, China (Table 1). Serum from six subjects with positive ImmunoCAP values (>0.35 kU_A/L) to dust mite and nine subjects with a clinical history of shellfish allergy, and six of whom had positive Rap v 2 specific IgE values, were used in this study. Two control healthy volunteers' subjects were used. This study was approved by the local ethics committee (file no. QD-PF-20190618685, Ocean University of China, Qingdao, China) and written consent was obtained from each subject.

Molluscan Samples. Fresh specimens of 16 different molluscan species were acquired from local markets and distributors across Qingdao, China, and each molluscan species is categorized by

Table 1. Clinical Characteristics of Mollusc-Sensitized, Mite-Allergic, and Healthy Control Subjects^c

serum ID	sex ^a (age)	specific IgE value		clinical presentation	
		Der f specific IgE (kU _A /L)	Rap v 2 specific IgE (O.D.) ^d	symptoms with ^b	symptoms ^c
Dust Mite Allergic and/or Sensitized Subjects					
D1	35 (M)	66.9		DM	Ar, U
D2	13 (M)	44.3		DM	U, V&D
D3	24 (M)	>100		DM	Ar
D4	28 (M)	>100		DM	Ar
D5	26 (F)	21.5		DM	Ar
D6	32 (F)	86.3		DM	Ar
Self-Reported Molluscan Shellfish Allergic and/or Sensitized Subjects					
M1	F (50)		1.036	SS	As, Ar, Dz, Uc,
M2	46 (M)		1.065	SS	As, Fe, Dz, Uc
M3	30 (F)		0.741	SS, DM	U, A
M4	24 (M)		0.728	SS	U, V&D, A
M5	49 (M)		0.640	SS	U, A
M6	16 (M)		1.124	SS, F	Ar, U, A
M7	36 (F)		ND	CL, O	U, Ar
M8	69 (M)		ND	CL, S	U, AD
M9	28 (M)		ND	CU	U, Ar
Healthy Individual Control Subjects					
C1	36 (F)			none	none
C2	26 (M)			none	none

^aM, male; F, female. ^bSS: sea snail; F: fish; DM: dust mite; CL: clam; O: oyster; S: scallop; CU: cuttlefish. ^cAr: allergic rhinitis; U: urticaria; Dz: dizziness; Uc: unconsciousness; As: anaphylactic shock; A: asthma; Fe: facial edema; AD: atopic dermatitis; V&D: vomiting and diarrhea. ^dNegative control for specific IgE (sera from healthy individuals): OD 0.16–0.31. The levels of IgE immunoglobulin against Rap v 2 protein were significantly increased in sea snail-allergic patients compared to a negative control. ^eND: not determined/unknown.

morphological characteristics, as listed in Table 2 and Figure S1. Columellar muscle was obtained from sea snail and abalone, adductor muscle from the scallop, clam, oyster, razor shell and cockle, mantle muscle from cuttlefish, and leg muscle from octopus. All muscle samples for extraction were stored at -80 °C prior to further use.

Different Mollusc and House Dust Mite (*D. farinae*) Extract. Each muscle sample of mollusc was cut into small pieces and homogenized with four volumes of 0.5 M NaCl in 0.01 M phosphate buffer (pH 6.8) overnight with constant mixing, followed by centrifugation at 12,000 rpm $\times$ 4 °C for 30 min, and the supernatant was clarified, filter sterilized, and stored at -80 °C in aliquots. The *D. farinae* extract was purchased from a commercial bio-reagent corporation (Biolead Biology Sci & Tech. Co. Ltd., Beijing, China). The total protein concentration of each extract was determined using a Bradford assay kit (Solarbio, Beijing, China) using bovine serum albumin (BSA) as a standard.

Table 2. on 16 Molluscan Species Used in This Study

common name	species	family	order	classification
sea snail	<i>Rapana venosa</i>	Muricidae	Stenoglossa	Gastropod
sea snail	<i>Turritella bacillum</i>	Turritellidae	Turritellinae	Gastropod
sea snail	<i>Reishia clavigera</i>	Muricidae	Neogastropoda	Gastropod
zoned horn snail	<i>Batillaria zonalis</i>	Batillariidae	Neotaenioglossa	Gastropod
bladder moon snail	<i>Neverita didyma</i>	Naticidae	Littorinimorpha	Gastropod
nassa mud snail	<i>Nassarius variciferus</i>	Nassariidae	Neogastropoda	Gastropod
abalone	<i>Haliotis discus hannah</i>	Haliotidae	Archaeogastropoda	Gastropod
scallop	<i>Argopecten irradians</i>	Pectinidae	Anisomyaria	Bivalvia
clam	<i>Ruditapes philippinarum</i>	Veneridae	Veneroida	Bivalvia
oyster	<i>Crassostrea gigas</i>	Ostreidae	Ostreida	Bivalvia
clam	<i>Macra antiquata</i>	Mactridae	Veneroida	Bivalvia
clam	<i>Dosinorbis japonica</i>	Veneridae	Venerida	Bivalvia
razor shell	<i>Solen strictus</i>	Solenidae	Adapedonta	Bivalvia
cockle	<i>Tegillarca granosa</i>	Arcidae	Arcida	Bivalvia
octopus	<i>Amphioctopus fangsiao</i>	Octopodidae	Octopoda	Cephalopod
cuttlefish	<i>Loligo japonica</i>	Sepiariidae	Sepiida	Cephalopod

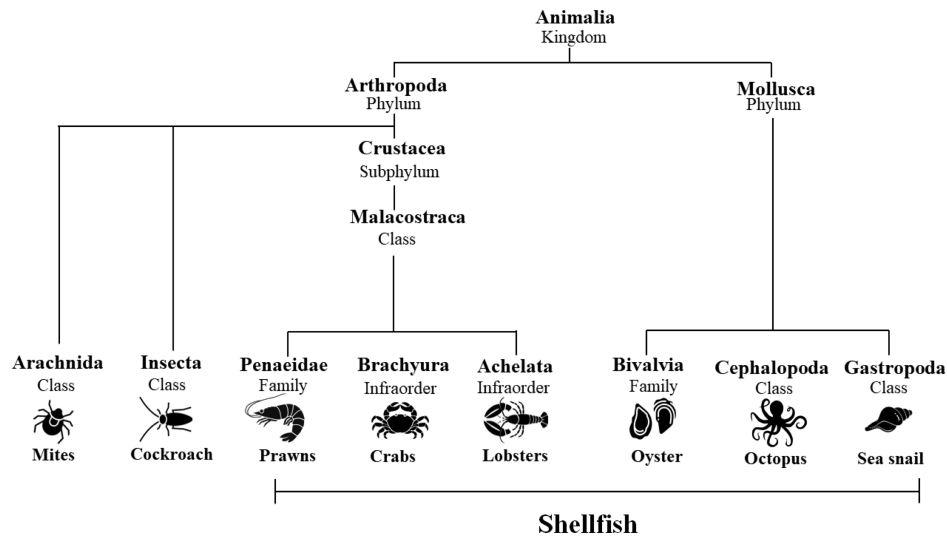


Figure 1. Taxonomic tree of edible crustacean and molluscan shellfish species and related invertebrates.

SDS-PAGE Analysis and Immunoblotting. SDS-PAGE was performed to separate different molluscs and mite extract, as described previously.²⁰ Each sample (10 µg/well) was mixed in a 1/4 ratio with bromophenol blue buffer, heated at 100 °C for 7 min, and subjected to electrophoresis using 4–12% Bis–Tris gels. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Richmond, CA, USA) or transferred onto PVDF membranes (Thermo Scientific, Waltham, WA, USA). For immune-detection of IgE binding proteins, immunoblotting of separated proteins was performed as described using patient sera diluted in 5% non-fat dry milk and 1% BSA in PBST (0.1% Tween 20, 1× PBS, pH 7.4).²⁰ Patient sera was diluted 1:4 followed by 1:2000 HRP-labeled goat anti-human IgE. Blots were incubated with an ECL chemiluminescent substrate and detected by ChemiDoc XRS+ (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to visualize the antibody-binding protein bands.

Mass Spectrometry. Mass spectrometry (MS) identification of proteins was performed using the method described previously.²⁶ Briefly, the protein bands corresponding to highly IgE-reactive proteins of mollusc and mite extracts were excised from SDS-PAGE gels, then pellet reduced, alkylated, and digested with trypsin for MALDI-TOF/MS. The MS data were extracted and exported in Mascot generic file format, the proteins were identified based on the National Center for Biotechnology Information non-redundant database (NCBInr) using the Matrix Science (MASCOT) search

engine (<http://www.matrixscience.com>) with all taxonomy selected. Mass spectra were acquired using a Bruker Ultraflex MALDI-TOF/TOF-MS (Bruker Daltonics GmbH, Bremen, Germany).

Purification of Natural PM (Rap v 2). Rap v 2 in the *Rapana venosa* muscle was purified from the myofibrillar protein fraction by salting out (10–20% saturation of ammonium sulfate) and further isolated by a ceramic hydroxyapatite type I column (Bio-Rad Laboratories, Hercules, CA), as reported previously.²⁰ The homogeneity and IgG/IgE binding activity of the purified preparation was supported by SDS-PAGE analysis and immunoblotting.

ELISA and Inhibition ELISA Assays. IgE ELISA was performed as reported previously.²⁶ Briefly, a 96-well ELISA plate were coated with 5 µg of different mollusc extracts (including six different gastropod molluscs, seven different bivalve molluscs, and two different cephalopod molluscs), and then detected IgE cross-reactivity between different molluscs using sea snail-sensitized sera diluted 1:10 and goat anti-human IgE-HRP diluted 1:4000 in 1% non-fat dry milk/0.1% Tween/PBS, respectively. For the inhibition of ELISA, an inhibition-ELISA was performed to analyze the IgG cross-reactivity to PM in the various mollusc extracts. Rabbit Anti-Rap v 2 polyclonal antibody (pAb) (diluted 1:5000) was pre-incubated with increasing concentration of inhibitors (cuttlefish, octopus, four various sea snail, abalone, oyster, clam, and cockle) solution (1–300 µg/mL) used as a primary antibody. In addition, IgE inhibition ELISA assays were carried out with sea snail-allergic and/or mite-allergic patients, using

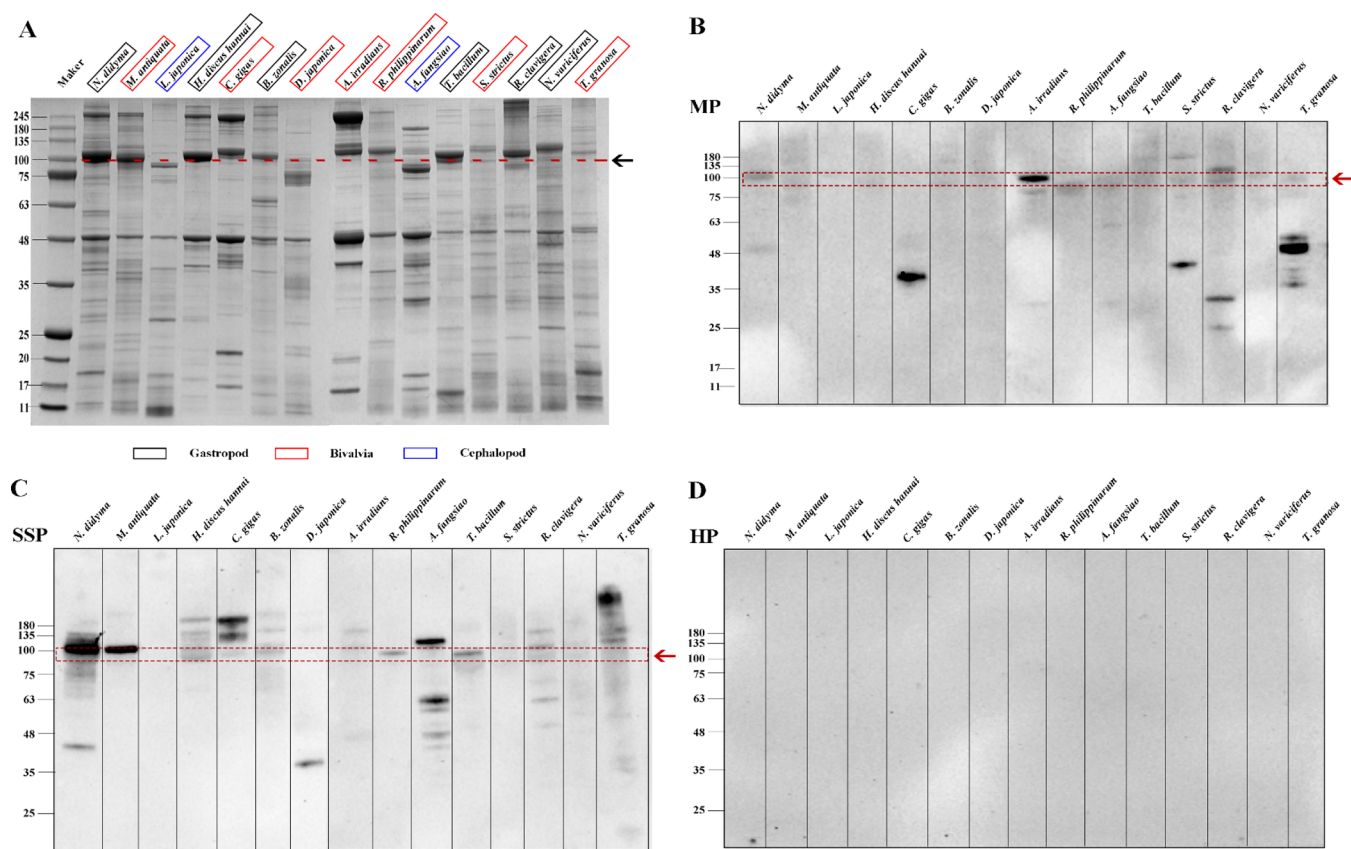


Figure 2. Analysis of SDS-PAGE of 15 mollusc extracts and IgE cross-reactivity with mollusc-sensitized patients. (A) SDS-PAGE of 15 different mollusc protein extracts. (B) IgE immunoblotting of the mollusc-sensitized patient sera pool (M6, M7, M8, and M9) to 15 different mollusc protein extracts. (C) IgE immunoblotting of the sea snail-sensitized patient sera pool (M2, M3, and M4) to 15 different mollusc protein extracts. (D) IgE immunoblotting of a healthy person sera pool (C1 and C2) to 15 different mollusc protein extracts. Fifteen different mollusc extracts, *N. didyma* protein extract; *M. antiquata* protein extract; *L. japonica* protein extract; *H. discus hannai* protein extract; *C. gigas* protein extract; *B. zonalis* protein extract; *D. japonica* protein extract; *A. irradians* protein extract; *R. philippinarum* protein extract; *A. fangsiao* protein extract; *T. bacillum* protein extract; *S. strictus* protein extract; *R. clavigera* protein extract; *N. variciferus* protein extract; *T. granosa* protein extract. MP, mollusc-sensitized patients; SSP, sea snail-sensitized patients; and HP, healthy individuals. Long arrows indicate PM.

Rap v 2 and/or dust mite extract as the solid phase, sea snail-allergic and/or mite-allergic human serum diluted five times was mixed with an equal volume of different mollusc extracts or/and Rap v 2 inhibitors for pre-incubation used as the primary antibody. Then, for both IgG and IgE inhibition ELISA, the HRP-conjugated goat anti-rabbit IgG (diluted 1:10,000) HRP-conjugated goat anti-human IgE (diluted 1:4000) acted as the secondary antibody to assess the relation of different mollusc and mollusc-mite cross-reactivity. Both ELISAs and inhibition ELISAs were carried out in triplicate, and the results were presented as means $\pm$ standard deviation (SD).

Analysis of the Amino Acid Sequence of Different Mollusc PM Alignment. To predict the capture antibody binding characteristics to mollusc PM, a protein sequence alignment was performed to compare different molluscs PM using the Multiple Sequence Alignment in Clustal Omega (EMBL-EBI). Evolutionary analyses relatedness to PM proteins were conducted in MEGA6 as reported previously.²⁰ This provides the bioinformatics data required to perform the cross-reactivity assessment studies.

Determination of the Allergenic Potential. The allergic potential of the identified cross-reactive allergen in HDM extracts was predicted using the Allermatch webtool (<http://www.allermatch.org>). A comparison of allergenic proteins in Allermatch is based on Comprehensive Protein Allergen Resource, UniProt, as well as WHO-IUIS allergen databases. The search was only performed on allergenic proteins in the 80 amino acid sliding window alignments that had more than 35% of identity in the window.²⁷

Statistical Analysis. The Mann–Whitney *U* test in conjunction with an ANOVA unpaired *t*-test was used to compare serum IgE

reactivity and overall IgG/IgE inhibition ELISA assay, and Spearman's correlation test was used to assess the correlation between individual specific IgG/IgE ELISA against different mollusc or mite extracts and allergen Rap v 2. At a *p* value of <0.05, differences were considered significant. Statistical analyses were performed using GraphPad Prism version 8.0 software (GraphPad, San Diego, CA).

RESULTS

Taxonomic Correlation between Shellfish and House Dust Mite. The marine shellfish, which causes an allergic reaction, typically refers to the two invertebrate phyla of molluscs and crustaceans (Figure 1). Marine molluscs can be classified into the three classes of bivalves, cephalopods, and gastropods, including several important consumed species such as sea snail, abalone, scallop, oyster, squid, and octopus, which belong to the phylum Mollusca. Crustaceans are, including prawn, crab, and lobster, classified as the phylum Arthropoda together with arachnids and insects. Cross-reactive allergens are homologous molecules that originate from numerous organisms and cause cross-reactivity between the same or different evolutionary species. Cross-reactivity between several cross-reactive allergens including TM and hemocyanin present in shellfish, HDM, and edible insects is frequently observed. In general, IgE cross-reactivities can occur between allergenic molecules in closely related species or well-preserved

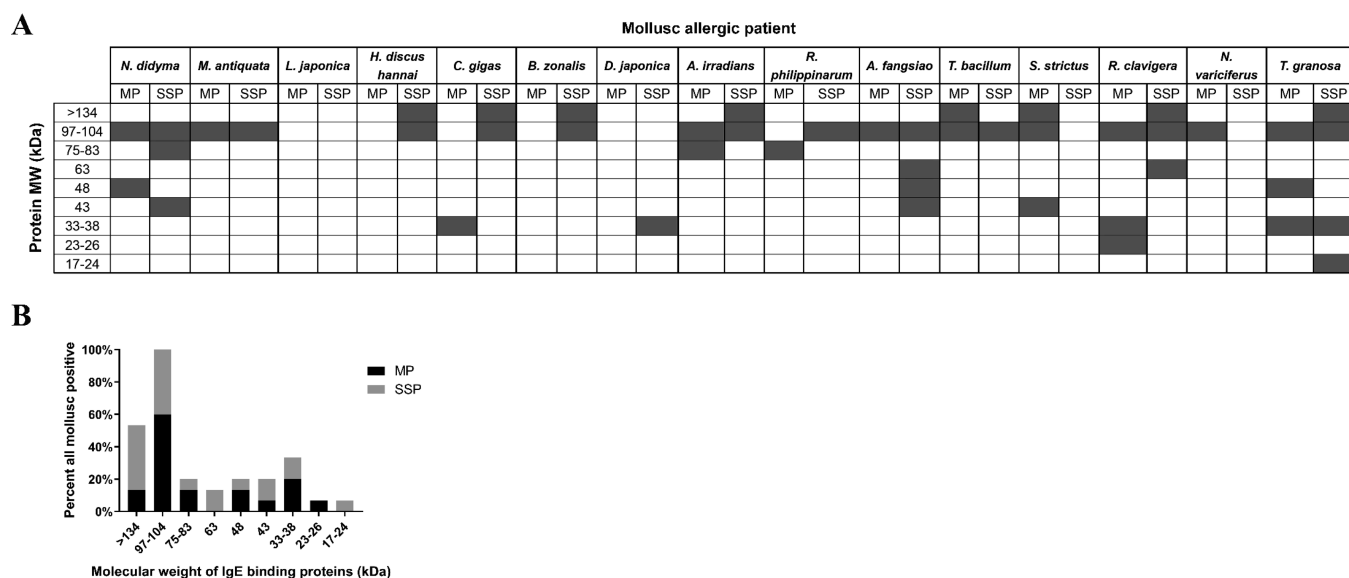


Figure 3. Allergogram analysis of IgE reactivity by an immunoblot to proteins in different mollusc extracts. (A) Panels show specific IgE-binding proteins from different mollusc groups. (B) Percentage of all mollusc reactivity toward different IgE-binding proteins. Filled portions represent positive proteins of IgE recognition to MP and SSP, respectively.

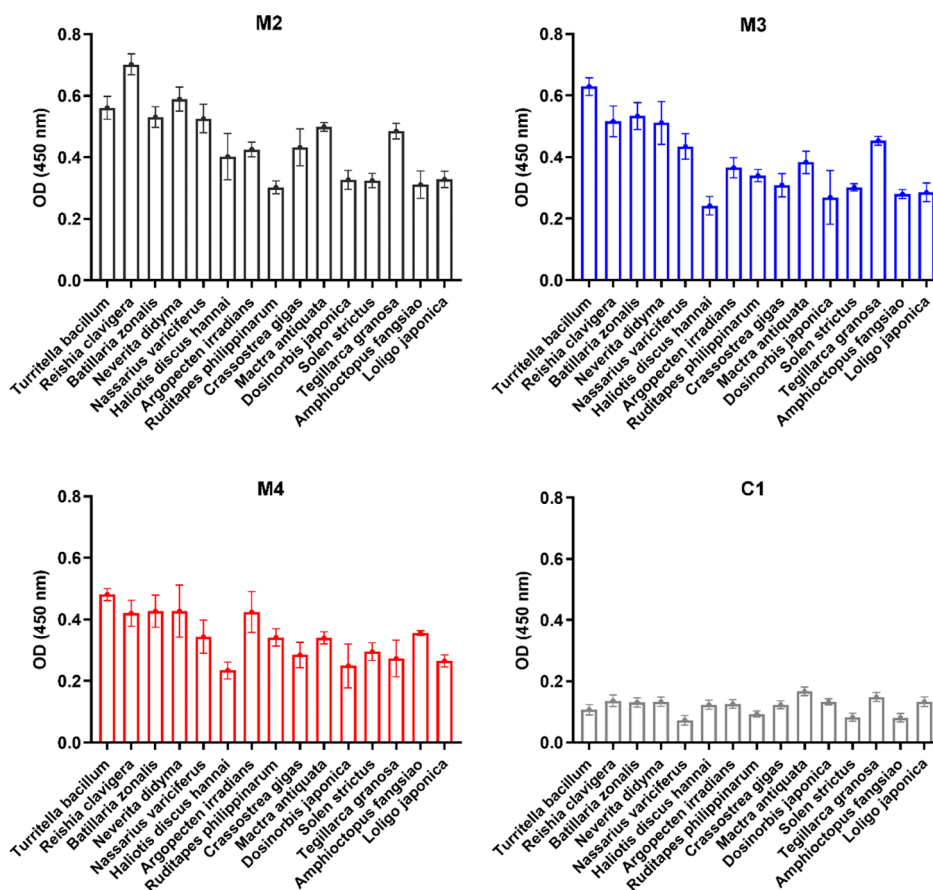


Figure 4. Indirect IgE ELISA analysis of different mollusc species. IgE ELISA assays were performed on 15 different mollusc protein extracts with 3 sea-snail-sensitized patients' sera (M2, M3, and M4) and a negative serum (C1).

molecules with similar functions present in a wide range of different species. An evolutionary proximity between species and common allergens found among them might provide an explanation for the clinical cross-reactivity, which suggests that

some patients with a mollusc or HDM allergy may experience allergic reactions upon the consumption of other molluscs.

SDS-PAGE and IgE Reactivity to Mollusc Extracts.

Analysis of diverse mollusc extracts by SDS-PAGE revealed several protein bands with a molecular weight (MW) ranging

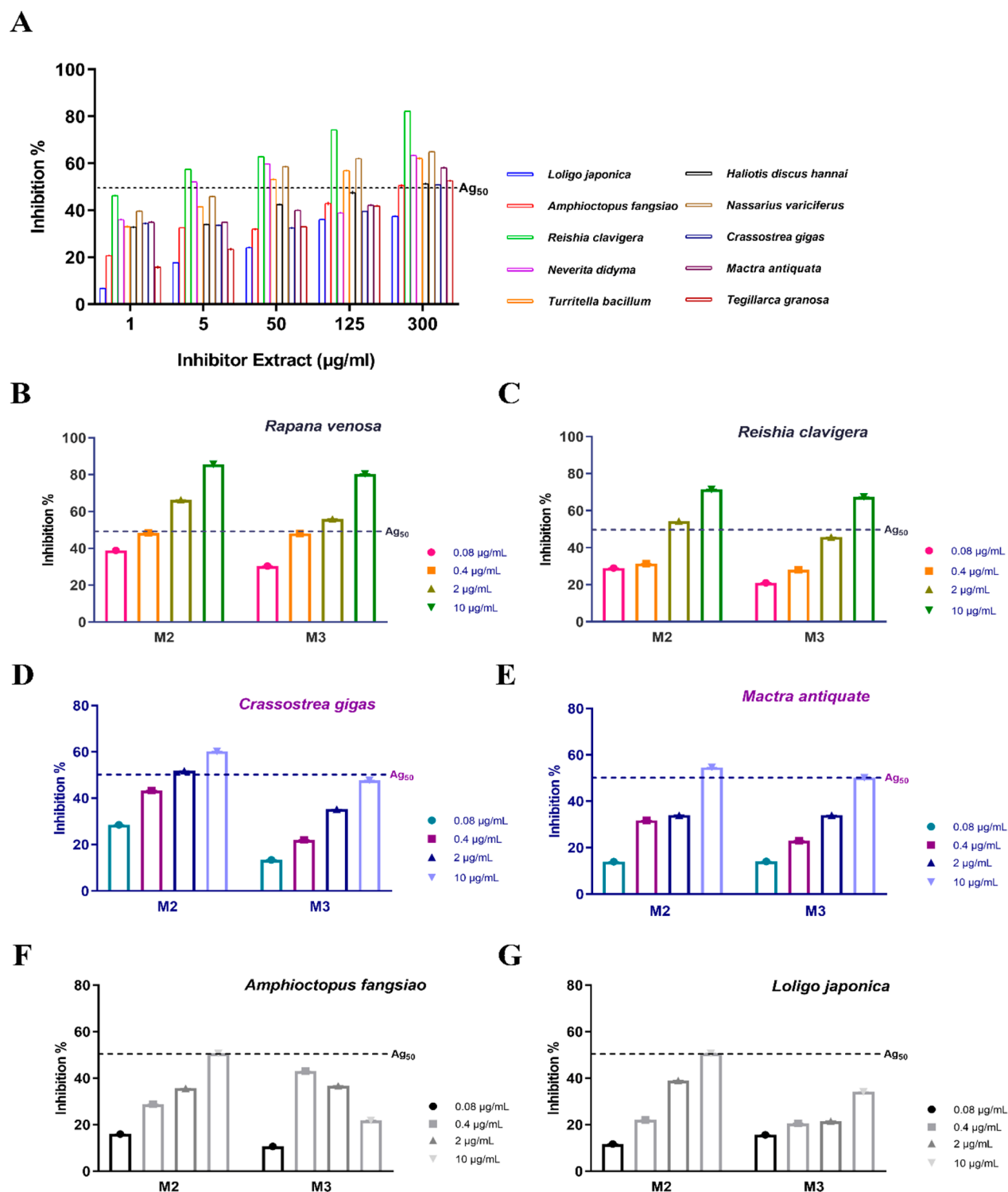


Figure 5. IgG/IgE inhibition ELISA analysis of different mollusc species. (A) IgG inhibition ELISA for the evaluation of anti-Rap v 2 pAb cross-reactivity against different mollusc protein extracts. Inhibition of IgE reactivity to Rap v 2. Sera from two sea snail-allergic patients (M2 and M3) were incubated with an increasing concentration of mollusc inhibitors (B, *R. venosa* protein extract; C, *R. clavigera* protein extract; D, *C. gigas* protein extract; E, *M. antiquate* protein extract; F, *A. fangsiao* protein extract; and G, *L. japonica* protein extract).

from 10 to at least 245 kDa (Figure 2A). Profiles of protein extracts differ obviously from different mollusc extract groups including gastropod, bivalves, and cephalopod. Overall, except for mollusc *Dosinorbis japonica*, the MW protein bands ranging from 97–104 and 48 to 33–38 kDa were similar for gastropod and bivalve groups, but protein profiles with a MW below the 25 kDa region were different in those of mollusc extracts.

Moreover, compared with the other two mollusc groups, the distribution and characteristics of the protein bands of *Amphioctopus fangsiao* and *Loligo japonica* belonging to the cephalopod are more closer, *L. japonica* was weaker than *A. fangsiao* in terms of the intensity of the protein bands.

IgE-reactive proteins of each mollusc extracts were examined by immunoblotting using samples from mollusc- and specific

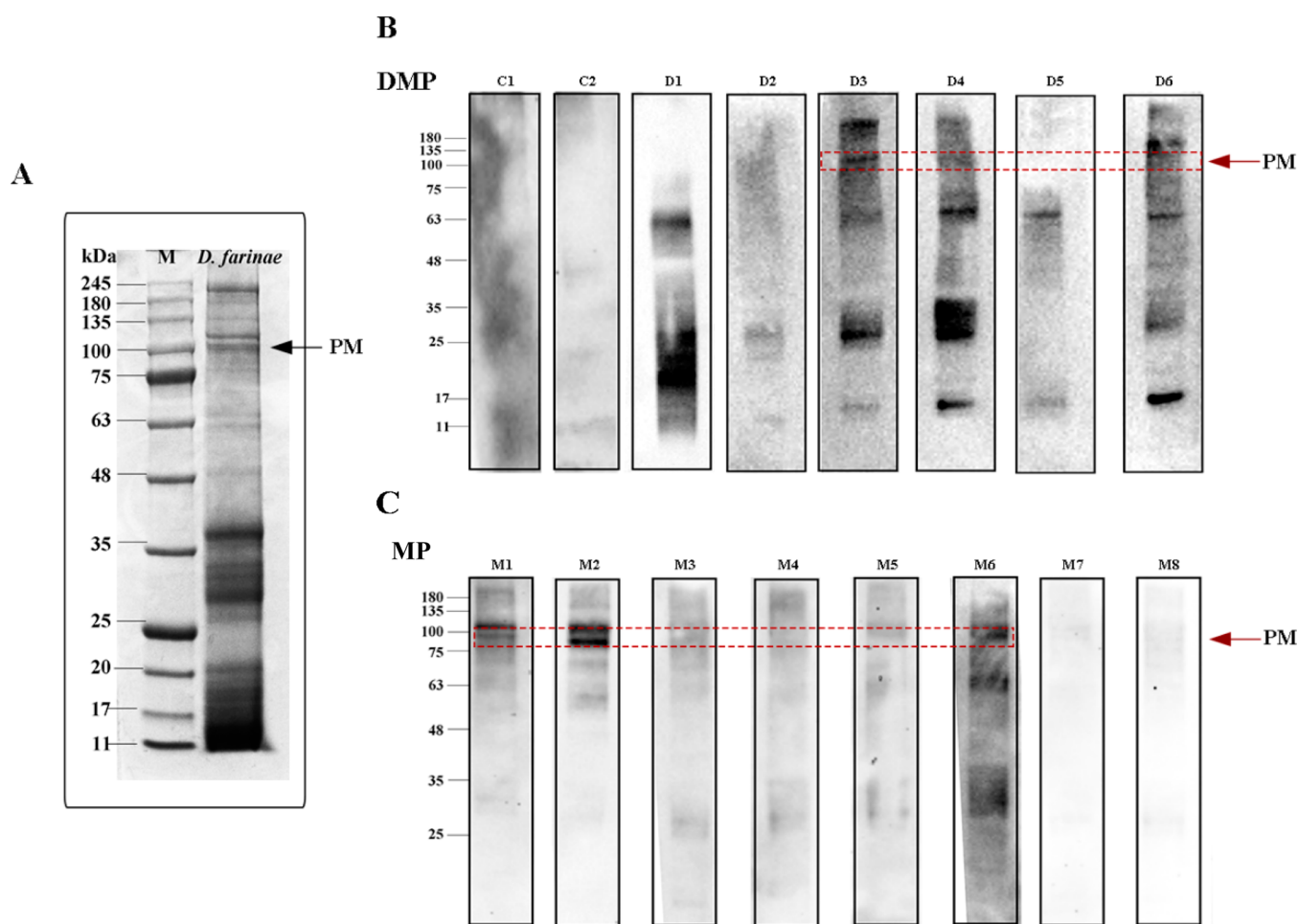


Figure 6. Analysis of the *D. farinae* protein extract and IgE reactivity with dust mite and mollusc allergic patients. (A) SDS-PAGE of the *D. farinae* protein extract. (B) IgE immunoassay of dust mite allergic patient sera (DMP, D1 to D6; HP, C1 to C2) to the *D. farinae* protein extract. (C) IgE immunoassay of mollusc-sensitized patient sera (MP, M1 to M8) to the *D. farinae* protein extract. DMP, dust mite-sensitized patients; MP, mollusc-sensitized patients; HP, healthy individuals.

sea snail-sensitized patients (Figure 2B,C). The results are summarized in allergograms to analyze the IgE recognition data from these sera (Figure 3A,B). A pooled serum from mollusc-sensitized patients (M6, M7, M8, and M9) revealed significant IgE reactive bands in all three mollusc groups. The most frequently recognized band was observed for the protein with a MW between 97 and 104 kDa (PM, 60% in all molluscs), but there was a weaker intensity of IgE binding within proteins. Besides that, several strongly IgE-reactive protein bands were found in 33–38 kDa (TM, 20% in all molluscs) and 48 kDa regions (13% in all molluscs) in mollusc species. On the other hand, further analysis of the cross-reactive proteins between gastropod, bivalve, and cephalopod three mollusc groups was performed by immunoblotting against all mollusc extracts using a sea snail-sensitized pooled serum (M2, M3, and M4). Interestingly, the IgE binding recognized more proteins and more intensity in specific sea snail-sensitized serum (SSP) than in mollusc-sensitized serum (MP). The IgE-reactive bands in the PM region were recognized at a high frequency (73% in all molluscs), especially in the gastropod extracts. Moreover, there were also other IgE-binding proteins detected in 33–38 kDa (TM, 13% in all molluscs), 43 kDa (AK, 13% in all molluscs), 63 kDa (13% in all molluscs), and so forth. In addition, there was no significant

IgE binding to any of the mollusc extracts for a non-atopic control serum (Figure 2D).

Different Mollusc Cross-Reactivity Study. IgE cross-reactivity was determined by using the IgE ELISA assays for different mollusc extracts with sera from three sea snail allergic patients (M2, M3, and M4). As indicated in Figure 4, IgE reactivity of different mollusc extracts with certain levels of IgE binding is observed in all three sea snail allergic patients. As expected, the level of IgE binding was generally the highest when coating extract species were the same as the serum from the species. Gastropod extracts showed higher IgE cross-reactivity than other mollusc extracts and the maximal IgE reactivity to *Turritella bacillum*, *Reishia clavigera*, *Batillaria zonalis*, and *Neverita didyma* was observed in three sea snail allergic patients, respectively. M2 and M3 showed a lower IgE reactivity to cephalopod extracts, including *A. fangsiao* and *L. japonica* extracts, than mostly bivalve species extracts, but M4 had higher IgE reactivity to the *A. fangsiao*'s extract than some bivalve species extracts. Minimal IgE binding was obtained with the non-atopic control sera (C1). In total, sea snail allergic patients consistently showed higher IgE reactivity with mollusc extracts from gastropod species than other mollusc groups.

PM Cross-Reactivity among Different Mollusc Species. The cross-reactive protein MW at 97–104 kDa in the

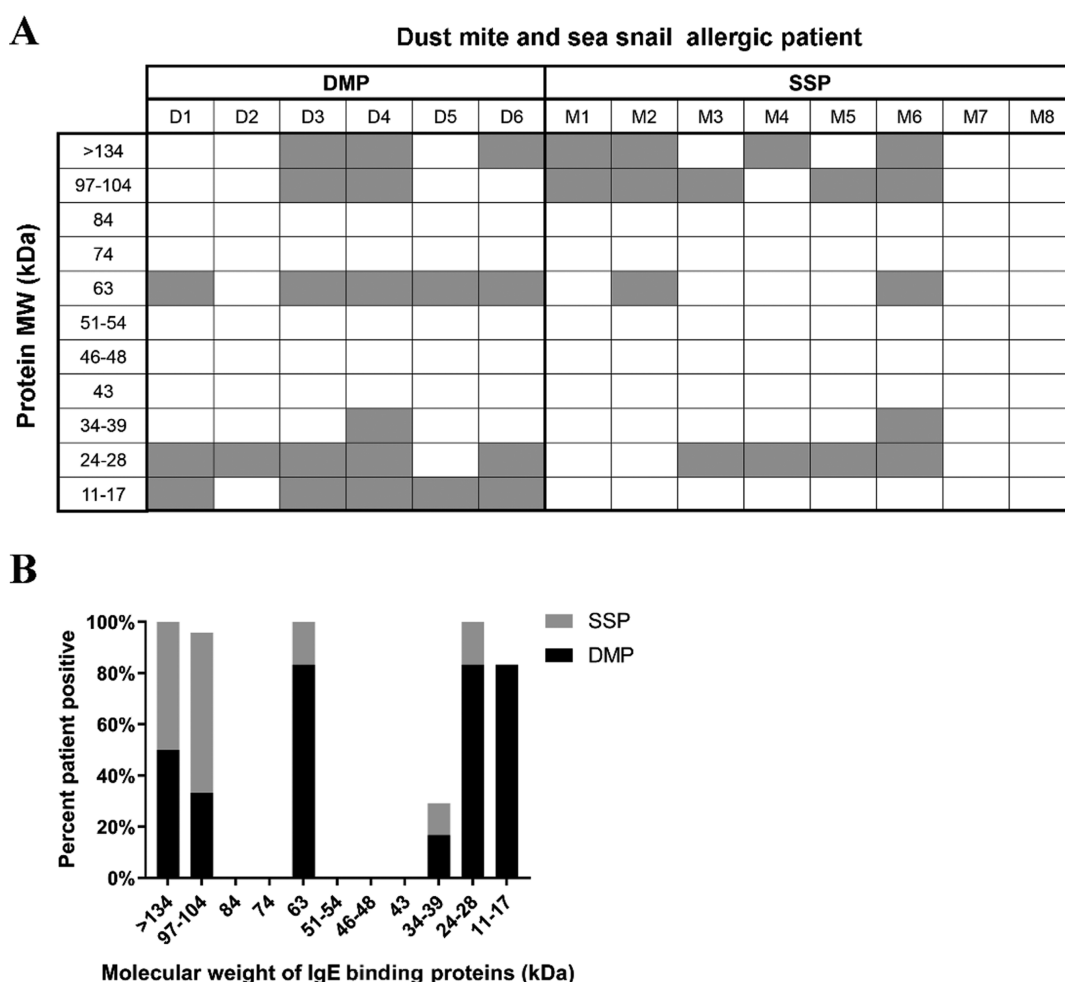


Figure 7. Allergogram analysis of IgE reactivity by an immunoblot to a *D. farinae* protein extract. (A) Specific IgE-binding proteins from dust mite *D. farinae*. (B) Percentage of patients with reactivity to IgE-binding *D. farinae* protein. Filled portions represent allergens recognized by DMP and MP.

PM region was frequently observed by IgE immunoblotting. Inhibition IgG ELISA further validated the results of immunoblotting, and the ability of diverse mollusc extracts to inhibit IgG binding was assessed by using polyclonal antiserum (Figure 5A). All mollusc extracts caused an apparent dose-dependent inhibition of the anti-Rap v 2 antibody, and besides inhibitor *L. japonica*, the inhibition rates were more than 50% at a maximum concentration of inhibitors (300 μ g/mL). In addition, inhibition IgE ELISA was used to further assess the cross-reactivity between sea snail PM Rap v 2 and different mollusc extracts using Rap v 2-sensitized serum based on the result of IgE ELISA assays. Rap v 2-sensitized serum (M1 and M2) were pre-incubated with different mollusc extracts and examined for IgE reactivity with allergen Rap v 2, maximum inhibitions of IgE reactivity reached 50% inhibition at three group mollusc extracts in the assays. Rap v 2 exceeding 65% inhibition was obtained at a final concentration of 10 μ g/mL of *R. venosa* and *R. clavigera* extracts in two sea snail allergic patients, indicating that gastropod extracts are significantly cross-reactive (Figure 5B,C). Besides that, bivalve extracts (*Crassostrea gigas* and *Macrura antiquata*) showed a weaker inhibition of IgE reactivity to Rap v 2 (45–60%) at the maximum inhibition (Figure 5D,E); however, cephalopod extracts only showed a limited inhibition of IgE reactivity (21–51%) at a maximum concentration of inhibitors (Figure 5F,G).

IgG/IgE cross-reactivity to mollusc PM may be attributed to molecular differences in the primary structure of PM among different mollusc groups (Figure S2A). The gastropods' PM were closely related to the *R. venosa* PM with >70% sequence identity. However, when compared to bivalve and cephalopods' PM, the percent of sequence identity was only ranged between 64.69 and 69.08% due to relatively distant phylogenetic relationships (Figure S2B,C).

SDS-PAGE and IgE Reactivity to Dust Mite Extract.

The electrophoretic separation of the mite protein extract from *D. farinae* was determined by SDS-PAGE, which showed several bands ranging from 11 to 245 kDa (Figure 6A). All six dust mite allergic patients (D1 to D6) were tested by immunoblotting and reacted with positive IgE reactivity to the *D. farinae* protein extract (Figure 6B). As shown in allergograms, the immunoblotting of mite allergic sera demonstrated positive IgE reactivity at the 11–17 (like epididymal protein), 24–28 (cysteine protease), and 63 kDa (α -amylase) protein bands along with a little band with MW ranging from >134, 34–39 (TM) and 97–104 kDa (PM). Five out of six patients (83%) showed positive signals toward *D. farinae* proteins like epididymal protein, cysteine protease, and α -amylase regions. In addition, no positive band to the *D. farinae* protein extract was detected when using non-allergic control sera (C1 and C2).

Identification and Analysis of Cross-Reactive Protein PM Involved in the Cross-Reactivity of Mite–Mollusc.

Additional immunoblotting was conducted using a mollusc-sensitized serum to identify IgE-binding components involved in cross-reactivity between *D. farinae* and mollusc species (Figure 6C). Multiple cross-reactive bands (at 24–28, 34–39, 63, 97–104, and >135 kDa) were detected in the *D. farinae* protein extract (Figure 7A). Two (33%) dust mite-sensitized and five (62%) sea snail-sensitized patients demonstrated IgE binding to PM in the 97–104 kDa regions (Figure 7B). The MW at 98 kDa of the most frequently recognized cross-reactive bands from the dust mite extract was selected and excised from SDS-PAGE gel to further characterize proteins by MS. Table 3

Table 3. Start and End Position Numbers, Calculated Molecular Weights, and Amino Acid Sequences of Peptides Identified for the 98 kDa IgE Cross-Reactive Band in the *D. farinae* Extract Using Mass Spectrometry

MW of protein from SDS-PAGE	start–end	Mr, calcd	peptide sequence
98 kDa	34–53	1947.90	RLEDKIRLLSDDLESEREMR
	60–71	1375.57	KAELQIQVMSLG
	74–89	1738.76	LEEAEGSSESVTENMK
	100–117	2155.10	KLLEDVHIESEETAHHLR
	119–136	2022.98	KHQAAIQEMQDQLDQLQK
	172–186	1827.97	NVEKLEYTVHELNIK
	204–219	1895.00	LSQENTELIKEVHEVK
	220–229	1164.63	LQLDNANHLK
	230–240	1301.66	TQIAQQLEDTR
	297–315	2116.01	SKYEAELQAHADVEVELRR
	321–334	1677.85	ISEYEEQLEALLNK
	364–381	2074.43	QQLEKRVAQLEKINLDLK
	382–396	1790.89	NKLEEVTMLMEQAQK
	428–437	1102.59	KLTDLLAEAK
	446–455	1137.60	RIHEQEIEIK
	456–469	1692.84	RLENERDELSAAYK
	485–493	1011.61	LIAELAQVR
	500–512	1541.84	LAQKDEIEALRK
	513–524	1691.87	KQYQIEIEQLNMR
	589–602	1582.84	QLQQAQVQLGVTQR
	604–615	1476.67	CQALQAELEEMR
	616–623	913.50	IALEQANR
	627–638	1407.71	QAEQLHEEAVVR
	639–653	1585.87	VNELTTINVNLASAK
	656–672	1979.91	LESEFSALQADYDEVHK
	683–689	715.45	KLTIELK
	693–701	1143.58	DLLEEQR
	723–736	1299.67	IEEVEANALAGGKR
	745–757	1514.76	IRDVEIEVEEERR
	773–785	1593.84	VKELLQNEEDHK
	786–800	1829.96	QIQLQEMTDKLNK
	807–821	1822.79	QMMEQEGMSQQNLTR

describes the results of identified effective peptides mapped to the NCBI peptide database from *D. farinae*, the matched peptides had over 95% identity with aligned amino acid sequences of PM from *D. farinae* (GenBank: AIO08864.1). Briefly, the mass spectrometry analysis identified a high frequently cross-reactive protein in the *Dermatophagoides farinae* extract: a band of 98 kDa as cross-reactive protein Der f 11 (PM). Besides, the allergenic potential of the identified cross-reactive protein was predicted using Allermatch. A

protein can be considered a potentially cross-reactive allergen when it shows more than 35% identity with a known allergen within a window of 80 amino acids or more. The results of Table 4 indicate that the identified PM could be a potentially cross-reactive allergen based on the above-mentioned criteria. Der f 11 matches the corresponding three mite species and two gastropod mollusc allergen PM, with a sequence identity between 36 and 97.9%. None of PM had been previously reported as a cross-reactive allergen between the mite and mollusc.

Mite–Mollusc Cross-Reactivity Study. To validate the IgE cross-reactivity between three mollusc species and *D. farinae* mite, an inhibition ELISA was performed, using dust mite-allergic patient serum. Sera were pre-incubated with sea snail (*R. clavigera*), oyster (*C. gigas*), and octopus (*A. fangsiao*) protein extracts and *R. venosa* PM (Rap v 2) as inhibitors, respectively, and determined to immunological cross-reactivity with the *D. farinae* protein extract. In inhibition ELISA shown in Figure 8, we observed a tendency for a more pronounced IgE reactivity to sea snail than to the other two molluscs (oyster and octopus). Only in D4 among the four serum subjects, all the dust-mite extract inhibited >50% serum IgE reactivity to the sea snail, oyster, and octopus at the highest concentration (50 μ g/mL). However, the dust mite extract effectively also inhibited the reaction to the sea snail even for the serum subject from D1 and D3, indicating sea snail extracts showed a greater inhibition of IgE binding than oyster and octopus extracts (Figure 8A–C).

PM Cross-Reactivity between *D. farinae* and Sea Snail. On the other hand, IgE cross-reactivity among the *D. farinae* extract and sea snail PM Rap v 2 was further assessed by inhibition ELISA using Rap v 2-sensitized sera based on the results of the above studies (Figure 8D). In the case of serum subjects from patients D1, D3, and D4, Rap v 2 and *R. clavigera* extracts as inhibitors produced very similar inhibition profiles, with the percentage inhibition of IgE binding to the dust mite extract being 50.69, 59.37, and 53.91%, respectively, at the highest concentrations of the inhibitor used.

IgE inhibition ELISA assays to the *D. farinae* protein extract. Four dust mite-sensitized patients' serum (D1, D2, D3, and D4) were mixed with an increasing concentration of inhibitors (A, *R. clavigera*; B, *C. gigas*; C, *A. fangsiao*; and D, Rap v 2) for pre-incubation.

DISCUSSION

Marine mollusc are common allergenic foods that cause clinical food allergy.²⁸ Clinical cross-reactivity among molluscs is common, similar to crustaceans, due to cross-sensitization within the same category, as seen in mollusc allergic individuals.²⁹ Research has shown that 50% of patients with mollusc allergy are estimated to be allergic to food in the same category, 10–20% of patients with mollusc allergy will react to other animals in the other categories.^{30,31} The second well-known cross-reactivity is between dust mites and molluscs. It has been speculated that a larger prevalence of shellfish sensitization is considerably connected with HDM sensitization and allergy through cross-reactivity among HDM and shellfish TM.^{32,33} However, this does not seem to rely on allergen TM as only 7% of patients in one study showed IgE binding to TM; therefore, other molluscan allergens may play a role in cross-sensitization except for TM.³⁴ Hitherto, numerous mollusc allergens have not been identified and characterized yet, and few studies have been conducted to investigate cross-

Table 4. Cross-Reactive Allergen PM in HDM Analyzed by means of Allermatch^a

protein	allergen	sequence identity in Allermatch				
		80 AA sliding window analysis		full sequence alignment		E
		# hits >35% identity	% hits >35% identity	%	overlap (AA)	
PM	Der p 11	797	100	97.90	876	0
	Blo t 11	797	100	89.8	876	0
	Ani s 2	796	99.87	50.90	871	1.10×10^{-182}
	Rap v 2	541	67.88	37.4	840	2.10×10^{-113}
	<i>H. discusdiscus</i> PM	376	47.18	36	842	1.50×10^{-107}

^aExpected value (E) describes the number of hits one can expect to see by chance when searching a database of specific size. Der p (*Dermatophagoides pteronyssinus*); Blo t (*Blomia tropicalis*); Ani s (*Anisakis simplex*); Rap v (*Rapana venosa*).

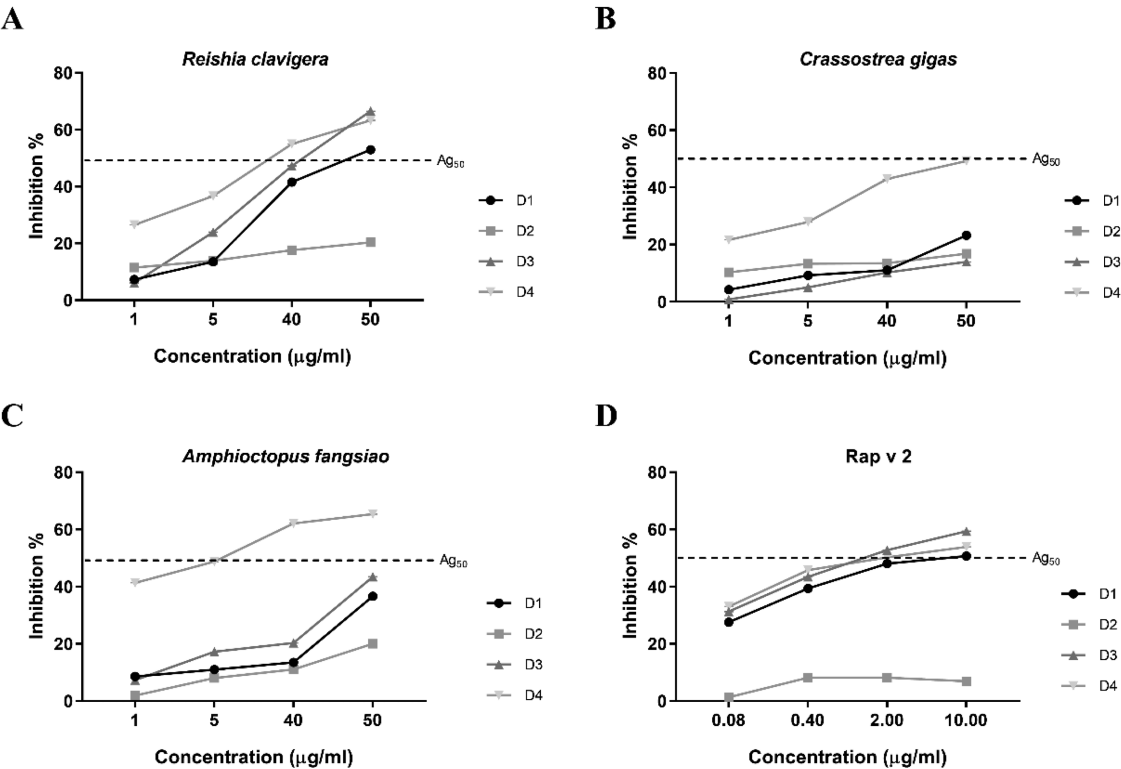


Figure 8. Cross-reactivity analysis between different mollusc species and mite.

reactivity involving mollusc species and mite–mollusc, as well as other cross-reactive allergens besides TM. As a result, we attempted to recognize more cross-reactive allergens in commonly consumed mollusc food items in Qingdao, China, as well as examine the frequency of recognition by patient IgE and cross-reactivity. Meanwhile, the IgE cross-reactivity and cross-reactive allergens of dust mite and mollusc extracts were also determined and recognized. Rap v 2 was further assessed for cross-reactivity with the mollusc group and dust mite PMs by structure-based methods and immunological assays.

Diagnosis of mollusc allergy is often difficult to identify in ingested species because of the wide variety of mollusc species available in different regions of the world. Furthermore, cross-reactivity with mollusc species make a diagnosis of mollusc allergy more complicated.^{35,36} Emoto et al. demonstrated IgE cross-reactivity to 4 gastropods and 7 bivalves by using a serum from 10 shellfish allergic patients and established a cross-reactivity in all species tested.³⁷ Moreover, in the clinical survey of mollusc allergic groups including scallops, clams, oysters, and mussels, among them, 34 individuals had positive reactions to one species, 13 individuals reacted to two species,

5 individuals reacted to three species, and 15 individuals reacted to all four mollusc species.⁹ TM was a major cross-reactive allergen for the reported cross-reactivity among molluscs, but it is also possible that the cross-reactivity between mollusc groups might involve other unidentified allergenic components present in molluscs. It is important to note that IgE sensitization patterns to different cross-reactive allergenic components are variable according to the geographic location and dietary habits. Molluscs such as sea snail, abalone, and octopus are usually eaten directly as sashimi without cooking to experience the unique taste and flavor of seafood in Korea, Japan, and the coastal areas of China.³⁸ In this study, we found many potential allergens in the mollusc group, such as 33–38 (TM), 43 (AK), 97–104 kDa (PM) protein bands, and so forth, the TM and AK proteins of these were involved in the cross-reactivity between different mollusc groups, but only 13% of all molluscs had IgE-binding capabilities to them. In particular, IgE immunoblotting revealed that 97–104 kDa in the PM region were recognized at a high frequency in different mollusc extracts. The correlation of IgE cross-reactivity detected by ELISA was significantly consistent especially

between the same mollusc species. Moreover, in Japan, Suzuki et al. also demonstrated that PM was a cross-reactive allergen in some molluscs by using Japanese sera from allergic subjects.¹⁹ In fact, similar to TMs, the amino acid identity between *R. venosa* PM and other known mollusc PMs could be as high as 78.58%, it was also found that PM was a major allergen-causing cross-allergic reaction in molluscs through the inhibition of IgG and IgE ELISA assays, suggesting that PM could be a potential cross-reactive allergen in molluscs.

On the other hand, the diagnosis of mollusc allergy might be hampered by the high degree of cross-reactivity between different mollusc allergens present in insects, mites, and other parasite-contaminated mollusc foods, and the observed positive sIgEs might not have any clinical implication.^{6,39} The “mite-shellfish-mollusc-syndrome” is a rare variant of food allergy in which the HDMs are the primary IgE-sensitizing agents, while shellfish/mollusc can induce food allergy, as well as anaphylaxis, even at first ingestion.⁴⁰ Clinically relevant cross-reactivity has also been often observed for gastropod mollusc and HDM. A survey in France showed that 169 children were tested for skin pricking, of which 31% of children allergic to HDM were allergic to snails.¹³ In one case, it was also noted that 70 patients were sensitized to molluscan shellfish, 90% of which were also sensitized to dust mites.⁴¹ As a matter of fact, we also found out that mollusc species involved in cross-reactivities with HDM, as measured by ELISA assay and immunoblotting. Allergenic molecules involved in shellfish allergy are cross-reactive with allergen components from HDM, especially certain proteins with a role in muscular contraction such as TM.⁴² However, some clinical researchers pointed out that TM is not a major cross-reactive allergen in HDM and snail cross-reactive allergies, or even involved in snail allergy, and that other non-TM allergens may play a dominant role in the cross-reactivity between HDM and molluscs.⁴³ Like the TM, PM is a protein associated with muscular contraction described in diverse invertebrates and is also a major allergen in molluscs and mites (such as Rap v 2, Der f 11 and Der p 11).^{20,44} As a high MW allergen, PM has been suggested to play an important role in allergic asthma caused by dust mites.⁴⁵ Interestingly, in this work, multiple IgE-binding components involved in cross-reactivity between *D. farinae* and mollusc species, as well as PMs were recognized and confirmed by mass spectrometry in the *D. farinae* extract and inhibition ELISA assay, suggesting that the PM might be a cross-reactive allergen implicated in mite–mollusc cross-reactivity. Allermatch results also indicated that 80 amino acid windows showed over 35% similarity with HDM PM to gastropod mollusc PM. This increases the chance of potential binding sites for IgE antibodies and hence the chance of clinical allergic cross-reactivity. Moreover, there seem to exist differences in mite–mollusc cross-reactivity depending on climate, a previous study revealed that TM and ubiquitin were responsible for mite–shellfish cross-reactivity from both continental dry and humid climates, while α -actinin and AK are involved in dry- and humid-climate groups, respectively.⁴⁶ Shen et al. indicated that environmental factors, lifestyles, and dietary habits play critical roles in allergen sensitization, IgE cross-reactivity to mite–shellfish-induced allergies and allergic disease development.⁴⁷ As a result of the differences in geographical exposure to dust mite allergens and dietary variables, people living in different climatic zones may have different cross-reactive allergenic components of mite–mollusc.

In summary, the presented work further demonstrated that some mollusc species display different degrees of cross-reactivity when exposed to other mollusc-sensitized individuals. Asthma appears to be a typical symptom after inhalation of dust mites and ingestion of certain mollusc such as snail and limpet, perhaps reflecting that there is a certain relationship between dust mites and mollusc allergies,^{48,49} and our results from inhibition assays have also supported this opinion that molluscs and mite have cross-reactivity with each other. Moreover, we observed for the first time in this study that PM as a cross-reactive allergen is involved in the cross-reactivity of different mollusc groups and mite–mollusc in the Chinese population. Thereby, our findings could be adapted to diagnosis and immunotherapy of mollusc allergy, as well as the early detection of risk factors for developing mollusc food allergy in dust mite allergic patients. Nevertheless, more clinical research on mollusc allergy and the study of allergen epitopes is encouraged, in our opinion, to better understand the cross-reactions of mollusc allergic individuals and to describe the risk of ingesting mollusc food.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.1c05421>.

Sixteen molluscan species used in this study and multiple sequence alignment between different mollusc PM allergens (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Xiang Gao – Department of Allergy, The Affiliated Hospital of Qingdao University, Qingdao, Shandong Province 266003, P. R. China; Phone: +86-532-82911937; Email: gaoxiang2002@163.com

Zhenxing Li – College of Food Science and Engineering, Ocean University of China, Qingdao, Shandong Province 266003, P. R. China; orcid.org/0000-0002-7866-681X; Phone: +86-532-82032389; Email: lizhenxing@ouc.edu.cn; Fax: +86-532-82032389

Authors

Chuang Yu – College of Food Science and Engineering, Ocean University of China, Qingdao, Shandong Province 266003, P. R. China

Xue Ding – College of Food Science and Engineering, Ocean University of China, Qingdao, Shandong Province 266003, P. R. China

Hong Lin – College of Food Science and Engineering, Ocean University of China, Qingdao, Shandong Province 266003, P. R. China

Mati Ullah Khan – College of Food Science and Engineering, Ocean University of China, Qingdao, Shandong Province 266003, P. R. China

Hang Lin – Department of Allergy, The Affiliated Hospital of Qingdao University, Qingdao, Shandong Province 266003, P. R. China

Xuwen Dang – College of Food Science and Engineering, Ocean University of China, Qingdao, Shandong Province 266003, P. R. China

Complete contact information is available at: <https://pubs.acs.org/10.1021/acs.jafc.1c05421>

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MW, molecular weight; HDM, house dust mites; TM, tropomyosin; PM, paramyosin; AK, arginine kinase; MHC, myosin heavy chain; TIM, triosephosphate isomerase; Rap v 2, *Rapana venosa* paramyosin; IgE, immunoglobulin E; WHO/IUIS, World Health Organization and International Union of Immunological Societies

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