

Cloning, Expression, and Epitope Identification of Myosin Light Chain 1: An Allergen in Mud Crab

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Supporting Information

ABSTRACT: Mud crab (*Scylla paramamosain*) is a commonly consumed seafood as a result of its high nutritional value; however, it is associated with food allergy. The current understanding of crab allergens remains insufficient. In the present study, an 18 kDa protein was purified from crab muscle and confirmed to be myosin light chain 1 (MLC1) by matrix-assisted laser desorption/ionization–tandem time-of-flight mass spectrometry. Total RNA was isolated and amplified to obtain a MLC1 open reading frame of 462 bp, encoding 154 amino acids. A structural analysis revealed that recombinant MLC1 (rMLC1) expressed in *Escherichia coli* contained α -helix and random coil. Moreover, rMLC1 displayed strong immunoactivity by dot blot and a basophil activation test. Furthermore, seven allergenic epitopes of MLC1 were predicted, and five critical epitope regions were identified by an inhibition enzyme-linked immunosorbent assay and human mast cell degranulation assay. This comprehensive research of an allergen helps to conduct component-resolved diagnoses and immunotherapies related to crab allergies.

KEYWORDS: allergenic epitopes, expression, immunoinformatic tools, myosin light chain 1, *Scylla paramamosain*

INTRODUCTION

According to recent statistics, the total output of crustacean products was 6.914 million tons in 2017;¹ however, such increased consumption has resulted in an augmented incidence of allergic diseases in coastal areas.² Crustacean allergies can cause an immunoglobulin E (IgE)-mediated type I hypersensitivity reaction and represent a long-lasting disorder, which typically persists throughout life.³ Hence, the identification and characterization of both major and minor crustacean allergens is critical for the generation of reliable diagnostic tests and therapeutics.⁴ Currently, over six crustacean allergens have been identified, which include tropomyosin (TM),⁵ arginine kinase,⁶ sarcoplasmic calcium-binding protein,⁷ triose phosphate isomerase,⁸ and myosin light chain (MLC).⁹

MLC has been identified as a minor allergen in *Litopenaeus vannamei* (Lit v 3)⁹ and *Crangon crangon* (Cra c 5).¹⁰ Moreover, shrimp MLC is associated with a frequency of allergic sensitization ranging from 19 to 55%, and IgE-binding studies indicate that the recognition of MLC is higher in adults compared to children.¹¹ In addition, it was reported that these major and minor allergens are the most relevant in shellfish.¹² Although several allergens have also been explored from the edible parts of mud crab,^{13–15} seldom reports have focused on MLC in crab to date. The total output of mud crab (*Scylla paramamosain*) reached 151 976 tons, with a national marine fishing output of 79 491 tons in 2017.¹ As a result of its high production and consumption, crab has gradually become the predominant cause of allergic reactions; thus, there is need for a more extensive study. On the basis of the above research status, mud crab MLC is worthy of further investigation.

As a minor allergen, the abundance of MLC is substantially lower than primary allergens in the muscle. There are two classes of MLC, which are referred to as the essential light chain (MLC1) and the regulatory light chain (MLC2) (also referred to as α and β chains). Thus, certain minor allergenic proteins may be underrepresented in diagnostic or therapeutic allergen extracts as a result of a low concentration in the source material or unfavorable conditions during extract preparation.¹⁰ Hu et al. developed a method of obtaining high yields of recombinant sarcoplasmic calcium binding protein (rSCP) that compensate for the difficulty associated with purification and the low content in crab extract.¹⁵ It was also reported that recombinant allergens are typically used as an effective tool for diagnostic studies, which reveals the correlation between IgE reactivity to certain components and the clinical status of patients.^{16–18} Thus, the use of genetic engineering technology is necessary to produce recombinant allergens that create a more comprehensive understanding of relevant crab allergens and enable a more extensive component-resolved diagnostic study of crab allergies.

The antigenicity of an allergen depends upon its epitopes.¹⁹ An epitope is usually located on the local region of the allergen surface that can be recognized by a specific antibody. The epitope mapping of shrimp allergens uses previously reported overlapping synthetic peptides,²⁰ which provide theoretical reference for the epitope analysis of crab allergens.

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In comparison to phage display or overlapping synthetic peptide approaches, epitope prediction greatly reduced the experimental cost and time.^{21,22} Immunoinformatics using computer technology and the improvement of biological databases have been demonstrated to be an effective method of predicting allergen epitopes.²³ Bian et al. used immunoinformatics to identify class-II-restricted T cell epitopes to design subunit vaccines.²¹ Therefore, the crab allergen epitope can be explored by immunoinformatic tools to further analyze its differences with other allergens.

In this study, we aimed to identify and elucidate the characteristics of a crab allergen. To this end, the native protein was purified and confirmed by matrix-assisted laser desorption/ionization–mass spectrometry (MALDI–MS). Moreover, the recombinant protein was cloned and expressed by genetic engineering; the genetic sequence was analyzed by bioinformatic methods; the secondary structure was detected by a circular dichroism (CD) spectrum; and the immunoactivity was validated by dot blot and a basophil activation test (BAT). The allergenic epitopes were predicted using immunoinformatic tools and synthesized *in vitro*, after which they were identified with an inhibition enzyme-linked immunosorbent assay (iELISA) and human laboratory of allergic diseases 2 (LAD2) mast cell degranulation assay. Ultimately, the recognition of this crab allergen and the allergenic epitopes can be used as diagnostic and safe immunotherapeutic agents for subjects with shellfish allergies.

MATERIALS AND METHODS

Materials. Mud crabs were purchased at Jimei Market (Xiamen, Fujian, China). An Easstep Super Total RNA Extraction Kit and DNA Purification Kit were purchased from TaKaRa (Takara Bio, Kyoto, Japan). The peptides were synthesized by Cellmano Biotech (Hefei, Anhui, China). Horseradish-peroxidase-labeled goat anti-human IgE antibodies and goat anti-rabbit immunoglobulin G (IgG) antibodies were purchased from Southern Biotech (Birmingham, AL, U.S.A.). LAD-2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.).

Patient Sera. Sera were collected from 10 crab-sensitive patients and 2 non-allergic individuals (numbers 1–12) at the Women and Children's Hospital Affiliated to Xiamen University (human ethical approval KY-2018-018 and KY-2019-014). Blood donors provided signed informed consent. The specificity of IgE levels to crab (f23) was assessed *in vitro* using ImmunoCAP (Phadia AB, Rapskatan, Uppsala, Sweden); samples with a value of ≥ 0.35 kU/L were stored at -80°C until use; and food allergy-free serum was used as the negative control. Table S1 of the Supporting Information showed the individual sensitive profiles and clinical symptoms, which were used in the present study. The serum pool was prepared by mixing serum of numbers 1–10 in Table S1 of the Supporting Information, equal to 500 μL /tube without dilution.

Immunoassay of Myofibrillar Protein. *S. paramamosain* muscle was minced and homogenized with 10 volumes of ice-cold 20 mM PBS buffer (pH 7.4). The homogenate was then centrifuged at 8000g for 10 min at 4°C . The precipitate was resuspended in 10 volumes of PBS, mashed, centrifuged for precipitation, and then repeated 4 more times. The last obtained precipitation was dissolved in 20 mM Tris–HCl (pH 7.5) containing 0.5 M NaCl, and the solution was termed myofibrillar protein. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and western blot using human serum were performed.

Native Protein Purification. The native protein was eluted (flow rate of 1 mL/min) with three linear gradients (0–0.15, 0.15–0.3, and 0.3–0.5 M NaCl) on a Q-Sepharose column. The protein concentration was estimated at 280 nm; the protein was concentrated by a Millipore concentration tube (Merck KGaA, Darmstadt, Germany); and the tube had a molecular weight cut-off of 3 kDa.

The protein was added in the inner side of tube and centrifuged at 1000 rpm and 4°C ; the protein concentration was estimated at 280 nm every 10 min; the outer liquid of lower concentration was discarded; and the inner liquid of higher concentration was collected. SDS–PAGE and western blot were performed using rabbit anti-*Procambarus clarkii* MLC1 polyclonal antibodies ($1:10^5$ dilution) that were conserved by our laboratory according to the reported method.²⁴ Furthermore, the single protein band was sent to Shanghai Applied Protein Technology Co., Ltd. for analysis by matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF).

Primer Design and Cloning of the MLC1 Open Reading Frame (ORF). Total RNA was extracted from the crab muscle and reversed transcribed into cDNA, which served as a template for polymerase chain reaction (PCR). The primers for the coding sequence and promoter of the crab MLC1 gene were designed and synthesized on the basis of the published MLC1 sequences of four shrimp species (*P. clarkii*, *C. crangon*, *Penaeus japonicus*, and *Palaemon varians*). PCR amplification was performed using a forward primer (F_{MLC1} , 5'-ATGGCCGCGGATCTCAGTGCTCGTG-3') and reverse primer (R_{MLC1} , 5'-TTAAAGCAGCTGCGTCAACTTCTTT-3'). A universal DNA purification kit was applied to purify the PCR products, cloned into the pEASY-T1 vector (TransGene, Beijing, China), and sequenced (Xiamen, China).

MLC1 Sequence Analysis. The amino acid sequence, protein molecular weight, isoelectric point, and calcium binding site of the MLC1 gene were analyzed and predicted by ExPASy. The multiple sequence alignment was performed with shrimp MLC1 by DNAMAN, which is highly integrated software for molecular biology. A phylogenetic tree analysis was executed in MEGA 5.0 using the neighbor-joining (NJ) method.

MLC1 Structure Prediction. The PSIPRED online server (<http://bioinf.cs.ucl.ac.uk/psipred/>) was used to predict the secondary structure of MLC1.²⁵ The tertiary structure was modeled using SWISS-MODEL (<http://swissmodel.expasy.org/>), and the Protein Data Bank (PDB) file was viewed with PyMol software.

rMLC1 Expression. The pEASY-T1-MLC1 plasmid and pET-28a vector were digested with *EcoR* I and *Not* I and transformed into *Escherichia coli* BL21 (DE3) cells. After verification with sequencing, the expression strain, BL21-pET-28a-MLC1, was induced by isopropyl- β -D-thiogalactoside (IPTG) after 16 h at 16°C . The expression products were analyzed by SDS–PAGE, western blot, and mass spectroscopy. The recombinant MLC1 (rMLC1) was purified using nickel–nitrilotriacetic acid (Ni–NTA) resin according to the method described by Mao et al.¹⁴

rMLC1 Characterization. The secondary structure of rMLC1 was examined by measuring the CD spectra (190–260 nm) using a CD spectrophotometer (Applied Photophysics, Ltd., Surrey, U.K.). More specific experimental steps were described by Hu et al.¹⁵

The specific IgE-binding activity of rMLC1 was detected by dot blot using crab-allergic patient sera as the primary antibody, using the method described by Liu et al.²⁶ The dot blot results were quantized and shown as a histogram, according to the method of Wai et al.²⁷

rMLC1 BAT Analysis. The serum verified by dot blot was used to further detect the effect of allergen on the effector cells of the patients with BAT. As for the allergen concentration (50 $\mu\text{g}/\text{mL}$), we mainly refer to the paper by Yang et al.²⁸ The effect of rMLC1-induced basophil activation was measured by detecting the level of CD203c and CD63 upregulation using flow cytometry.²⁹ Allergen-induced CD203c and CD63 upregulation was calculated using the mean fluorescence intensities (MFIs) obtained with stimulated (MFI_{stim}) and unstimulated (MFI_{con}) cells and was expressed as the stimulation index (SI), defined as $\text{MFI}_{\text{stim}}/\text{MFI}_{\text{con}}$.²⁹

Immunoinformatic Prediction and Peptide Synthesis. To improve the prediction accuracy, the MLC1 sequences were analyzed using five immunoinformatic tools, including DNASTAR Proten, BepiPred 1.0, ABCpred, Immunomedicine Group, and NetMHC II 2.3 server-based computational approaches.

The predicted epitopes were primarily based on hydrophilicity, flexibility, accessibility, and antigenicity of the amino acid sequences. Finally, the results of the five immunoinformatic tools were combined,

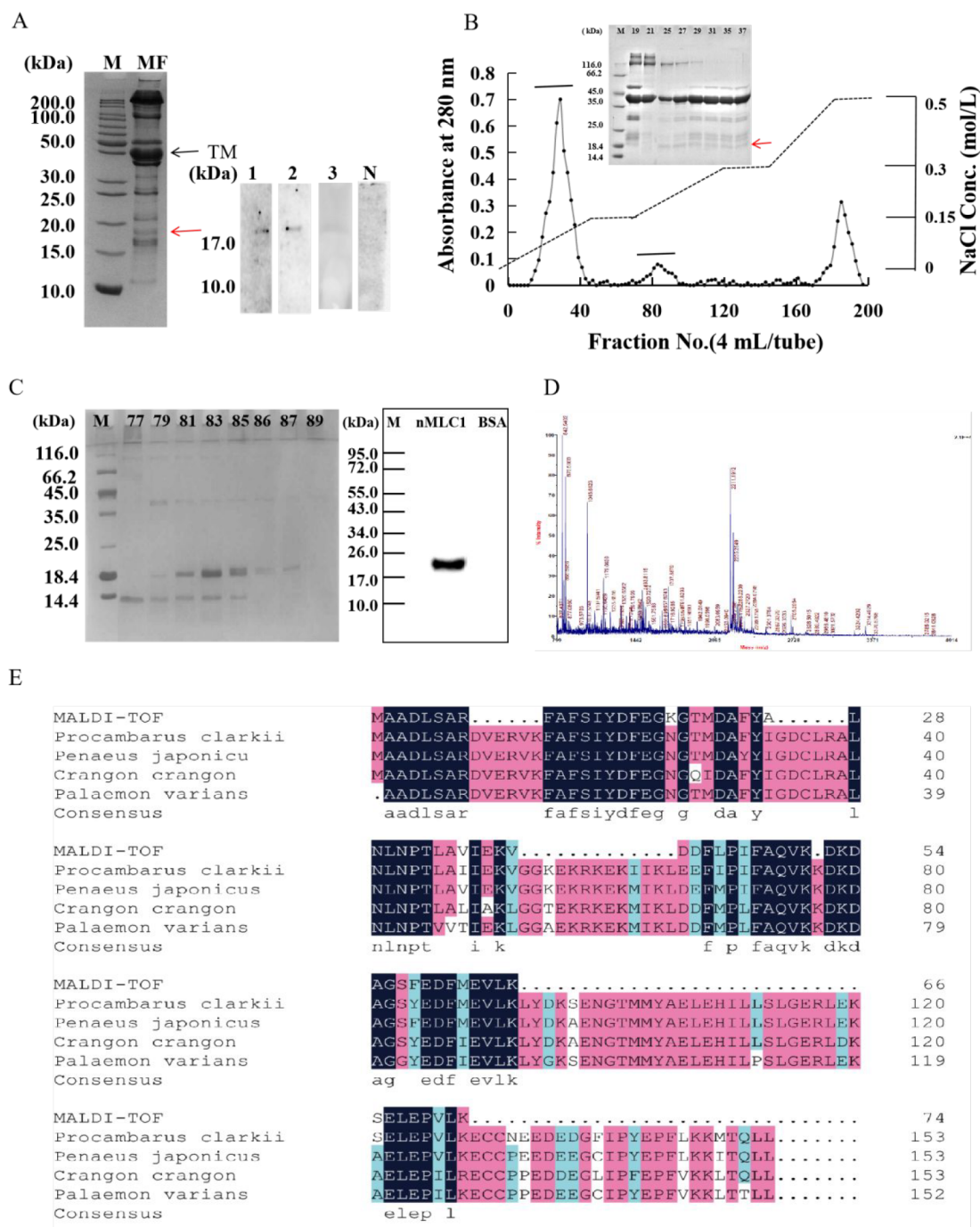


Figure 1. Purification and identification of the 18 kDa protein isolated from mud crab. (A) SDS-PAGE and an IgE-immunoassay analysis of myofibrillar protein from *S. paramamosain*. Lane M, protein marker; lane MF, myofibrillar protein. Strips 1–3 were three different sensitive individual sera, and the profile were showed in Table S1 of the Supporting Information. N = sera of healthy patients (negative control, numbers 11 and 12 in Table S1 of the Supporting Information). (B) Q-Sepharose chromatography. The numbers on the top of the lanes correspond to the fraction number. (C) SDS-PAGE (silver staining) and western blot analysis of the purified protein. The numbers on the top of the lanes correspond to the fraction number. (D) 18 kDa protein was confirmed by MALDI-TOF. (E) Sequence alignment results of the mass spectrometry peptides and matching protein (*P. clarkii* MLC1, *P. japonicus* MLC1, *C. crangon* MLC1, and *P. varians* MLC1).

and the predicted epitopes were synthesized and stored at -20°C until use.

Analysis of Epitope Peptides by iELISA. An iELISA was performed to analyze the IgE-binding activity of epitope peptides using a serum pool (Table S1 of the Supporting Information) according to the method described by Mei et al.,³⁰ with some modification. The peptide concentration ($2\text{ }\mu\text{g/mL}$) that had been explored as the most appropriate concentration was prepared and pre-incubated with an equal volume ($100\text{ }\mu\text{L}$) of sera (1:5 dilution) for 16 h at 4°C . The

positive control consisted of rMLC1 without peptide, and the negative control was coated with coating buffer. The inhibition rates were calculated as follows:

$$\{1 - [(\text{experimental group} - \text{negative control}) / (\text{positive control} - \text{negative control})]\} \times 100\%$$

LAD2 Cell Degranulation Assay of Epitope Peptides. The human LAD2 mast cell degranulation assay was performed to further

analyze the effect of epitope peptides according to the method described by Fu et al.³¹ Stimulation with PBS (pH 7.4) was used as negative control, and stimulation with rMLC1 was used as positive control. The percentage of β -hexosaminidase activity measured in the supernatant samples was compared to the total levels in the corresponding cell lysates.³²

Statistical Analysis. Data from the studies were presented as the mean $\pm$ standard deviation (SD). A one-way repeated-measures analysis of variance (ANOVA) with Duncan's test was used to compare quantitative outcomes, and $p < 0.05$ was considered to be statistically significant.

RESULTS

Purification and Identification of the 18 kDa Protein.

Myofibrillar protein from *S. paramamosain* was extracted and analyzed by SDS–PAGE. As shown in Figure 1A, there are many bands in myofibrillar protein, including the major allergen TM and a band near 18 kDa. Meanwhile, the 18 kDa band reacted with the crab-sensitive serum, which led to speculation that the 18 kDa protein was an allergen in mud crab.

The 18 kDa target protein was eluted at 0.15–0.3 M NaCl (Figure 1B) and separated as a single band with silver staining, which revealed that the content of the 18 kDa protein was extremely low (Figure 1C). Moreover, the protein was concentrated, and the IgG-binding activity was analyzed by western blot, which revealed a strong binding activity with the rabbit anti-*P. clarkii* MLC1 polyclonal antibody (Figure 1C).

The 18 kDa protein was also confirmed by MALDI–TOF–MS, and the peptide mass fingerprinting of the purified protein yielded multiple peaks that were compared to the National Center for Biotechnology Information (NCBI) database (panels D and E of Figure 1 and Table S2 of the Supporting Information). Searches against the entire NCBI database were performed using Mascot. A search for homologous proteins in the NCBI protein database showed that the seven peptides from the 18 kDa protein had over 80% identity with the sequences of *P. clarkii* MLC1 (AFP95338.1), *P. japonicus* MLC1 (ADD70028.1), *C. crangon* MLC1 (ACR43477.1), and *P. varians* MLC1 (ACR54116.1). Hence, the 18 kDa protein was determined to be a native MLC1 (nMLC1) in mud crab.

Cloning and Sequence Analysis of the MLC1 Gene. PCR analysis generated a band of an expected size of approximately 500 bp using the crab cDNA as a template (Figure 2A), and its nucleotide sequence has been submitted to GenBank (MK749844.1). The MLC1 gene was cloned into the pEASY-T1 vector, and positive clones were obtained by PCR with MLC1-specific primers (Figure 2B).

The sequence analysis indicated that the MLC1 gene contained a 462 bp ORF, which encoded a 154 amino acid protein with a calculated molecular mass of 17.42 kDa and an isoelectric point of 4.69. The total average hydrophilicity index was -0.304 , and the instability index in solution was 29.49, which classifies the protein as stable. Furthermore, this protein did not contain any Trp residue; however, the Leu residue content was the highest. As shown in Figure 2C, there is a N-glycosylation site at 99–101 residues, which was identified by the conserved Asn-X-Ser/Thr/Cys motif (marked with a solid black box). Moreover, the amino acid residues, which were located in the red box, represented the calcium binding sites.

The homology analysis using BLAST and DNAMAN demonstrated that the MLC1 protein shared a high homology with several MLC1 proteins of crustacean aquatic species. Figure 2D shows that the amino acid sequence of crab MLC1 was 89,

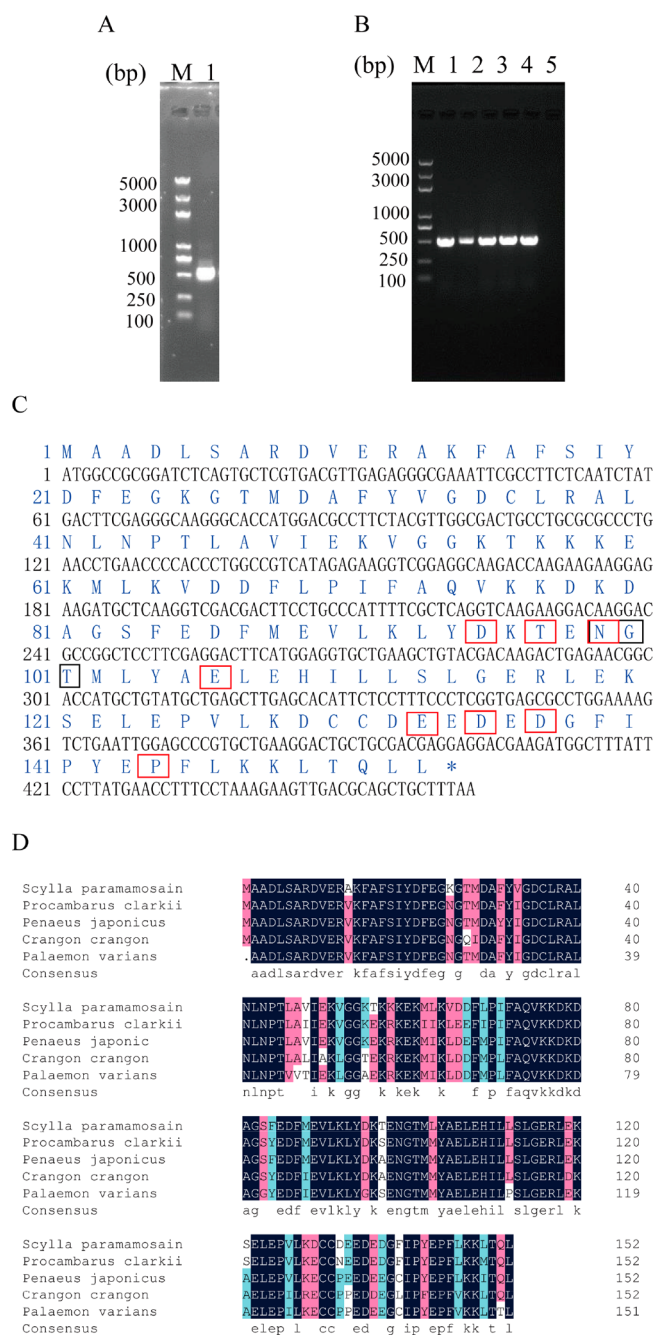


Figure 2. Agarose gel electrophoresis and amino acid sequence alignment of crab MLC1. (A) Agarose gel (1.0%) electrophoresis analysis of the PCR products of crab MLC1 cDNA. Lane M, DL5000 DNA marker; lane 1, ORF product. (B) PCR confirmation of the cloning plasmid, pEASY-T1-MLC1. Lane M, DL5000 DNA marker; lane 1–5, MLC1 fragments. (C) ORF and the deduced amino acid sequence of MLC1. The ORF contained 462 bp, and the deduced protein was composed of 154 amino acids. (D) Amino acid sequence alignment of MLC1 for the crustacean aquatic species from GenBank. The species are on the left, and the GenBank accession numbers of the sequence are as follows: MK749844.1, AFP95338.1, ADD70028.1, ACR43477.1, and ACR54116.1.

88, 80, and 79% identical to the sequences of *P. clarkii*, *P. japonicus*, *C. crangon*, and *P. varians*, respectively.

A phylogenetic tree was constructed to illustrate the relationship between the MLC1 protein of *S. paramamosain* and nine other higher aquatic species (Figure 3A). A total of six

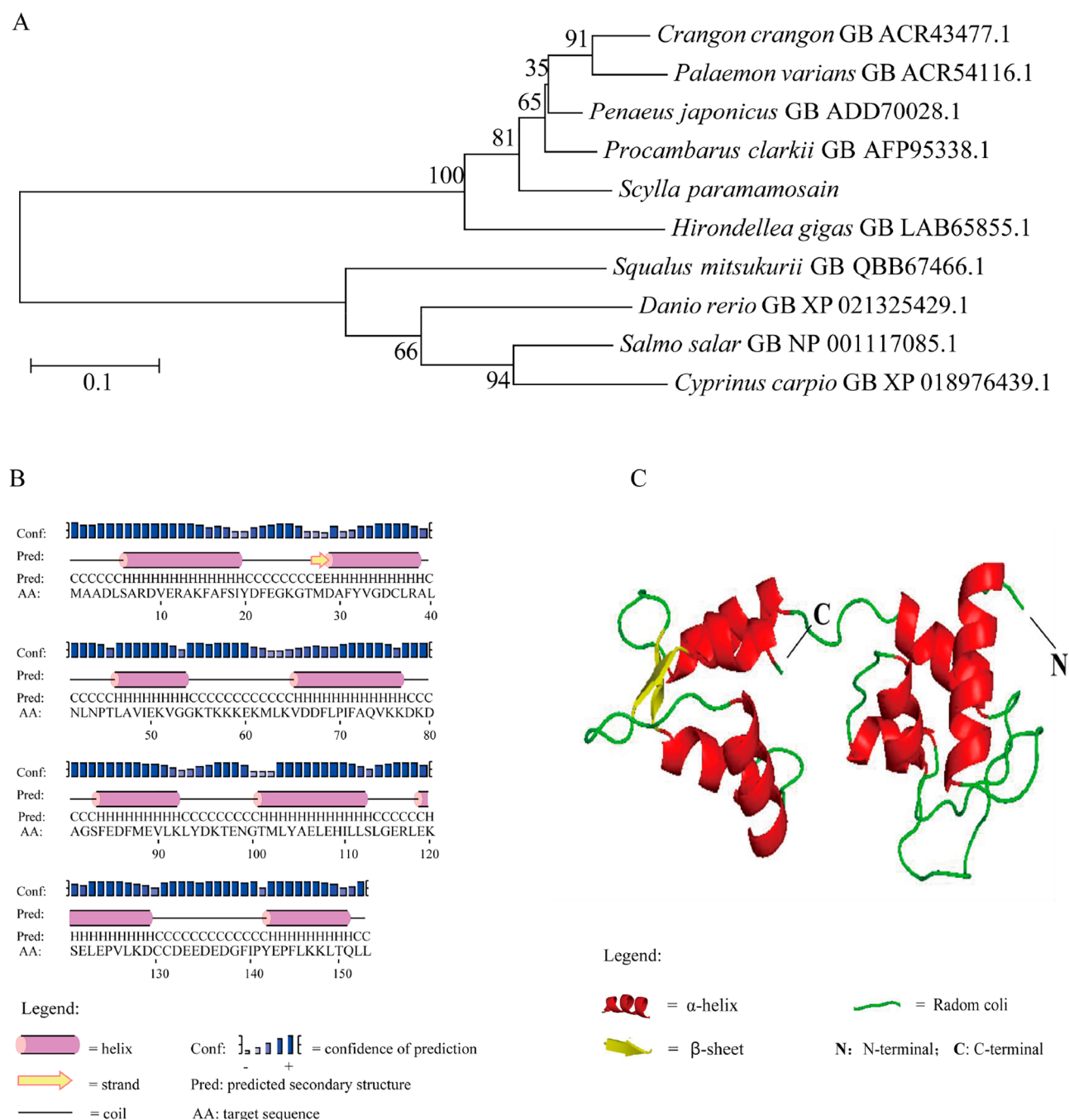


Figure 3. Phylogenetic tree analysis and structure simulation of crab MLC1. (A) Phylogenetic tree based on the amino acid sequences of MLC1. (B) Secondary structure was predicted for MLC1. (C) 3D model of MLC1 using the myosin light chain alkali from insects (PDB ID 5w1a.1.B) as a template.

clusters were identified: (1) crustacean, (2) shellfish, (3) Heterodontidae, (4) Schromberidae, (5) Salmonidae, and (6) Cyprinidae. Sequence alignment indicated that the crab MLC1 protein was more closely related to *P. clarkii* and *P. japonicus*, which belonged to the crustacean branch.

Secondary Structure Prediction and Three-Dimensional (3D) Modeling of MLC1. The secondary structure prediction showed eight α -helices and random coils with a short β -sheet (Figure 3B) in MLC1. A 3D structural model was constructed by SWISS-MODEL. The crystal structure of the myosin light chain alkali from insects (PDB ID 5w1a.1.B, showing a 57.53% identity) was selected as the template to build

3D structures on the web. It also consisted of α -helices and random coils (Figure 3C), which was consistent with the prediction of the secondary structure.

Expression, Purification, and Identification of rMLC1. rMLC1 was expressed as a soluble protein in *E. coli* BL21 induced by 0.5 mmol/L IPTG. SDS-PAGE analysis of the extracted total proteins generated a prominent band with a molecular mass of 24 kDa and was not detected in the non-induced control vector (Figure 4A). The 24 kDa molecular weight was higher than nMLC1 (18 kDa) because the original termination codon TAA was removed when the primer was designed, leading to the unterminated translation, so that the

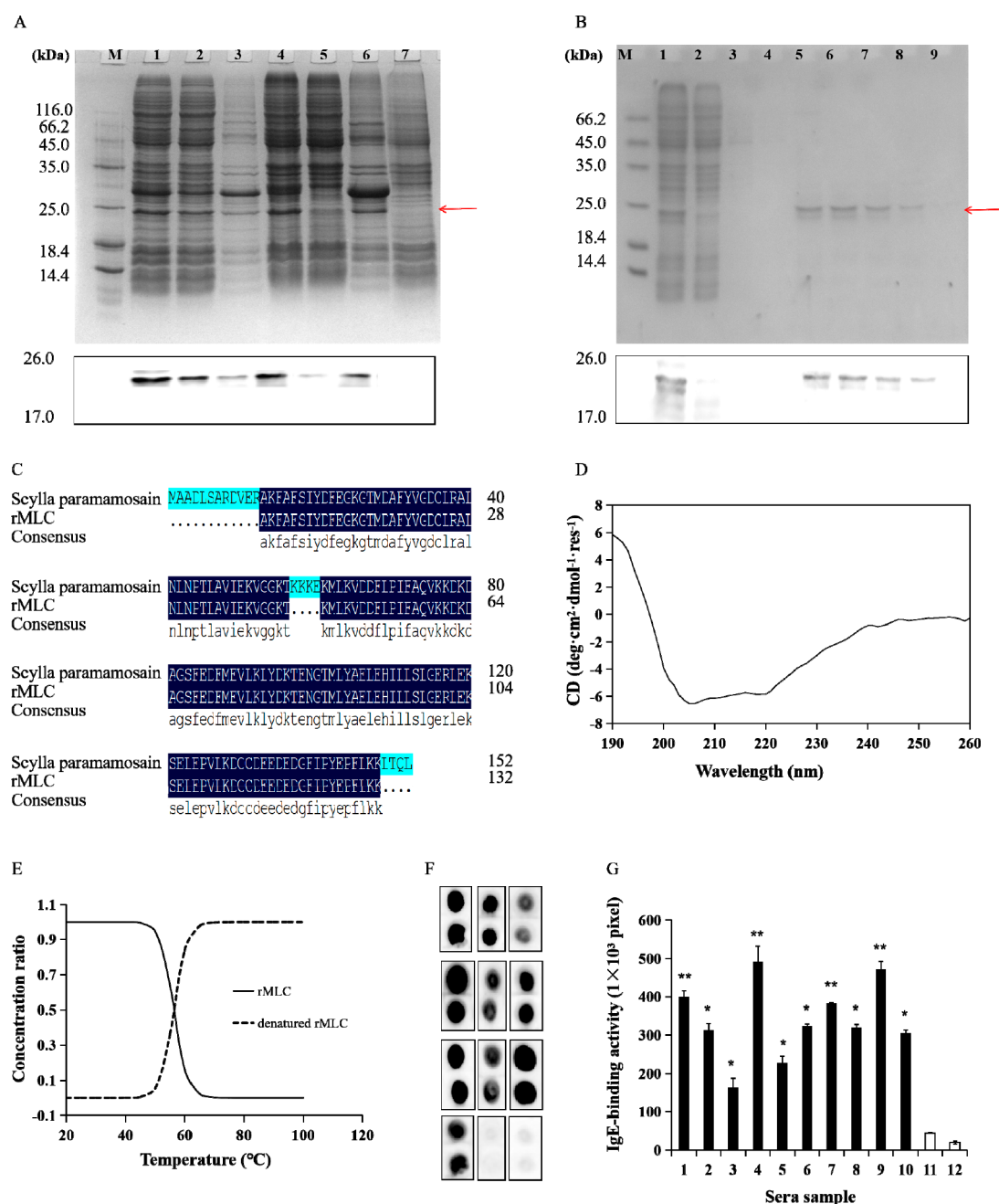


Figure 4. Expression, purification, identification, and characterization analysis of rMLC1. (A) SDS–PAGE analysis and western blot verification of rMLC1 expressed in *E. coli*. Lane M, protein marker; lane 1–3, ultrasonicated bacterial fluid, supernatants, and sedimentation of the strain induced in 16 h at 16 °C; lanes 4–6, ultrasonicated bacterial fluid, supernatants, and sedimentation of the strain induced in 16 h at 37 °C; lane 7, empty vector of pET-28a. The red arrow represents the target protein. (B) SDS–PAGE analysis and western blot verification of purified rMLC1 using Ni–NTA. Lane M, protein marker; lane 1, sample before purification; lanes 2–9, eluted fractions. The red arrow represents the target protein. (C) Map identification of MS/MS. (D) Secondary structural analysis of rMLC1 by CD spectroscopy. (E) Determination of the T_m value of rMLC1. (F) Analysis of rMLC1 IgE-binding activity by dot blot using crab-allergic patient serum. (G) Intensity of the dots shown on the nitrocellulose membranes. The quantification of grayscale dots was analyzed using ImageJ software.

whole expressed sequence length had 214 amino acids; hence, the 24 kDa protein was closed to the predicted size. The western blot analysis demonstrated that the 24 kDa protein band specifically reacted with the anti-*P. clarkii* MLC1 polyclonal antibody (Figure 4A). Using a purified Ni–NTA resin, the 24 kDa protein band was eluted after adsorption to obtain a single band (Figure 4B). The 24 kDa protein was confirmed by liquid chromatography–tandem mass spectrometry (LC–MS/MS), and the supporting peptides had 86% protein similarity with the

amino acid sequence of crab MLC1 (Figure 4C). These findings illustrate that rMLC1 was successfully expressed and further instructed the accuracy of the MLC1 amino acid sequence by cloning in crab.

Characterization of rMLC1. The CD spectroscopic analysis (Figure 4D) showed that rMLC1 exhibited an α -helical structure (consistent with panels B and C of Figure 3), in which the curve showed a characteristic negative maximum at approximately 210 and 220 nm. According to Global 3.0

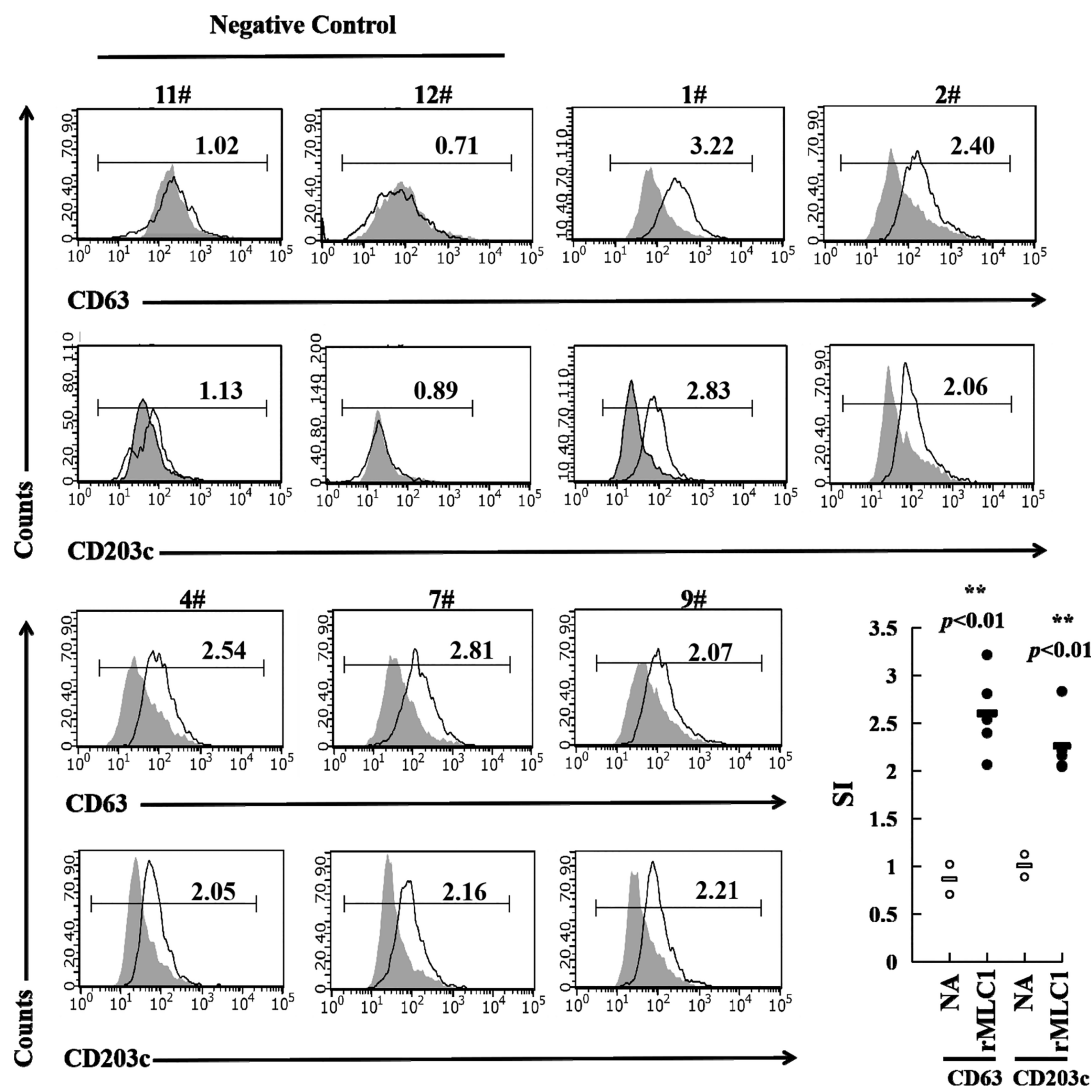


Figure 5. Validation effect on rMLC1 analyzed by BAT. Gray and white represent the cell population stimulated by PBS and rMLC1, respectively. NA represents healthy individuals, and rMLC1 indicates sensitive patients. The horizontal line represents the average values of SI by PBS or rMLC1 stimulation. (**) $p < 0.01$ compared to the negative control.

software (Figure 4E), the thermal denaturation temperature (T_m value) was 56.3 ± 0.7 °C. As the temperature increased from 20 to 100 °C, the secondary structures of rMLC1 changed gradually and were especially significantly altered in the exceeded T_m value.

The IgE-binding activity of rMLC1 was confirmed by dot blot, which had a strong binding activity with the sera of allergic patients (Figure 4F). Further quantification of positive grayscale dots was performed using ImageJ software, which revealed a significant difference compared to the non-allergic individuals (Figure 4G).

Immunogenicity of rMLC1 Analyzed by BAT. The specific IgE reaction of the serum (numbers 1–12) to rMLC1 had been verified; however, whether this sensitization is clinically relevant remains to be further explored. Hence, BAT was used to analyze the apparent correlation by the reactivity of basophils from voluntary donors. Figure 5 shows that, in comparison to the NA controls, rMLC1 stimulation could induce a significantly higher SI level of CD63⁺ ($p < 0.01$) and CD203c⁺ basophils ($p < 0.01$) in five patients. These results indicate that rMLC1 was able to induce basophil activation *in vitro*, and the serum can be used in future follow-up experiments.

Prediction of Allergenic Epitopes in MLC1. The secondary structure, surface accessibility, and fragment flexibility are important features for predicting antigenic epitopes, and the existence of highly hydrophilic regions also provides strong evidence for epitope identification. Thus, immunoinformatic tools (e.g., DNASTAR Protean system, BepiPred 1.0 server, ABCpred server, Immunomedicine Group, and NetMHC II 2.3 server) were used to analyze authentic epitopes. As shown in Figure 6A, the hydrophilic regions covered the majority of the MLC1 sequences, indicating high hydrophilicity. In addition, the surface accessibility and flexibility analysis predicted flexible, stretched, and easily exposed regions. Moreover, the antigenic indexes were calculated to show the potential allergenicity of the protein regions (panels B and C of Figure 6), and the predicted antigenic epitopes are presented in Table S3 of the Supporting Information. Finally, seven MLC1 peptides were acquired as the potential allergenic epitopes in mud crab by combining the results of the five immunoinformatic tools. These peptides were termed P1–P7 and synthesized for further verification (Table 1).

Verification of Epitope Peptides by iELISA. To verify the allergenicity of the predicted epitopes, an iELISA assay was

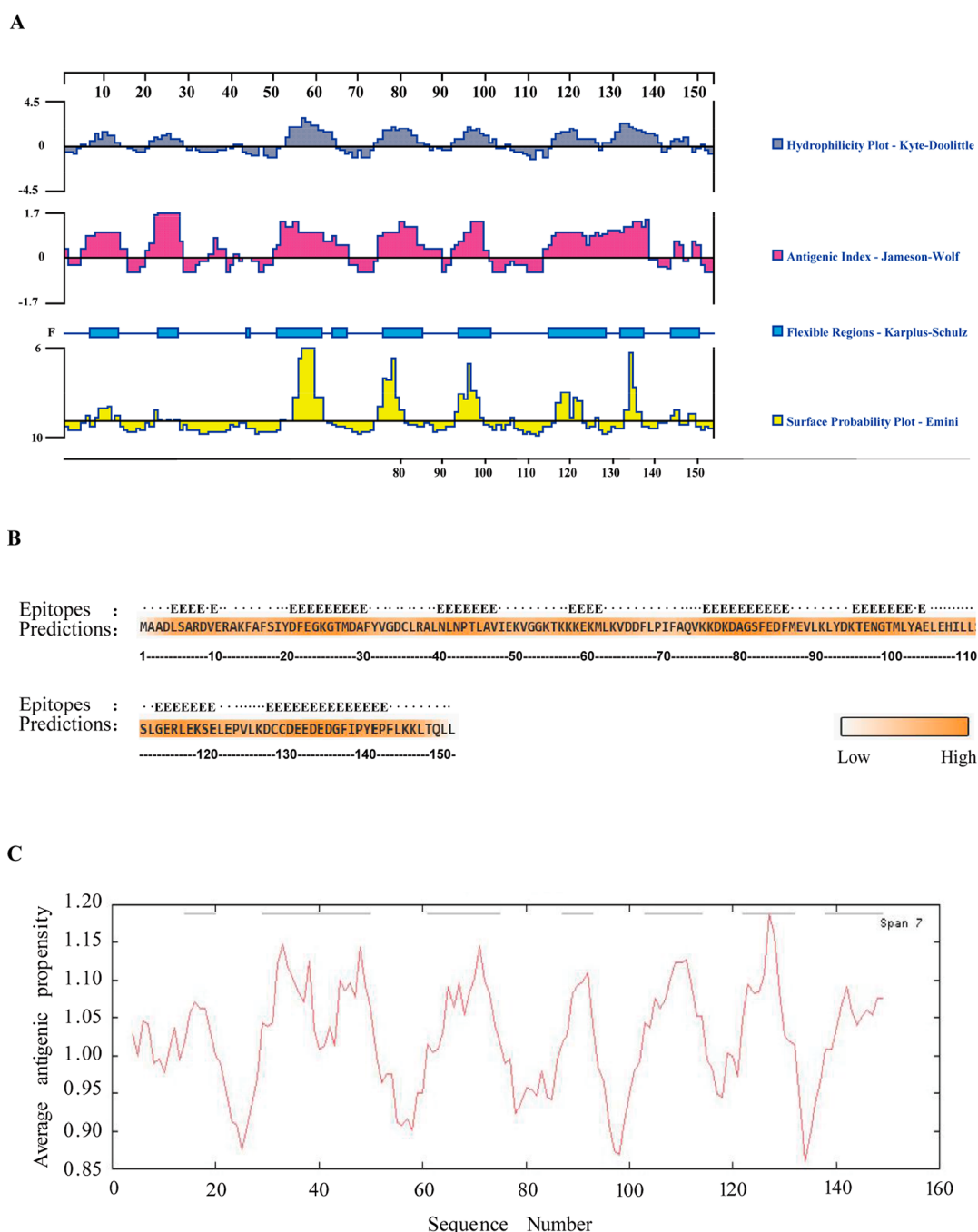


Figure 6. Prediction of allergenic epitopes in crab MLC1 using immunoinformatic tools. (A) Prediction of MLC1 allergenic epitopes using the DNASTAR Protean system. (B) Prediction of MLC1 allergenic epitopes using BepiPred 1.0. (C) Prediction of MLC1 allergenic epitopes by the Immunomedicine Group.

Table 1. Seven Allergenic Epitopes Were Selected Using Five Immunoinformatic Tools

number	amino acid sequence	position	length of sequence
peptide 1 (P1)	ARDVERAKFAFSI	7–19	13
peptide 2 (P2)	DCLRALNLP TLA	35–47	13
peptide 3 (P3)	KVGKTKKKKEK	51–61	11
peptide 4 (P4)	DDFLPIFAQVKKDKD	66–80	15
peptide 5 (P5)	KTENGTMLYAE	96–106	11
peptide 6 (P6)	HILLSLGERLEK	109–120	12
peptide 7 (P7)	DEDFIPYEPFLK	135–147	13

executed on the basis of the IgE competitive interaction between the epitope peptides and purified whole allergen protein (rMLC1) from the crab-allergic sera. As shown in Figure 7A, the peptides revealed highly inhibited effects, in which the inhibition rates varied from 64.44 to 81.01%. Among them, P7 displayed the highest inhibition efficiency (81.01%), followed by P3 (78.23%). Therefore, seven of the predicted peptides demonstrated the potential to cause an allergic reaction, suggesting that they were the critical epitope regions.

Validation of Epitope Peptides by the Mast Cell Degranulation Assay. IgE-mediated food allergies are mainly caused by a mast cell response; therefore, a mast cell

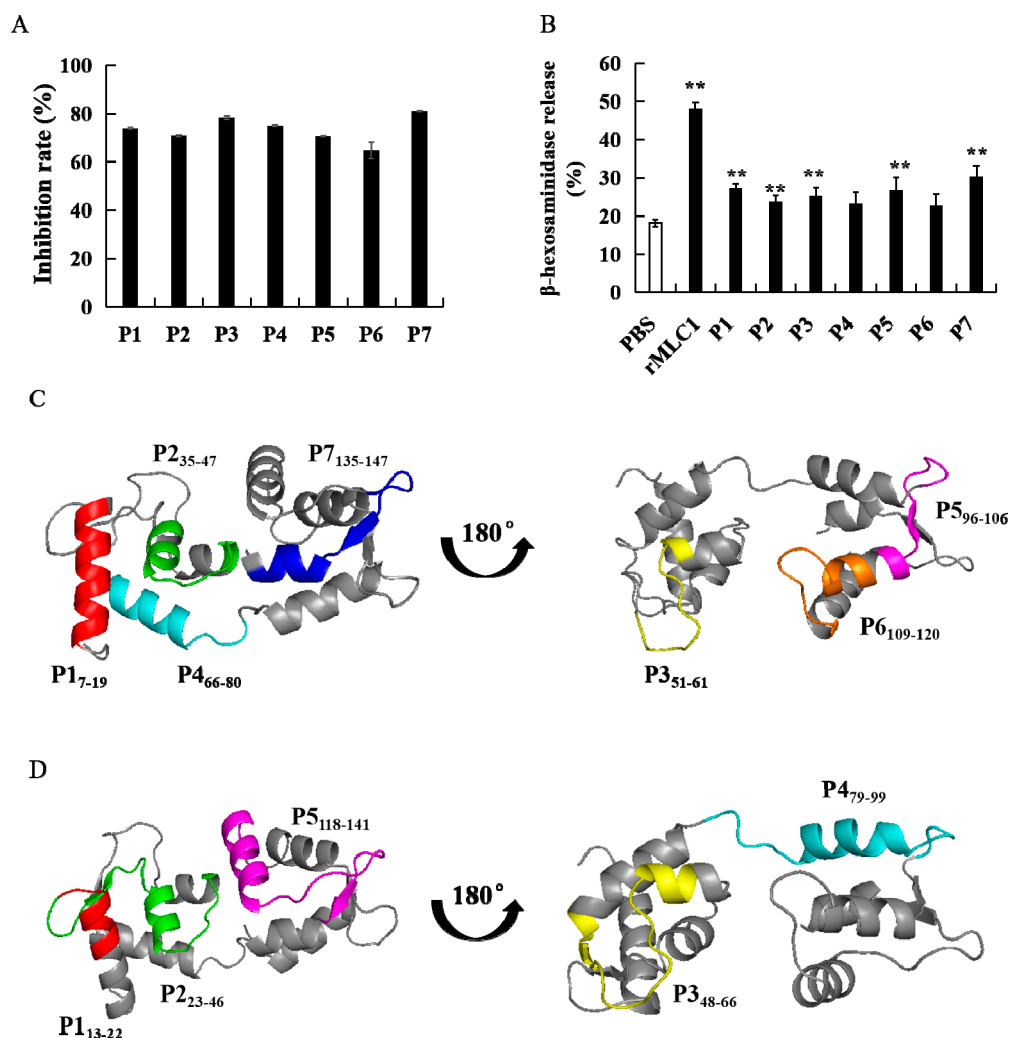


Figure 7. Antigenicity analysis and structural position of the epitope peptides. (A) Validation of allergenic epitope peptides by iELISA, with rMLC1 in the solid phase. IgE binding of the serum pool was determined with different epitope peptides. All data were presented as the mean $\pm$ SD ($n = 3$). (B) Validation of the allergenic epitope peptides using a LAD2 cell degranulation assay. PBS was used as a negative control, and rMLC1 was used as a positive control. All data are represented as the mean $\pm$ SD ($n = 3$). (***) $p < 0.01$ compared to the negative control. (C) 3D structural position of the allergenic epitopes in the mud crab MLC1 protein. (D) 3D structural position of the allergenic epitopes in the shrimp MLC1 protein.

degranulation test is typically used to detect allergen or epitope allergenicity. To determine whether the predicted MLC1 epitope peptides could induce the degranulation of human mast cells (LAD2 cells), the release of β -hexosaminidase enzyme was analyzed. As shown in Figure 7B, the ability of rMLC1 (48.14%) and all peptides to induce a response in LAD2 cells was analyzed. In comparison to PBS (18.28%), P7 displayed the highest degranulation efficiency (30.24%), and peptides that revealed a significant difference ($p < 0.01$) were considered as potential allergenic epitopes. As a consequence, P1, P2, P3, P5, and P7 of MLC1 resulted in significant degranulation, which suggested that they were the critical epitope regions.

DISCUSSION

Crustaceans, particularly shrimp and crab species, are considered to be highly allergenic foods and have become the main allergic food for both adults and children in China.³³ To date, shrimp have been more extensively studied in allergic reactions, and thus, several major and minor allergens have been identified and cloned.^{4,12} There is a relative lack of a

comprehensive review of crab-associated allergens because some potential allergens have yet to be identified.

It has been reported that two types of MLC belong to the calmodulin-like protein family that bind Ca^{2+} through their EF-hand domain, which are termed the essential light chain (MLC1) and the regulatory light chain (MLC2).⁴ However, the relationship between the two types remain unclear, except for the primary sequence differences. In our present purification experiment, 0.3 mg of purified nMLC1 was obtained from 170 g of crab muscle. In comparison to the content of crayfish nMLC1 (5.6 mg was acquired from 140 g of muscle),³⁴ the abundance of crab nMLC1 was extremely low and purification was difficult. Therefore, rMLC1 was acquired, including sequence information and the whole protein, using genetic engineering technology.

Most food allergens share similar physicochemical characteristics, in that they are glycoproteins, relatively stable to heat and acid–alkali, and also may retain their allergenicity following digestion.⁶ Zhang et al. analyzed the allergenic properties of nMLC1 in *P. clarkii*, confirming that MLC1 was a glycoprotein based on two N-glycosylated sites (Asn–Gly–Thr), was highly

resistant to heat, alkali, and digestion, and retained a weak IgE-binding activity when the secondary structure was altered.³⁴ In the present study, one identical potential N-glycosylated site (Asn-Gly-Thr) was identified by analyzing the amino acid sequence of crab MLC1, suggesting that crab MLC1 is a glycoprotein. In addition, the CD results showed that rMLC1 displayed a typical α -helix structure and higher T_m value, of which the characters were the same as nMLC1 from crayfish,³⁴ and further indicated that rMLC1 was suitable for subsequent experimental studies.

Recombinant allergen research has primarily focused on the identification of novel allergens from previous unreported species³⁵ and has investigated the development of novel allergy-diagnostic platforms to avoid challenges with risky foods.³⁶ BAT with specific allergens has been shown to be a useful diagnostic test for food allergens, which can more rapidly and effectively protect patients from the risk of inducing a severe allergic reaction, especially when skin tests and specific IgE (sIgE) are inconclusive.³⁷ Because the severe clinical symptoms of crab allergies are more suitable regarding basophilic activation, the BAT method of investigating the sensitization of crab allergy *in vitro* is reliable and easily applicable compared to sIgE.²⁸ rMLC1 was able to induce the significant upregulation of CD63 and CD203c, which showed obvious immunogenicity by BAT. Therefore, the successful expression of crab rMLC1 could play an important role in creating intact data and act as a useful tool for diagnostic studies.

Recombinant allergens have also been used for IgE epitope mapping studies.⁴ The acquisition of rMLC1 sequence information can lay a foundation for the exploration of its epitopes. An epitope is defined as the specific chemical group in an antigen molecule that determines the specificity of an antigen. On the basis of the continuity of the amino acid sequence, epitopes are classified as either linear or conformational. According to the report, linear epitopes are involved with immunoreactivity during exposure to gastrointestinal food allergens, because food allergens first interact with the gastrointestinal tract in the body.³⁸ Therefore, linear epitopes are considered to be important and worthy of attention. To research linear epitope mapping, immunoinformatics have already been shown to be an effective method of predicting allergen epitopes.²³ Competitive-binding methods, including iELISA and inhibitory dot blot assay, are rapid, efficient, and an economical means of epitope identification.²⁹ The cross-linking of IgE antibodies requires IgE epitopes on an allergen molecule to activate effector cells (i.e., mast cells).³⁹ As a consequence, a mast cell degranulation assay is a more straightforward method and has greater biological relevance compared to competitive-binding methods.³¹

In this study, seven allergenic crab MLC1 epitopes were predicted and identified via iELISA and human LAD2 mast cell degranulation assay. Subsequently, for the visualization of these epitopes, a surface representation was performed through homology modeling. Figure 7C presents the predicted allergenic crab MLC1 epitopes and allergenic shrimp MLC1 epitopes that have been previously reported by microarray analysis for IgE binding to overlapping synthetic peptides spanning the sequences (exhibited in Figure 7D).²⁰ It was obvious that the predicted allergenic epitopes in crab MLC1 (P1, P2, P3, and P7) were included in the reported shrimp MLC1 epitopes,²⁰ indicating that immunoinformatic methods are reliable and accurate. Crustacean allergens display a high degree of conservation and amino acid sequence identity, leading to the

presence of shared IgE epitopes, which is the primary reason for clinical and immunological cross-reactivity among these species.⁴ The reason for the poor sensitization of P4 and P6 compared to other peptides may be the lack of a high overlapping ratio with shrimp MLC1. Moreover, N- and O-linked allergenic glycoproteins, which have common core structures, are associated with the induction of an IgE response in allergic individuals.⁴⁰ In comparison to the P4 and P6 peptides, P5 continues to have a stronger allergenicity, even if there is no overlapping region between P5 and shrimp MLC1. It is speculated that the reason for this is that the amino acid positions of P5 (96–106) contain N-glycosylation sites (99–101).⁴⁰ Therefore, the relationship between allergenicity and crab MLC1 glycoproteins should be explored in greater depth. In addition, key amino acids in the crab MLC1 epitopes must be further researched, which can expect to reduce allergenicity via the molecular modification of various allergens.

In conclusion, MLC1 was identified to be a novel allergen in mud crab. rMLC1 was obtained by genetic engineering, which showed a typical α -helix structure and strong immunoactivity by dot blot and BAT. Furthermore, the epitope peptides were analyzed, and five critical epitope regions (P1, P2, P3, P5, and P7) that easily cause food allergies were identified with iELISA and LAD2 cell degranulation assay. Increased information regarding crab allergens and allergenic epitopes could be widely applied to allergen investigation as well as diagnostic and safe immunotherapeutic agents for shellfish allergies.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b04294.

Specific IgE levels and symptoms of crab-sensitive individuals (Table S1), effective peptides obtained by MALDI-TOF analysis (Table S2), and allergenic epitopes predicted by five immunoinformatic tools (Table S3) (PDF)

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Notes

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■ ABBREVIATIONS USED

BAT, basophil activation test; CD, circular dichroism; ELISA, enzyme-linked immunosorbent assay; iELISA, inhibition enzyme-linked immunosorbent assay; IgE, immunoglobulin E; IPTG, isopropyl- β -D-thiogalactoside; LAD2, laboratory of allergic diseases 2; MFI, mean fluorescence intensity; MLC1,

myosin light chain 1; nMLC1, native myosin light chain 1; ORF, open reading frame; PBS, phosphate-buffered saline; rMLC1, recombinant myosin light chain 1; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TM, tropomyosin; 3D, three-dimensional

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Table S1. Specific IgE levels and symptoms of the crab sensitive individuals.

Serum No.	Age (years)	Sex ^a	Specific IgE (kU/L) ^b	Symptoms
1	35	M	212.9	Pruritus and urticaria
2	34	F	65.2	Anaphylactic rhinitis
3	27	M	14.7	- ^c
4	31	F	295.1	Chronic urticaria
5	18	F	36.0	- ^c
6	31	F	54.3	Edema
7	37	M	90.9	Nausea
8	24	M	50.8	Allergic purpura
9	6	F	253.6	Sleep apnea
10	23	F	60.5	Atopic dermatitis
11 ^d	23	F	0.1	- ^c
12 ^d	28	M	0.1	- ^c

^a M, male; F, female. ^b Specific IgE ≥ 0.35 (kU/L) is defined as sensitive serum. ^c means no clinical symptoms. ^d Healthy patients' sera (negative control).

Table S2. Effective peptides obtained by MALDI-TOF analysis.

Calc. Mass	Obsrv. Mass	$\pm$ Da	Amino acid sequence	Position
850.4087	850.4821	0.0734	MAADLSAR	1-8
1323.6256	1323.6964	0.0708	FAFSIYDFEGK	15-25
1504.6569	1504.7358	0.0798	GTMDAFYVGDCLR	26-38
1395.8206	1395.8718	0.0512	ALNLTNPTLAVIEK	39-51
1391.7570	1391.7985	0.0415	VDDFLPIFAQVK	65-76
1730.7942	1730.8627	0.0685	DKDAGSFEDFMEVLK	78-92
914.5193	914.5245	0.0052	SELEPVLK	121-128

Table S3. Allergenic epitopes predicted by five immunoinformatic tools.

Tools	Amino acid regions
DNAStar protean	7-13, 23-27, 53-61, 76-85, 94-100, 116-126, 132-137, 144-147
BepiPred 1.0 server	5-10, 21-29, 41-47, 58-61, 77-86, 96-104, 115-121, 131-144
ABCpred	3-19, 29-46, 45-61, 52-68, 68-84, 78-94, 90-106, 104-120, 116-132, 135-141
Immunomedicine Group	14-20, 29-50, 61-75, 87-93, 103-114, 122-132, 138-149
NetMHC II	35-42, 66-77, 109-118