

Cloning, Expression, and the Effects of Processing on Sarcoplasmic-Calcium-Binding Protein: An Important Allergen in Mud Crab

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S Supporting Information

ABSTRACT: Shellfish allergy is a prevalent, long-lasting disorder usually persisting throughout life. However, the allergen information is incomprehensive in crab. This study aimed to identify a novel allergen in crab, show its potential in diagnosis and reduce the allergenicity by food processing. A 21-kDa protein was purified from *Scylla paramamosain* and confirmed as sarcoplasmic calcium binding protein (SCP) by matrix-assisted laser desorption ionization-time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF-MS). Total RNA was isolated from crab muscle, and a rapid amplification of cDNA was performed to obtain an ORF of 579 bp that coded for 193 amino acid residues. According to the results of circular dichroism analysis and ELISA assay, the recombinant SCP (rSCP) expressed in *Escherichia coli* showed similar physicochemical and immunoreactive properties to native SCP (nSCP). Additionally, the extensive cross reactivity of SCP among different species and the bidirectional IgE cross-reactivity between nSCP and rSCP were detected by iELISA. The allergenicity of rSCP was reduced via Maillard reaction or enzymatic cross-linking reaction, which was confirmed by the results of scanning electron microscopy, dot blot, and digestion assay. A straightforward and reproducible way was developed to obtain high yields of rSCP that maintains structural integrity and full IgE reactivity, which could compensate the low specific IgE-titers of most patient sera for future diagnosis. Furthermore, the Maillard reaction and enzymatic cross-linking reaction were effective approaches for the production of hypoallergenic seafood.

KEYWORDS: *Scylla paramamosain*, sarcoplasmic calcium binding protein, expression, diagnosis, allergenicity reducing

INTRODUCTION

The popularity of shellfish has been increasing worldwide, with a consequent increase in adverse reactions that can be allergic or toxic. Shellfish allergy is a long-lasting disorder which causes an allergen-specific immunologic response mediated by IgE antibody.^{1,2} Arginine kinase (AK) and tropomyosin (TM) have been identified as two major allergens of crab and shrimp.^{3–5} Recently, myosin light chain,⁶ hemocyanin,⁷ and sarcoplasmic calcium binding protein (SCP) have been reported to be novel allergens of shellfish.

SCP has been identified as an allergen in *Penaeus monodon* (Pen m 4)⁸ and *Crangon crangon* (Cra c 4),⁹ and the IgE-binding activity of SCP has further been demonstrated at the molecular level in *Litopenaeus vannamei* (Lit v 4).¹⁰ It has been reported that TM and SCP sensitization is associated with clinical reactivity to shrimp.¹¹ In the previously paper, SCP in the crude extracts of mud crab could not be detected by rabbit anticrayfish SCP polyclonal antibody.¹² Therefore, more evidence are needed for the confirmation of the existence and allergenicity of SCP in mud crab.

Recombinant allergens are useful tools for diagnostic studies. They also have the potential to be starting points for dissection of the molecular details whereby allergens effectively cross-link IgE/FcεRI complexes.^{13–15} It has been proposed that only a

few recombinant allergens may be sufficient to detect the vast majority of allergen-specific sensitizations.^{16–18} Component-resolved diagnostics (CRD) is pushing allergology in the coming Era of “Precision Medicine”, an approach integrating novel individual genetic or molecular data for improved geno-/phenotyping and proper selection of personal treatments.¹⁹ It was reported utilization of rJug r 5 for CRD in walnut allergy is of particular importance for the detection of patients with monosensitization to Jug r 5.²⁰ However, the diagnosis of shellfish allergy meets a challenge for the lacking of molecular information in crab allergen.

Food processing has been reported to affect the allergenicity of food products.^{21,22} Maillard reaction and enzymatic cross-linking reaction are efficient methods for reducing the allergenicity of allergens, even in a complex food matrix.^{23–26} It has been found that the IgE/IgG binding properties of hazelnut allergen Cor a 11 could be decreased, resulting from the aggregation promoted by glycation.²⁷ Enzymatic cross-linking reaction of AK by tyrosine/cafeic acid had a high

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potential in mitigating IgE-binding activity and allergenicity.²⁸ The main objective of this work was to identify the physicochemical properties and allergenicity of native SCP in crab and investigate the potential of recombinant SCP (rSCP) in diagnostics that will compensate for the difficulties in purification of native protein and the low sensitivity of allergen in the crude extract. Additionally, the effect of Millard reaction and enzymatic cross-linking reaction on the functional properties and allergenicity of SCP were further evaluated, which might provide information for the developing of hypoallergenic food.

MATERIALS AND METHODS

Chemicals. The tyrosinase from mushroom and 8-anilino-1-naphthalenesulfonic acid (ANS) was purchased from Sigma-Aldrich (Seelze, Germany). Xylose was purchased from Macklin (Shanghai, China). The goat antirabbit IgG antibody or goat antihuman IgE antibody was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD). The Eastep Super Total RNA Extraction Kit (Promega Biological, Shanghai) and the Gene Racer kit (Invitrogen, Karlsruhe, Germany) were used for the gene clone.

Human Sera. Sera were obtained from 22 crustacean-allergic patients (nos. 1–22) provided by the Xiamen Second Hospital (human ethical approval number is XSH2012-EAN019, Xiamen, China). The patients had convincing histories of crab anaphylaxis with clear crab-exposure-related symptoms. Their specific IgE antibodies to crab or/and shrimp were quantified in vitro with ImmunoCAP (Phadia AB, Uppsala, Sweden) in Table 1. All sera were stored at -30°C until analysis.

Myosinogen Immunoassay and Protein Purification. Muscle of *S. paramamosain*, *Eriocheir sinensis*, *Procambarus clarkia*, *Litopenaeus*

vannamei, *Crangon crangon*, *Penaeus monodon*, and *Portunus pelagicus* were minced and homogenized with five volumes of ice-cold 20 mM Tris-HCl buffer (pH 7.0). Then the homogenate was centrifuged at 7200g for 20 min at 4°C , and the supernatant was myosinogen.

The purification of the 21-kDa protein was performed in accordance to Mao et al.,²⁹ with some modifications. In brief, ammonium sulfate was added to the myosinogen of mud crab with increasing saturation from 70% to 90% to precipitate the protein, which was then dialyzed using 20 mM Tris-HCl buffer (pH 7.0) overnight before loading onto a Q-Sepharose column. The elution was carried out with three linear gradients of 0–0.15, 0.15–0.3, and 0.3–0.5 M NaCl at a flow rate of 0.8 mL/min. Fractions that contain the 21-kDa protein were collected and subjected to a Sephacryl S-200 HR gel column, which was pre-equilibrated with Tris-HCl buffer, at a flow rate of 0.4 mL/min. Samples were loaded in 12% SDS-PAGE, and the gels were stained for protein. The potential target protein bands were excised from the Coomassie Brilliant Blue R-250 stained gel and analyzed by Shanghai Applied Protein Technology Company Limited (Shanghai, China).

IgE-immunoblot was performed to validate the target protein. Briefly, protein samples were electrophoretically transferred to a nitrocellulose membrane and blocked with 5% skim milk in TBST (20 mM Tris-HCl, pH 7.0, containing 0.15 M NaCl and 0.05% Tween-20). After washing, the membrane was incubated with the allergenic patients' serum pool (1:4 dilution) as the primary antibody, IgE antibody (diluted 1:20 000) was used as the secondary antibody and the results were visualized by enhanced chemiluminescence (ECL). For dot blot, the purified protein was directly blotted on nitrocellulose membrane and incubated with pooled human sera after blocking.¹² The purified protein was also used to generate a specific polyclonal antibody by subcutaneously injected of adult female New Zealand white rabbit, following the method of Liu et al.³⁰ The specific polyclonal antibody (1:40 000 dilution) was then recruited as primary antibody in the IgG-immunoblot performed below. HRP-labeled goat antirabbit IgG antibody (1:20 000 dilution) was used as the secondary antibody and the results were also visualized by ECL.

Gene Cloning, Structural Modeling, and Recombinant Expression. Total RNA was isolated from muscle tissue of *S. paramamosain* using the Eastep Super Total RNA Extraction Kit. A rapid amplification of cDNA ends (5' RACE) to obtain the 5' cDNA sequence using the Gene Racer kit. 5'-AACCAGAAGATCATGTCC-AACCTC-3' and 5'-GCGTTGGCGGACTCGTCGGGGTTG-3' were used as forward and reverse gene-specific internal primers deduced from the already published SCP cDNA sequence (Acc. No. AEG79568, Acc. No. ACR43475, Acc. No. ACM89179, and Acc. No. BAL72725.1). Full-length cDNA was obtained by PCR using 5'-terminal primer (5'-CTTGTCTCATCAGTAGCGAGCTTG-3') deduced from the results of the 5' RACE and 3'-terminal primer (5'-CAAGACTGTCATTGGTTCGCTGTTTC-3'). Full-length PCR products were purified, and the cDNA was cloned into the pCR4-TOPO vector, expanded in DH5 α cells. Multiple sequence alignment in NCBI of SCP were compared. The analysis tool provided by ExPASy-SIB bioinformatics resource database (<http://www.expasy.org/tools/>) suggested SCP basic properties. DNA STAR Protean and Disco Tope 2.0 Server were used to predict the possible antigen epitopes. The 3D structure of *Amphioxus* SCP (<http://www.rcsb.org/pdb/>, PDB 2SAS) was modeled with swiss model (<http://swissmodel.expasy.org/>) as the reference. The positions of the identified epitopes were assigned to the 3D structure of SCP with pyMOL software (DeLano Scientific, San Carlos, CA).

For the production of rSCP, the primer pair (forward, 5'-TACATATGATGGCTTACTCTTGGGA-3' and reverse, 5'-TAGTCGACTTACTGCACCTCCTTCA-3') was used for PCR amplification. The underlined nucleotides denoted digestion sites for the restriction enzymes Nde I and SalI, respectively. Restriction enzyme digested PCR products were ligated into Nde I and SalI restriction sites in pET-22b. The pET-22b-SCP plasmid was chemically transformed into *E. coli Rosetta*. Single colonies were picked and incubated in LB broth, then induced with 0.5 mM isopropyl-B-D-thiogalactopyranoside for 8 h at 30°C . Cell pellets were

Table 1. Specific IgE Levels in Patients in Relation to Crab or/and Shrimp by ImmunoCAP Testing^a

no.	ID	age (years)	sex	specific IgE (kU/L) crab or/and shrimp	symptom
1	7542	3	M	0.47	bronchial asthma
2	9010	84	M	9.60	allergic purpura
3	2112	29	F	0.70	edema
4	6847	5	M	3.60	bronchitis
5	3703	10	M	1.10	bronchial asthma
6	6432	52	F	1.80	cough
7	2250	4	M	0.80	chronic urticaria
8	6324	85	M	0.47	unknown
9	3362	43	F	0.70	unknown
10	4714	23	F	27.0	type I diabetes
11	7708	16	F	1.80	chronic urticaria
12	5779	16	M	2.0	cough
13	0441	31	M	3.50	sleep apnea
14	4026	49	M	2.20	bronchial asthma
15	0015	69	M	1.80	lungs infected
16	2052	22	F	2.0	sleep apnea
17	5354	3	F	1.10	bronchopneumonia
18	8637	1	M	0.47	respiratory tract infection
19	2238	1	F	28.0	health checkup
20	6907	75	M	2.20	chronic obstructive pulmonary disease
21	5796	83	M	48.50	poor breathing
22	2910	16	M	35.0	atopic dermatitis

^aA serum with the specific IgE > 0.35 (KU/L) is defined as positive. Nos. 1–18, patients' sera positive to crab SCP; nos. 19–22, patients' sera positive to crab and shrimp SCP; M, male; F, female.

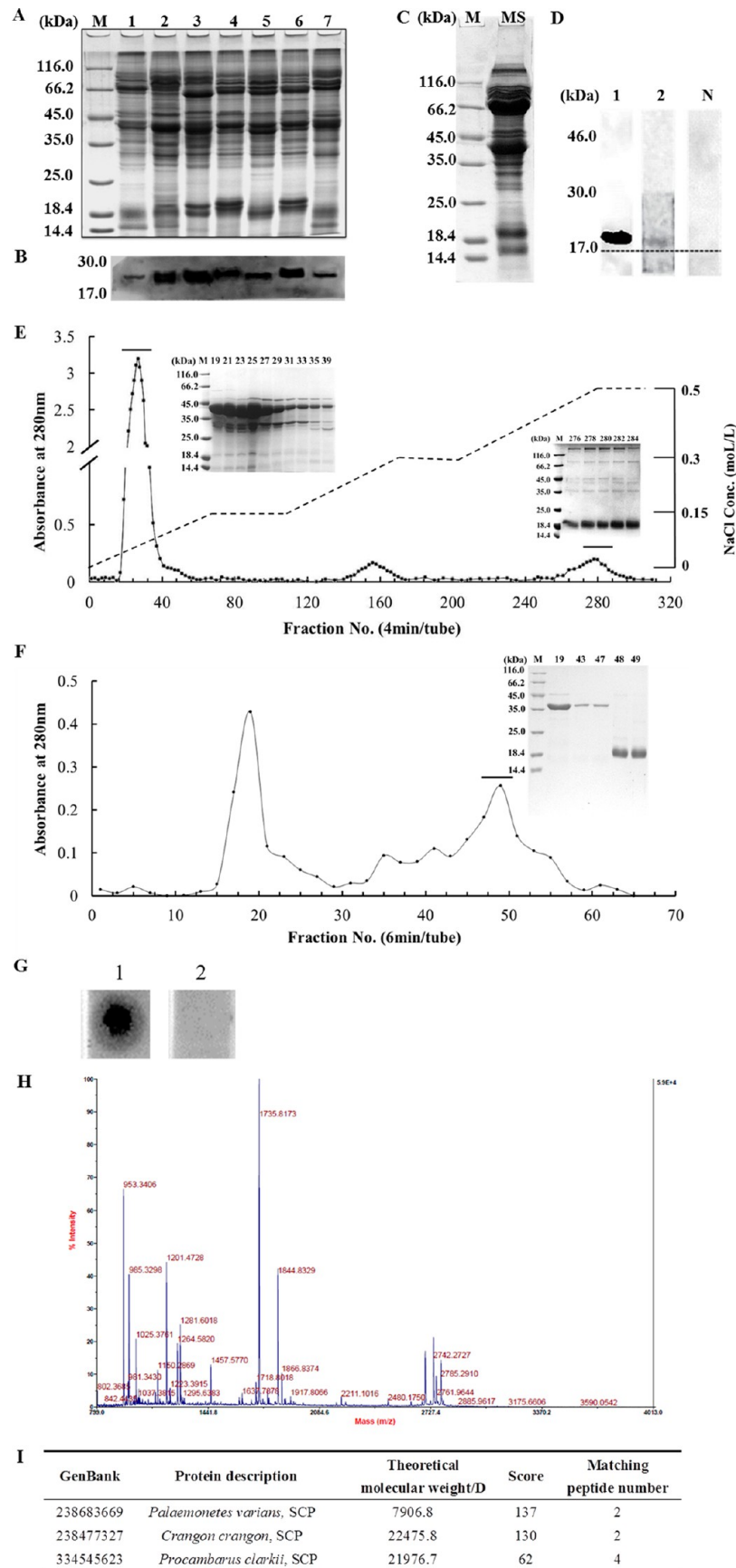


Figure 1. Purification and identification of the 21-kDa protein of crab: (A,B) SDS-PAGE (A) and IgE-immunoblot (B) analysis of myosinogen of different shellfishes (1) *Scylla paramamosain*, (2) *Eriocheir sinensis*, (3) *Procambarus clarkia*, (4) *Litopenaeus vannamei*, (5) *Crangon crangon*, (6) *Penaeus monodon*, (7) *Portunus pelagicus*. Serum Nos. 19–22 were used as a pooled serum; M, protein marker. (C,D) SDS-PAGE (C) and IgE-

Figure 1. continued

immunoblot (D) analysis of myosinogen from *Scylla paramamosain*. MS: SDS-PAGE analysis of myosinogen; (1, 2) immunoblotting profiles of myosinogen, serum nos. 1–2 and 3–4 were used as a pooled serum; N, healthy patients' sera (negative control). (E) Q-Sepharose chromatography. The numbers on the tops of the lanes corresponded to the fraction tube number. (F) Sephacryl S-200 HR gel chromatography. (G) Dot blot analysis of the purified protein: (1) sera of crustacean-allergic patients' pool ($n = 5$, serum nos. 4–8), (2) negative control. (H) Map of mass spectrometry/mass spectrometry (MS/MS). (I) Mascot search results.

harvested by centrifugated, resuspended, and sonicated. Recombinant protein was purified using Sephacryl-200 gel filtration column.

Characterization of nSCP and rSCP. For the investigation of pH and thermal stability, nSCP and rSCP (protein concentration is 0.25 mg/mL) were incubated in buffers at different pH values (1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0) for 1 h or in different temperatures (4 °C, 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90 °C, and 100 °C) for 30 min, followed by SDS-PAGE and IgG-immunoblot analysis.⁵ The samples incubated in pH 7.0 or 4 °C were recognized as controls.

The secondary structure of nSCP was examined by measuring the far-ultraviolet circular dichroism (CD) spectra (180–260 nm) using a CD spectrophotometer (Applied Photo Physics Ltd., Surrey, U.K.) and compared with that of rSCP.³¹ Each spectrum represents a scan at 25 °C with the protein concentration adjusted to 0.25 mg/mL. The effects of temperature on nSCP and rSCP secondary structure were detected from the CD spectra. The operating parameters were as follows: scan rate, interval, bandwidth, temperature range, and heating rate were set to 100 nm/min, 0.25 s, 1.0 nm, 20–100 °C, and 1 °C/min, respectively.

Inhibition enzyme linked immunosorbent assay (iELISA) were performed as described by Fei et al.²⁸ Each well of a polystyrene 96-well ELISA plate (Nunc Maxisorb, Denmark) was coated with the protein (0.2 µg) and incubated at 4 °C overnight or at 37 °C for 2 h. Inhibitors were prepared in 10-fold serial dilutions and preincubated with the same volume of human sera for 2 h. For inhibition ELISA analysis, different SCPs from *L. vannamei* (Lit v 4), *P. monodon* (Pen m 4), *P. pelagicus* (named Por p), and rSCP were purified (data not shown) and tested. The cross-inhibition between nSCP and rSCP were also evaluated.

Enzymatic Cross-Linking Reaction and Maillard Reaction.

The activity of the tyrosinase (1900 nKat/mL) was assayed using 3,4-dihydroxy-L-phenylalanine as the substrate.²⁸ For the cross-linking reaction of rSCP, tyrosinase (400 nKat/g) was incubated with rSCP with 0.25 mM caffeic acid as a mediator at 37 °C for 30 min (the product was named CL-SCP). Maillard reaction was performed as the method of Zhao et al.,³² with modifications: samples were mixed at an SCP-to-xylose ratio of 1:4 (w/w) and incubated at 100 °C for 70 min (the product was named MR-SCP). MR-SCP was dialyzed against 20 mM PBS (pH 7.4) at 4 °C for 16 h and then stored at –30 °C until use.

The processed products were coated with gold and the morphology were observed using a scanning electron microscope (SEM, Phenom Pro, Eindhoven, Holland). The SEM analysis was performed under the following conditions: Mag = $\times 1.0$ k, Vacc = 5 kV, WD = 271 µm, and Signal Name = SE (M). iELISA was performed to analysis the cross-inhibition between rSCP and modified SCPs as described above. The surface hydrophobicities of the samples (0.25 mg/mL) were determined using ANS as the fluorescence probe.³¹ For in vitro stimulated digestion analysis, the final concentration of each sample (rSCP, MR-SCP, and CL-SCP) was adjusted to 0.50 mg/mL, and the simulated gastric fluids (pepsin) were prepared as described previously.⁵ Samples treated in the same way without enzymes were used as a control. SDS-PAGE and dot blot analysis were carried out to determine the digestibility and IgG-binding activity of the samples.

RESULTS

Isolation and Identification of the 21-kDa Protein. The 21-kDa protein was detectable in the myosinogen of all the seven-tested species, which showed positive reaction with

shellfish patients' serum pool (Figure 1A,B), despite the slight difference in their molecular weights. The 21-kDa protein in mud crab can be recognized by the sera of shellfish allergic patients, indicating that the protein has the potential to be an allergen. The target protein was eluted at 0.3–0.5 M NaCl (Figure 1E) on Q-Sepharose column and then separated on Sephacryl S-200 HR gel column as a single band with a molecular weight of 21-kDa in SDS-PAGE (Figure 1F). The IgE-binding activity of the 21-kDa target protein was detected using dot blot, which had a positive result to allergic patients' sera while had a negative result to nonallergic individual. It is notable that the abundance of 21-kDa in mud crab is low (approximately 5.1%) analyzed by the Gel-PRO, and only 1 mg of 21-kDa was obtained from 150 g of crab muscle in the present study.

The purified 21-kDa protein was then identified by matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS). The peptide mass fingerprinting of the purified protein gave multiple peaks ranging from 800 to 4000 Da (Figure 1H). The peaks were compared with the NCBI database. The Mascot score standard ($p < 0.05$) results listed three proteins with high score were *Palaemonetes varians* SCP (Acc. No. ACR54113.1), *C. crangon* SCP (Acc. No. ACR43475.1), and *P. clarkii* (Acc. No. AEG79569.1) SCP, respectively, indicating that the 21-kDa protein obtained in the present study was SCP in mud crab.

Cloning and Expression of SCP. The gene of SCP was cloned, and the sequence was deposited in the GenBank database with accession number JQ860424.1. The cDNA of SCP was 986 bp in full length and the ORF was 579 bp which encodes 193 amino acids (Figure 2A), and the calculated isoelectric point based on the cloned sequence was 4.44. There was a potential N-glycosylation site in the amino acid sequence of SCP (marked with a dashed box), which was identified by the conserved motif Asn-X-Ser/Thr/Cys. The protein sequence alignment showed that the SCPs from different crustacean species shared high similarity. The amino acid sequence of crab SCP was 90%, 82%, 80%, 78%, 79%, and 85% identical to the sequence of *E. sinensis* SCP (Acc. No. AJG01360.1), *C. crangon* SCP, *L. Vannamei* SCP (Acc. No. ACM89179), *Marsupenaeus japonicus* SCP (Acc. No. BAL72726.1), *P. monodon* (Acc. No. BAL72725.1), and *P. clarkii* SCP, respectively (Figure 2B).

The 3D structure showed that SCP has five helix–loop–helix motifs (namely, EF-hand domains), formed two Ca^{2+} binding sites (Figure 2C). Four linear epitopes were predicted (amino acid residues 53–57, 72–76, 90–97, and 140–159, Figure 2D). Four predicted discontinuous regions (42–50, 52–53, 72–74, and 154) were molecularly mapped onto the SCP structure and formed two conformational epitopes (Figure 2E). The residue D₁₅₄ was predicted as an individual region of conformational epitopes and at the same time a member of the predicted linear epitope 140–159, it might be a potential key amino acid.

The recombined expression vector (pET-22b-SCP) was sequenced by standard sequencing (Invitrogen, China). The amino acid sequence of the recombinant protein was totally

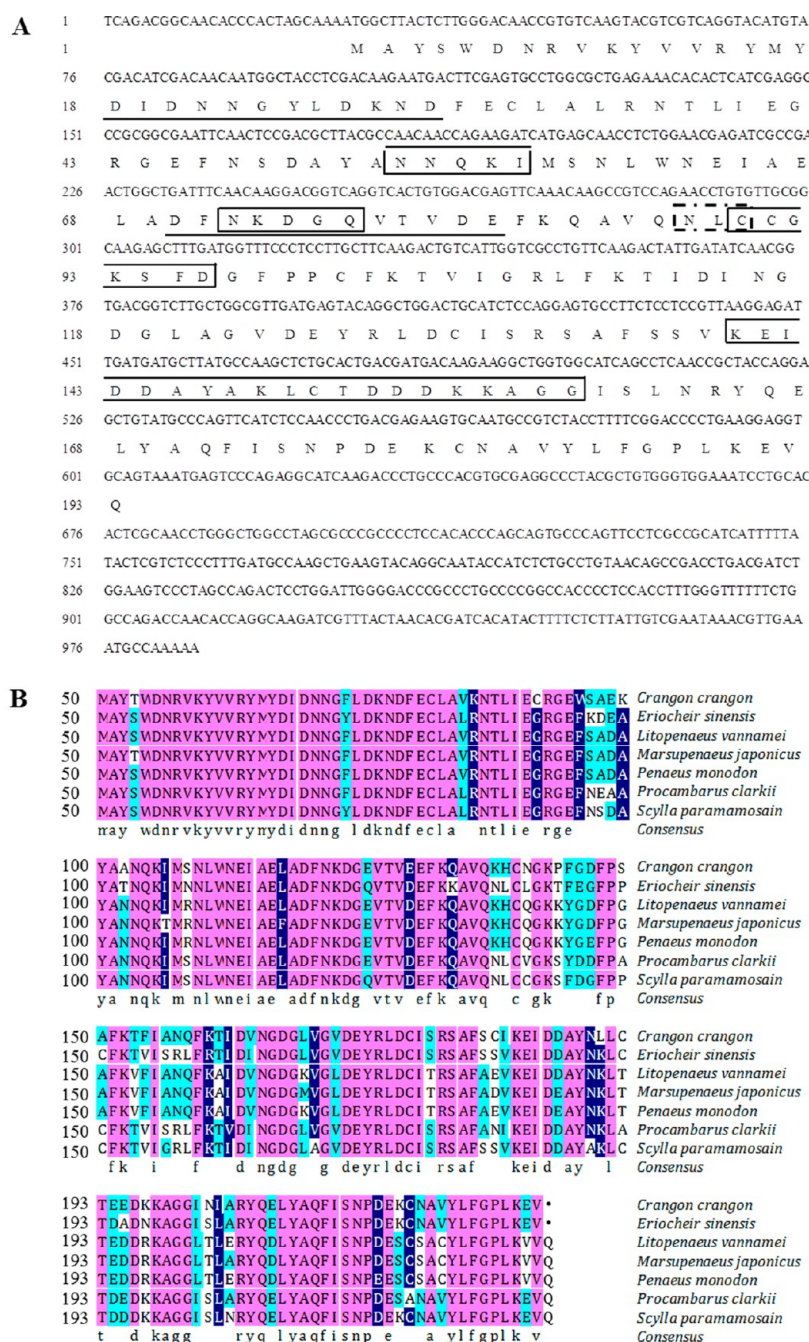


Figure 2. cDNA and deduced amino acid sequence of SCP and multiple sequences alignment results: (A) cDNA and deduced amino acid sequence of SCP. The N-glycosylation site is marked by a dashed box; the horizontal line represents the two calcium binding sites; and the box represents the predicted four linear epitopes. (B) Multiple sequence alignment of SCP between *Scylla paramamosain* and selected invertebrates. (C) Simulated three-dimensional structure of SCP. (D,E) Schematic diagram of SCP linear and conformational epitope regions. The regions of epitopes were labeled using numbers.

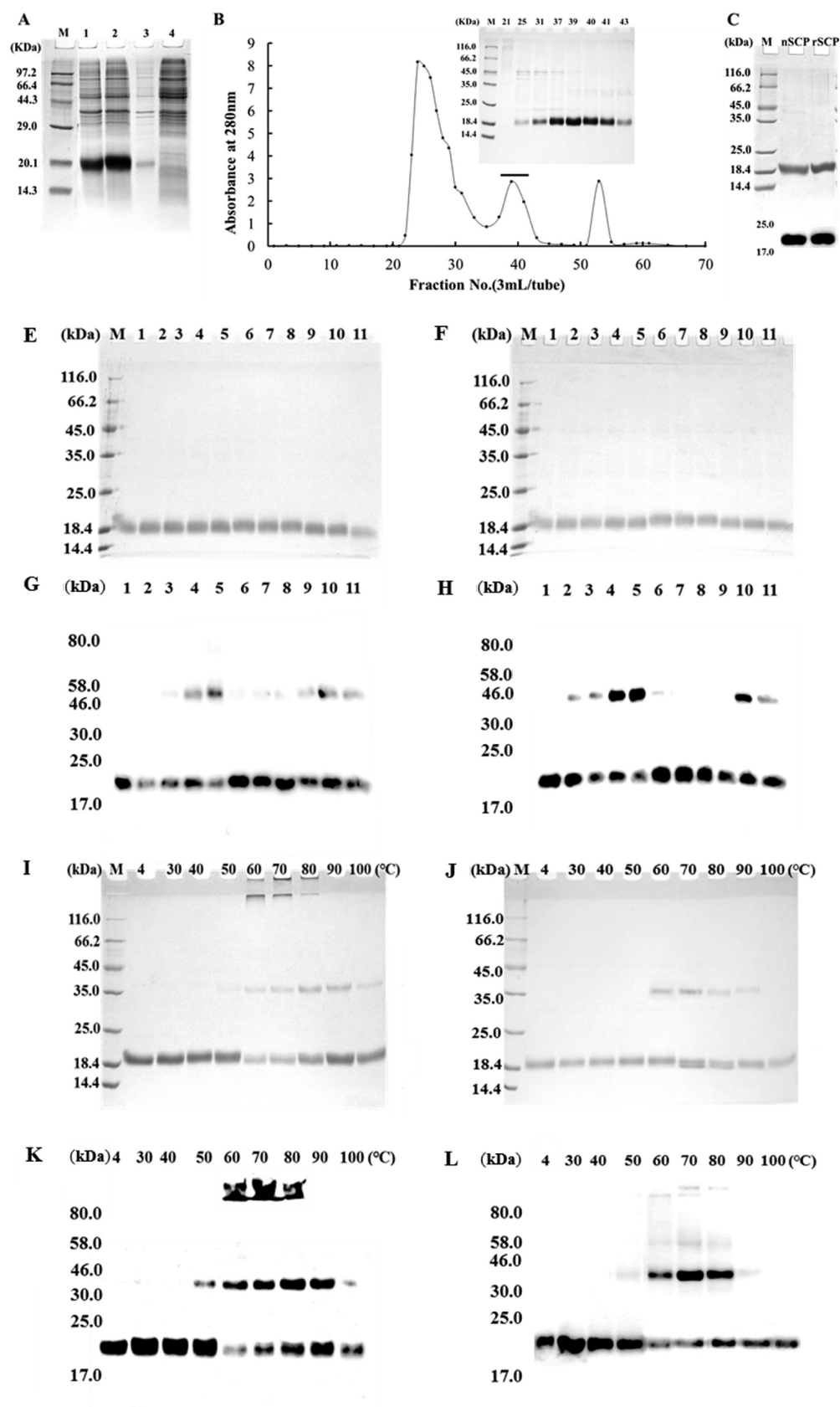


Figure 3. Expression of recombinant SCP and characteristics of SCPs: (A) SDS-PAGE analysis of rSCP. Lane 1, cell lysate; lane 2, lysate supernatant; lane 3, lysate pellet; lane 4, *Rosetta* (control). (B) Purification of rSCP by Sephacryl S-200 HR gel chromatography. (C,D) Specific analysis of anti-SCP polyclonal antibody by SDS-PAGE and immunoblot. (E–H) SDS-PAGE and IgG-immunoblot analysis of the pH stabilities of nSCP and rSCP. (I–L) SDS-PAGE and IgG-immunoblot analysis of the thermal stabilities of nSCP and rSCP.

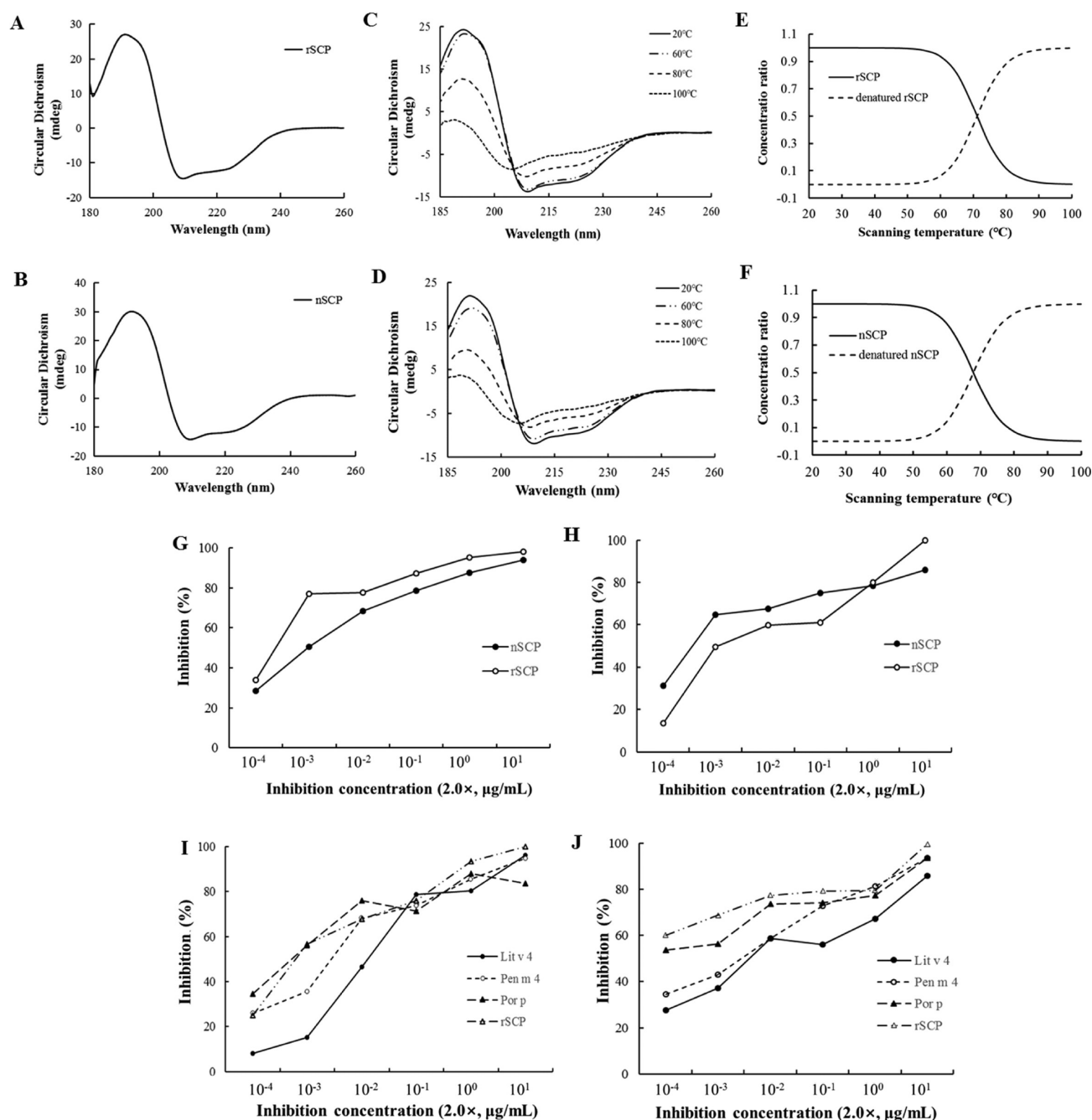


Figure 4. Circular dichroism spectroscopy and iELISA analysis: (A,B) secondary structure analysis of nSCP and rSCP by circular dichroism spectroscopy. (C,D) Effects of temperature on the secondary structures of nSCP and rSCP. (E,F) Determination of the thermal denaturation temperature values of nSCP and rSCP. (G,H) Inhibition ELISA: nSCP (G) and rSCP (H) on the solid phase; IgE-binding of serum pool (serum nos. 9–14) was determined upon dose-dependent inhibition with different SCPs. (I,J) IgE cross-reactivity among Lit v 4, Pen m 4, Por p and rSCP. iELISA was performed using Lit v 4 (I) and rSCP (J) on the solid phase; patient serum pool (serum nos. 19–22) was used.

matched with nSCP (Figure S1 and Table S1). rSCP was present in the supernatant as soluble protein (Figure 3A,B), which was consistent with nSCP in molecular weight and IgG binding activity (Figure 3C,D).

Physicochemical Characterization of SCP. In the pH stability analysis, both of the native and recombinant SCP were relatively stable in different pH (Figure 3E,F), while dimers with IgG-binding activity were formatted in strongly acidic (pH 2.0–5.0) and alkaline (pH 10.0–11.0) conditions (Figure 3G,H). With the temperature increasing, the IgG-binding activities of the original band of nSCP and rSCP decreased

slightly in 100 °C but the polymers retained immunological binding capacity. Both of the native and recombinant SCP were not degraded even when the temperature increased to 100 °C, while nSCP was easier aggregated than rSCP (Figure 3I–L). In above, nSCP and rSCP displayed similar pH and thermal stabilities, and heating had a significant effect on the polymerization of SCP rather than causing its degradation.

The results of CD spectroscopic analysis (Figure 4A,B) showed that nSCP and rSCP have α -helical structures. Both curves were consistent and showed characteristic minima at approximately 210 and 220 nm. With the temperature

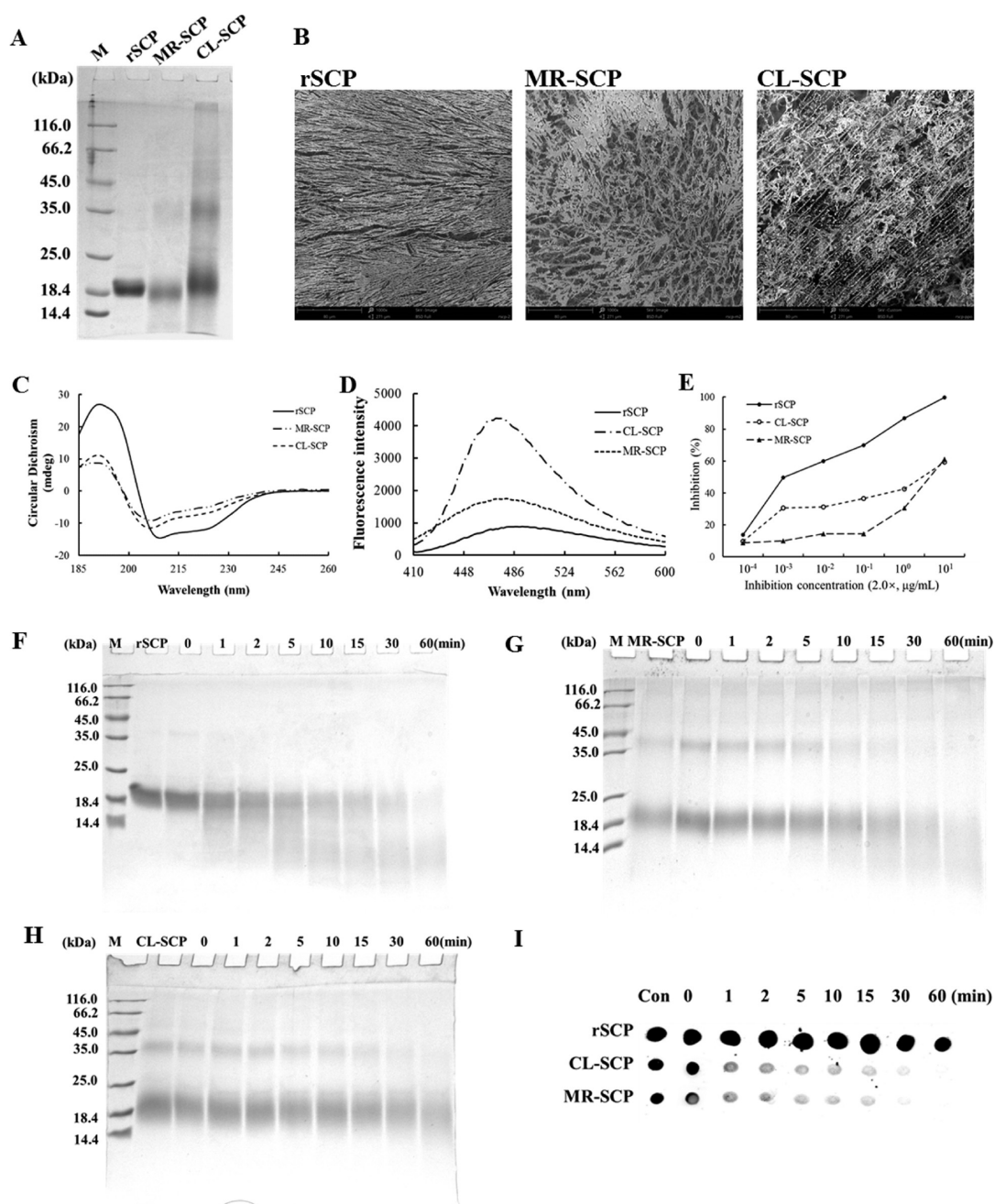


Figure 5. Cross-linking reaction and glycation of rSCP: (A) CL-SCP and MR-SCP were analyzed by SDS-PAGE, (B) scanning electron microscopy analysis, (C) circular dichroism spectroscopy analysis. Resulting spectra were recorded as the average of three scans. (D) Hydrophobicity analysis, (E) IgE cross-reactivity between rSCP and modified SCPs. IgE iELISA was performed using rSCP on the solid phase; patient serum pool (serum nos. 15–18) was used. (F–H) Digestion stability analysis, (I) dot blot analysis. Con: rSCP, MR-SCP, and CL-SCP were untreated with pepsin. Rabbit anti-SCP IgG was used as the primary antibody.

increasing from 20 to 100 °C, the secondary structures of SCP changed significantly (Figure 4C,D). The percentage of α -helix were reduced to 7.12% in nSCP and 8.75% in rSCP, while the β -sheet content in nSCP and rSCP increased to 11.65% and 14.47%, respectively. The thermal denaturation temperatures of nSCP and rSCP analyzed by Global 3.0 software were 69.5 and 70.8 °C, respectively (Figure 4E,F).

iELISA was performed to compare the IgE-binding capacity of rSCP and nSCP, the IC₅₀ of nSCP, rSCP inhibition to nSCP were 3.0 ng/mL, 0.5 ng/mL, while the IC₅₀ of nSCP, rSCP inhibition to rSCP were 1.0 ng/mL, 10.0 ng/mL (Figure 5G,H). The bidirectional IgE cross-reactivity that detected

between nSCP and rSCP indicating that rSCP can be used for further research instead of nSCP. In addition, iELISA was also employed to test the cross-reactivity of SCP from different species. The IC₅₀ of Lit v 4, Pen m 4, Por p, and rSCP inhibition to rSCP were 19.0 ng/mL, 4.0 ng/mL, 0.2 ng/mL, 0.03 ng/mL, respectively. The IC₅₀ of that inhibition to Lit v 4 were 34.0 ng/mL, 6.0 ng/mL, 1.0 ng/mL, 2.0 ng/mL, respectively. Whole proteins have an inhibitory effect to Lit v 4 or rSCP in 10-fold serial dilutions with inhibition rate was up to 80% at the highest inhibitor concentration (Figure 4I,J).

Cross-Linking Reaction and Glycation of rSCP. SDS-PAGE analysis showed that treatment with both xylose and

tyrosinase/cafeic acid caused the polymerization of rSCP. It was reported that the Maillard reaction promoted aggregate formation of the glycosylated protein.³² MR-SCP was extensively modified by glycosylation, with the main band dispersed and a small number of dimers formed, which was consistent with the previous description³³ that the Maillard reaction products were heterogeneous. CL-SCP was extensively cross-linked, with the protein molecular weight over 40 kDa (Figure 5A).

SEM showed the protein aggregates were composed of neat rows of rod-shaped proteins (Figure 5B), which intertwined to form a complex structure. Maillard reaction caused glycosylation led to a loose structure, while the polymerization of CL-SCP generated long-chain proteins resulting in a complex network. After tyrosine or xylose treatment, the positive and negative molar residue ellipticity peaks near 210 and 220 nm of MR-SCP or CL-SCP decreased compared with rSCP (Figure 5C). Further analyses showed that the percentages of α -helix were reduced to 6.74% in MR-SCP and 7.79% in CL-SCP. The contents of β -sheets, at around 198 nm, increased to 12.06% in MR-SCP and 10.95% in CL-SCP. The percentages of random coil, near 220 and 230 nm, increased to 4.09% in MR-SCP and 4.36% in CL-SCP. Furthermore, both MR-SCP and CL-SCP are highly hydrophobicity compared with rSCP (Figure 5D), and the fluorescence intensity peak showed a red shift, with CL-SCP appearing with the highest hydrophobicity.

For the investigation of iELISA, rSCP had greater inhibitory activity than CL-SCP and MR-SCP (Figure 5E). Compared with CL-SCP, MR-SCP has lower inhibitory activity. The IC₅₀ values for rSCP, CL-SCP, and MR-SCP were 8.0 ng/mL, 3.84 μ g/mL, 17.0 μ g/mL, respectively. It implies that modified SCP significantly reduced the activity of IgE-binding, and both the Maillard reaction and cross-linking may be effective methods for allergen desensitization.

The original band of rSCP was digested into small fragments by pepsin within 1 min and the fragments failed to degrade further at 15 min (Figure 5F,G). CL-SCP exhibited more resistance to digestion than rSCP, with the remaining macromolecular band within 15 min and displaying the original band up to 60 min (Figure 5H). MR-SCP was more easily degraded than CL-SCP, but it was relatively stable compared with rSCP. The IgG-binding activity of fragments of CL-SCP and MR-SCP decreased following digestion (Figure 5I), while a little change of rSCP digested fragments was detected.

DISCUSSION

Crustaceans are highly allergenic foods responsible for inducing allergic reactions in both children and adults. However, in spite of the high prevalence of crustacean allergy, there is a lack of full-scale knowledge of allergens and some potential allergens have not been characterized.

In our previous paper, 8 μ g of crude extracts from mud crab was analyzed by Western blot, which showed no IgG-binding activity using rabbit anticrayfish polymorphic SCP polyclonal antibody.¹² It was speculated that the negative result was caused by the low percentage of SCP in mud crab. Actually, the low percentage of SCP (5.1%) in the crude extracts of crab was proved by the Gel-PRO, the loaded quantity was thus adjusted to 20 μ g in the present study and the 21-kDa protein was eventually detectable (Figure 1A). In addition, SCP from mud crab was also recognized by the patients' sera and identified by matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF-MS). Given evidence to the existence of SCP in mud crab, it was

reported that minor allergens are essential in the diagnostics of food allergies, and diagnostic use of Jug r 5 (with a slight abundance has been detected in walnut extract) was proved to compensate for the low sensitivity of walnut extract for patients with birch pollen associated walnut allergy.²⁰ Therefore, SCP might also play an important role in mud crab sensitization which cannot be negligence.

A correctly folded protein was expressed to enable the production of rSCP (around 280 mg of rSCP was produced per liter medium) as a soluble protein in *E. coli*. It was utmost importance that the rSCP is essentially identical to the native protein in IgE/IgG-binding capacities and secondary structures. Moreover, at temperatures below 70 °C, both rSCP and nSCP showed no signs of degeneration but they were more inclined to form polymers, and the polymer content was highest when the temperature was around 70 °C. This corresponded to the effect of temperature on the secondary structures of rSCP and nSCP as detected by CD spectroscopy. The denaturation temperatures of rSCP and nSCP were around 70 °C, and their structure tend to be changed at the temperature over 70 °C.

Allergenicity was closely related to some specific peptide sequences, namely, allergy epitopes.³⁴ It was reported that N- and O-linked allergenic glycoproteins, which have common core structures, are associated with the induction of an IgE response in allergic patients.³⁵ The N-glycosylated sites of SCP partially overlapped the amino acid positions of a linear epitope (amino acid residues 90–97). Therefore, the relationship between the allergenicity and glycosylation of SCP warranted further research. Furthermore, the three-dimensional structure of SCP was constructed to predict the conformational epitopes (Figure 2C–E). The 52–53, 72–74 regions and amino acid residues (D₁₅₄) comprise both linear and conformational epitopes. IgE antibodies to certain epitopes of shrimp AK could be used as biomarkers for the prediction of clinical reactivity in subjects with sensitization to shrimp.³⁶ Therefore, future studies should assess the clinical reactivity in subjects with sensitization to crab using the predicted epitopes in the present study.

Few studies have been conducted on the effects of processing conditions on SCP. Tyrosinase forms new bonds between tyrosine residues and reactive benzoquinone intermediates in proteins, which may introduce both intra- and intermolecular covalent cross-links.³⁷ There is one tyrosine located in one of the linear epitope (amino acid residues 140–159) of SCP, as a consequence, the tyrosine in the epitope is involved in a cross-linking reaction, which might lead to the changing and masking of the allergic epitope and result in a significant decrease in the allergenicity of the cross-linked SCP. In addition, the results of SEM showed that the glycosylated samples were aggregated to form interconnected structures similar to a report on β -lactoglobulin.³⁸ The formation of such a network structure might block the access of immunoglobulins/enzymes to the epitopes/cleavage sites, which might decrease the immunoreactivity or increase the stability of MR-SCP.

The fluorescence intensity of CL-SCP was significantly higher than rSCP, while the fluorescence intensity of MR-SCP changed only slightly compared to rSCP. These results indicated that cross-linking of rSCP might have exposed previously buried hydrophobic regions to the protein surface, thus increasing the extent of hydrophobic interactions and promoting protein interactions and aggregation. The paper reported that there was a strong negative correlation between gastric pepsin activity and protein surface hydrophobicity.³⁹ It

may own to the increased hydrophobicity and formation of aggregation preventing access of the pepsin to its cleavage sites. Our similar observation of increased surface hydrophobicity and decreased digestibility after cross-linking can be explained by this model as well.

Food allergens sometimes remained allergenic during gastrointestinal-like digestion and caused anaphylaxis. In the present investigation, rSCP and MR-SCP were digested rapidly, although the cross-linked form of CL-SCP was resistant to digestion. After digestion, rSCP, MR-SCP, and CL-SCP were broken down into small peptides. Although rSCP appeared to be an easy target for gastric digestion, the IgG-binding activity of the small peptides retained a high potential for allergenicity. However, the IgG-binding activities of the small peptides of digested CL-SCP and MR-SCP were significantly reduced. Thus, Maillard reaction or cross-linking reaction seemed to modify, mask, or destroy the allergic epitopes, and then the effect remained after digestion. In our previous study, enzymatic cross-linking reaction was an effective way to reduce the allergenicity of TM, AK in crab.^{28,31} Furthermore, on the basis of the enzymatic cross-linking reaction and Maillard reaction, a kind of hypoallergenic crab meat product was developed and a Chinese patent was applied for (No. 2015108074188, No. 201610455642.X, and No. 201610182552.8).

It appears crucial to include rSCP in future diagnostic and therapeutic strategies. The present study indicates that rSCP has the potential to completely replace nSCP in food allergy research with the bidirectional IgE cross-reactivity between nSCP and rSCP. An association of tropomyosin sensitization accounts for the extensive cross reactivity observed among crustaceans, arthropods, and invertebrates,⁴⁰ and SCP in different shellfishes showed the extensive cross reactivity. In addition, *S. paramamosain* is the commonly consumed crab in western Pacific and especially in China, with 150 000 tons of aquaculture production and 84 000 tons of fishing production in 2015,⁴¹ *S. paramamosain* SCP study would representative among crustaceans, arthropods, and invertebrates. What's more, the low abundance of SCP makes it difficult to be purified and may not adequately presented in natural protein extracts, thus resulting in the low specific IgE-titers of most patient sera (Table 1). More importantly, we found 10 of 61 patients' IgE negatively reacted to crab extracts (Table S2) but with clinical allergy symptoms and their serum were positive to SCP detected by dot blot (Figure S2). It may due to the low sensitivity of crab extract for patients. However, it can clearly demonstrate the need for the identification of marker allergens for a more reliable diagnosis.

Taken together, a straightforward and reproducible way was developed to obtain high yields of rSCP that maintains its structural integrity and full IgE reactivity, so it may be used for future diagnostic. Moreover, Maillard reaction or enzymatic cross-linking reaction can be used for industrial production of hypoallergenic seafood. In future investigations, it is important to examine the effects of MR-SCP and CL-SCP in vivo.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b02381.

Additional experimental details (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

ANS, 8-anilino-1-naphthalenesulfonic acid; CD, circular dichroism; CL-SCP, cross-linked sarcoplasmic calcium binding protein with tyrosinase; CRD, component-resolved diagnostics; iELISA, inhibition enzyme linked immunosorbent assay; MR-SCP, Maillard reacted sarcoplasmic calcium binding protein with xylose; nSCP, native sarcoplasmic calcium binding protein; rSCP, recombinant sarcoplasmic calcium binding protein

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Supporting Information

Cloning, expression, and the effects of processing on sarcoplasmic-calcium-binding protein, an important allergen in mud crab

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Running title: Clone, expression and the effect of food processing on SCP.

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Supporting methods

LC-MS/MS analysis

LC-MS/MS analysis was performed using a LTQ mass spectrometer (Thermo El-njectron, Bremen, Germany) coupled with a nanoelectrospray ionization source. Samples were loaded onto a reversed-phase C18 column with a diameter of 75 $\mu\text{m} \times 15\text{ cm}$ and particle size of 3 μm . Separation of the peptides was performed using a gradient going from 5 to 40% acetonitrile in 0.1% formic acid over 60 min. The raw wiff map files were collected by MS/MS. Data processing and retrieval analysis using Protein Pilot Software v. 4.5 software (AB SCIEX, USA) based on Paragon algorithm. Peptides were searched using the following parameters: peptide tolerance, 2.0 amu; enzyme, trypsin (KR/P); enzyme limits, fully enzymatic (cleaves at both ends); Protein search unused score > 1.3 as a credible result, the peptide confidence is greater than 95% for reliable sequences. Sample identification of specific cases such as the results of the excel form.

Figure S1. Identification of the amino acid sequence of the purified recombinant protein. A, Protein sequence alignments of SCP from *S. paramamosain*; B, Peptide information and secondary mass spectrometry.

A	AFJ80778.1	MAYSWDNRVKYVFR	MYDIDNNGYLDKNDFECLALR	NTLIEGRGEFNSDA	50
	rSCP		MYDIDNNGYLDKNDFECLALR	GRGEFNSDA	31
	Consensus		ymydidnngyldkndfeclalr	grgefnsda	
	AFJ80778.1	YANNQRKIMSNLWNEIAELADFNKDGQVTVDFFKQAVQNLCCKGSFDFGFP			100
	rSCP	YANNQRKIMSNLWNEIAELADFNKDGQVTVDFFKQAVQNLCCKGSFDFGFP			81
	Consensus	yannqkimsnlwneiaeladfnkdqgvtvdefkqavqnlccgksfdgfpp			
	AFJ80778.1	CFKTVIGRLFKTIDINGDGLAGVDEYRLDCISRSASFSSVKEIDDAYAKLC			150
	rSCP	CFKTVIGRLFKTIDINGDGLAGVDEYRLDCISRSASFSSVKEIDDAYAKLC			131
	Consensus	cfkvtvigrifktidingdglagvdeyrlcdcisrsafssvkeiddayaklc			
	AFJ80778.1	TDDDKKAGGISLNRYQELYAQFISNPDEKCNNAVYLFGLKEV			192
	rSCP	TDDDKKAGGISLNRYQELYAQFISNPDEKCNNAVYLFGLKEV			173
	Consensus	tdddkkaggislnryqelyaqfisnpdekcnnavylfgplkev			

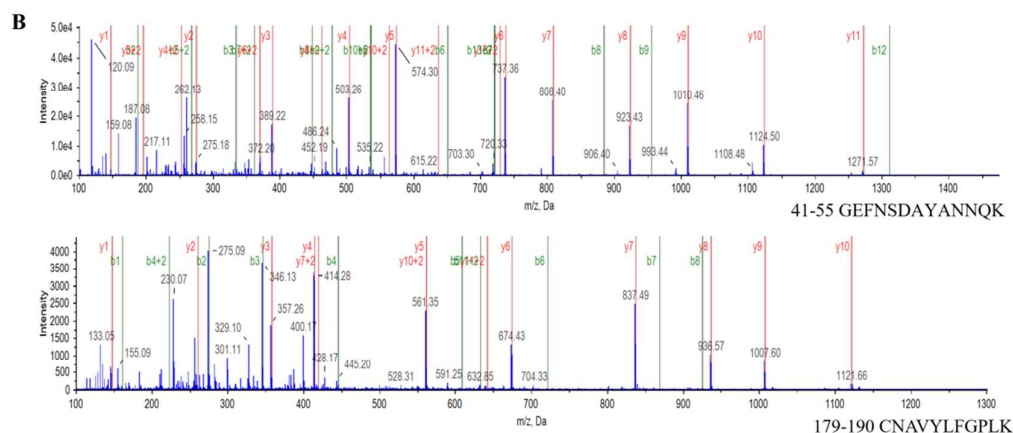


Table S1

Effective peptides from mass spectrometry (MS) compared with *S. paramamosain* SCP

Calc.Mass	Obsrv.Mass	$\pm$ da	Start Seq.	End Seq.	Sequence
3388.713	3388.916	0.186	14	35	YMYDIDNNGYLDKNDFECLALR
1668.807	1668.854	0.047	41	55	GRGEFNSDAYANNQK
4284.329	4284.629	0.300	56	92	IMSNLWNEIAELADFNKDGQVTVDEFKQAV QNLCCGK
1726.962	1727.074	0.112	93	107	SFDGFPPCFKTVIGR
2123.156	2123.222	0.066	108	126	LFKTIDINGDGLAGVDEYR
1369.69	1369.725	0.035	127	139	LDCISRSAFSSVK
1839.95	1840.026	0.076	140	155	EIDDAYAKLCTDDDKK
2612.456	2612.625	0.169	156	178	AGGISLNRYQELYAQFISNPDEK
1636.917	1637.006	0.089	179	190	CNAVYLFGLPK

Table S2.

Specific IgE levels in patients in relation to crab analysis by ImmunoCAP testing.

No.	ID	Age (years)	SEX	Specific IgE (kU/L)	Symptom
				Crab	
1	8860	31	M	< 0.35	Lung shadows
2	5526	31	M	< 0.35	Bronchial asthma
3	6571	1	M	< 0.35	Bronchial asthma,
4	1060	37	F	< 0.35	Cough variant asthma
5	6721	2	M	< 0.35	Bronchial asthma,
6	5862	3	F	< 0.35	Allergic purpura
7	9791	1	M	< 0.35	Cough
8	2393	<1	M	< 0.35	Congenital throat wheezing
9	8149	3	M	< 0.35	Edema
10	8725	3	M	< 0.35	Bronchitis

*A serum with the specific IgE > 0.35 (KU/L) is defined as positive. M, male; F, female.

