

Physicochemical characterization and identification of major linear epitopes of sarcoplasmic calcium-binding protein (SCP) allergen from Pacific oyster (*Crassostrea gigas*)

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Abstract

BACKGROUND

Food allergy is a serious public nutritional health problem that has attracted extensive worldwide attention. Shellfish allergy is a long-lasting disorder that has a lifelong impact on health. Sarcoplasmic calcium-binding protein (SCP) plays a vital role in cell and muscle functions and has been identified as an allergen in oyster.

RESULTS

In this study, recombinant SCP (rSCP) with a molecular mass of 21 kDa was produced and identified based on SCP amino acid sequencing of Pacific oyster (*Crassostrea gigas*), and was used as a follow-up experimental material. Its physicochemical characterization showed that purified rSCP is highly stable to heat and acid-alkali and trypsin digestion but less resistant to pepsin digestion. We established an animal sensitization model and rSCP displayed stronger Immunoglobulin E (IgE)-binding activity with rat serum in the rSCP + cholera toxin (CT) group compared with the CT group and a control group. Five epitope peptides were identified as linear immunodominant epitopes by indirect competitive enzyme-linked immunosorbent assay (icELISA) for the first time. We also found that conformational epitopes may play a major role in the immunoreactivity of SCP.

CONCLUSION

These results are significant for understanding hypersensitization of humans to oyster and offer available preventive measures and treatment programs in further research. © 2021 Society of Chemical Industry.

INTRODUCTION

With the rapid development of industrial technology, preferences for food types and diets have changed dramatically. The number of people with food allergies is also increasing. This ultimately affects people's quality of life and safety. Food allergies are mainly caused by the eight major sources of allergenic food proposed by the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO),¹ especially aquatic products. Fish and shellfish are popular because of their high nutritional value and flavor. A shellfish is defined as any edible marine invertebrate, including crustaceans and mollusks. It has been widely accepted that reactions to shellfish allergies continue throughout life, and they are often associated with severe symptoms, even life-threatening anaphylaxis,² which is different from allergies caused by eggs and milk.³ Ten kinds of shellfish allergens have been identified: tropomyosin (TM), arginine kinase (AK), myosin light chain (MLC), troponin C, triosephosphate isomerase (TIM), sarcoplasmic calcium-binding protein (SCP), fatty acid binding protein (FABP), hemocyanin, filamin (FLN c), and paramyosin (PM).⁴⁻¹³ Tropomyosin and SCP have been identified as allergens in oyster.¹⁴ The Pacific oyster, *Crassostrea gigas*, makes up about half of oysters consumed in the world.¹⁵ The increased consumption of oyster has been accompanied by more frequent allergic reactions.

Sarcoplasmic calcium-binding protein is an invertebrate EF-hand calcium-binding protein, which is expressed most strongly in skeletal muscles. It has been proposed that it fulfills a similar function in muscle relaxation to vertebrate parvalbumin (PV),¹⁶ which is also an EF-hand Ca^{2+} -binding protein established as a major fish allergen.¹⁷ Currently, SCP has been identified as an allergen in *Penaeus monodon* (*Pen m* 4) and *Crangon crangon* (*Cra c* 4),^{17, 18} and the Immunoglobulin E (IgE)-binding activity of SCP has further been demonstrated at the molecular level in *Litopenaeus vannamei* (*Lit v* 4).¹⁹ Sarcoplasmic calcium-binding protein is a major allergen that can be detected by the serum of more than 50% of shellfish allergic patients.^{9, 19} For some people, SCP may be more important than tropomyosin as a shellfish allergen.² As described above, SCP is an important and interesting allergen of shellfish and more in-depth research of oyster SCP should be conducted. Studies have shown that recombinant sarcoplasmic calcium-binding protein (rSCP) has the same IgE reactivity as natural SCP (nSCP).²⁰ Recombinant sarcoplasmic calcium-binding protein is a useful tool for studying shellfish allergens and in the diagnosis of shellfish allergy. We aimed to characterize and produce *C. gigas* rSCP to be used as diagnostic and immunotherapeutic agents for subjects with oyster allergy.

In this study, the stability of rSCP was analyzed by heat treatment, acid-alkali treatment, and simulated gastrointestinal digestion. The antiserum of brown Norway (BN) rats was prepared by the immunization of expressed rSCP and used to identify linear epitopes. The study also used several typical chemical reagents to change the specific structure of SCP to explore its allergenicity alternation.

MATERIALS AND METHODS

Expression, purification and identification of rSCP

According to the gene sequences of the SCP reported by UniProt (Gene name: CGI_10006806), the complete sequence of SCP was designed and synthesized by GenScript Inc., (Nanjing, China) and cloned into the pET-30a(+) vector, then transferred into *E.coli* BL21(DE3). The plates were cultured overnight to screen the positive clones and obtain the recombinant plasmids.

Three strains were selected and inoculated into 4 mL LB tubes containing 50 $\mu\text{g mL}^{-1}$ kanamycin (Biotopped Co. Ltd., Beijing, China). When the optical density at 600 nm (OD_{600}) reached 0.6 ~ 0.8, 0.5 $\text{mmol}\cdot\text{L}^{-1}$ isopropyl- β -D-thiogalactoside (IPTG) (Biotopped Co. Ltd., Beijing, China) was added and the cells were cultured at 15 °C for 16 h or 37 °C for 4 h. After determining the optimal expression condition, the bacteria cells were collected by centrifugation, resuspended in dissolving solution (20 $\text{mmol}\cdot\text{L}^{-1}$ Tris-HCl, 200 $\text{mmol}\cdot\text{L}^{-1}$ NaCl, pH 8.0) and the supernatant was collected by ultrasound and centrifugation. Next, the supernatant was loaded into a Ni^{2+} charged Hi-Trap chelating HP column of an ÄKTA-fast protein liquid chromatography (FPLC) system (GE Healthcare, Stockholm, Sweden). After washing the column, the bound protein was eluted with a linear 20–500 $\text{mmol}\cdot\text{L}^{-1}$ gradient of imidazole in the same buffer. To further purify the protein, the concentrated elution was applied onto a gel-filtration Hi-load Superdex-75 column equilibrated with a buffer containing 50 $\text{mmol}\cdot\text{L}^{-1}$ Tris-HCl, 100 $\text{mmol}\cdot\text{L}^{-1}$ NaCl, pH 7.5. All purification procedures described here were performed at 4 °C.

To analyze the accuracy of the rSCP further, the target protein was excised from sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and subjected to in-gel tryptic digestion. The digests were sent to the Institute of Biophysics, Chinese Academy of Science (Beijing, China) and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS, Bruker, Karlsruhe, Germany). The mass spectrum conditions were as follows: the wavelength was set at 337 nm and the acceleration voltage was 25 kV; the positive ion mode was used to obtain the data automatically. The peptide mass fingerprint of target protein was compared using the Mascot search tool in the NCBI database.

Extraction and purification of nSCP

Pacific oysters were purchased from a local market. The purification of nSCP was carried out based on the method described by Chen *et al.*⁵ with some modifications. The adductor muscle of oyster was chopped and dissolved in ten times volume of pre-cooled buffer A (50 $\text{mmol}\cdot\text{L}^{-1}$ NaCl, 2 $\text{mmol}\cdot\text{L}^{-1}$ NaHCO_3 , 10 $\text{mmol}\cdot\text{L}^{-1}$ Ethylene Diamine Tetraacetic Acid (EDTA)), which was homogenized by high-speed homogenizer and centrifuged (10 500 $\times$ g, 20 min, 4 °C) to get the supernatant. Then 75–90% ammonium sulfate salting out and centrifugation (10 500 $\times$ g, 40 min, 4 °C) were carried out to collect precipitation, and the precipitate was dissolved in a small amount of buffer B (10 $\text{mmol}\cdot\text{L}^{-1}$ Tris HCl, 0.1 $\text{mmol}\cdot\text{L}^{-1}$ EDTA, pH 8.0) and dialyzed in dialysate (10 $\text{mmol}\cdot\text{L}^{-1}$ Tris HCl, pH 8.0, 4 °C) for 16 h. Finally, the HiTrap-Q column (GE Healthcare, Stockholm, Sweden) was balanced with buffer B, and subsequently

used to loading sample. The adsorbed part was eluted with salt-containing buffer B and then dialyzed again to obtain purified protein. Protein was analyzed on SDS-PAGE and stored at -80°C until use.

Stability analysis of the rSCP

To investigate thermal stability, purified rSCP was incubated at different temperatures (0, 30, 40, 50, 60, 70, 80, 90, and 100°C) for 30 min. For pH stability, rSCP was incubated in buffers with different pH values (pH 1.0, 2.0, 4.0, 5.0, 7.0, 8.0, 10.0, and 11.0) for 1 h at room temperature. After incubation, all the samples were analyzed by SDS-PAGE. The sample incubated in 0°C or pH 8.0 was a control.

The *in vitro* stability analysis of the target protein was performed in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) following the method described by Huang *et al.*²¹ with some modifications. The total volume of the reaction solution was 1 mL. For gastric digestion, the ratio of pepsin to the target protein was 1:200 (w:w). An aliquot of $100\ \mu\text{L}$ of the reaction solution was removed after different time intervals (0, 1, 3, 7, 15, 30, 45, and 60 min), and the reaction was immediately terminated by the addition of $300\ \text{mmol}\cdot\text{L}^{-1}\ \text{Na}_2\text{CO}_3$. For intestinal digestion, the ratio of pancreatin to the target protein was 1:200 (w:w). The reactions were performed for 0, 5, 15, 30, 60, 120, 180, and 240 min, then they were immediately terminated by heating at 95°C for 5 min. A target protein sample dissolved in SGF/SIF with no corresponding enzyme was used as the undigested control. The final concentration of each sample was adjusted to $0.3\ \text{mg}\ \text{mL}^{-1}$ and analyzed by SDS-PAGE.

Secondary structure analysis of the rSCP

The secondary structure of rSCP was examined by measuring the far-ultraviolet circular dichroism (CD) spectra (180–260 nm) using a CD spectrophotometer (Applied Photophysics Ltd., Leatherhead, UK). Samples were measured in the region between 190 and 250 nm. The specific conditions were as follows: samples were scanned with a 0.2 nm step increment and 2.0 nm bandwidth, and the scanning speed was $200\ \text{nm}\ \text{min}^{-1}$. The baseline buffer spectrum was subtracted from each spectrum and the resultant values were converted to molar ellipticity (θ) using the built-in analysis function in the instrument's software. Experiments were performed in triplicate and their average values were taken as the final results.

Animal sensitization and tissue staining

Sixty $\times$ 6-week-old female specific pathogen free (SPF) BN rats were purchased and allowed to settle in for 7 days. The rats were then randomly divided into a phosphate buffered saline (PBS) control group, a cholera toxin (CT) (Beyotime Co. Ltd., Shanghai, China) group, and an rSCP + CT experimental group (20 rats in each group) to establish the animal sensitization model. Negative serum was obtained before immunization. Rats in the experimental group received 1 mL rSCP ($1\ \text{mg}\ \text{mL}^{-1}$) and $10\ \mu\text{g}$ CT on days 5, 13, 21, and 29 by gavage, on days 1, 9, 17, 25 by intraperitoneal injection, and blood samples were collected on days 9, 17, 25,

and 33, 4 h after the immunization to determine the polyclonal antibody levels by immunoblot analysis, during which five rats in each group were taken for testing. Rats in the CT group were only injected or gavaged with 10 μg CT (dissolved in 1 mL PBS) and those in the control group were only injected or gavaged with 1 mL PBS. Other conditions were no different from the experimental group. A serum pool from sensitized rats was made by mixing equal aliquots of serum IgE. On day 33, all sensitized rats were challenged with 1 mL rSCP (2 mg mL⁻¹) and 10 μg CT by intraperitoneal injection and gavage concurrently, and rats in the CT group were given 10 μg CT (dissolved in 1 mL PBS) by intraperitoneal injection and gavage simultaneously. The same operation was conducted in the control group. Small intestinal tissue and liver tissue were taken for hematoxylin–eosin (HE) staining to observe pathological changes and for toluidine blue (TB) staining to observe the degranulation of jejunal mast cells after challenge. The experiments were approved by the medical ethics committee at the China National Research Institute of Food and Fermentation Industries.

Epitope prediction

To predict the IgE linear epitope regions of oyster SCP more accurately, five computational methods based on immunoinformatics were used to analyze the complete amino acid sequence of SCP: the DNA star Protein system, ABCpred, Bepipred 1.0 Server, the Immunomedicine Group, and Immune Epitope Database (IEDB) Analysis Resource. In the DNASTar Protean system, the prediction of linear epitopes is mainly based on the hydrophilicity, surface accessibility, and flexibility and antigenicity of the amino acid sequence. BepiPred 1.0 Server could provide more direct epitope information according to the physicochemical properties of sequences. Through comprehensive analysis of prediction results and immunoinformatics parameters, we were able to select possible linear epitopes of oyster SCP. To further identify the predicted epitope peptides, they were synthesized by the Fmoc solid-phase method (GL Biochem Ltd., Shanghai, China). The purity and accuracy of the synthesized peptides were analyzed by high-performance liquid chromatography (HPLC) and electrospray ionization-mass spectrometry (ESI-MS) (purity $\geq 98\%$).

Immunoactivity assay

A direct enzyme-linked immunosorbent assay (ELISA) was conducted following the method described by Fang *et al.*²² The rSCP was diluted to 25 μg mL⁻¹ with carbonate buffer solution (CBS), 100 μL of which was placed in a 96-well plate (Corning Inc., NY, USA) at 4 °C overnight. The 96-well plate was washed with PBS-Tween 20 (PBST) for four times, then incubated with 350 μL 5% skim milk (Beyotime Co. Ltd., Shanghai, China) for 1 h at room temperature. The pooled, sensitized BN rats' serum diluted with 2% skimmed milk (100 μL , diluted 1:100) was added into the well as a primary antibody, and the goat anti-rat IgE-HRP antibody (100 μL , diluted 1:1000, Abnova, NY, USA) was incubated as a secondary antibody. 3, 3', 5, 5'-Tetramethylbenzidine (TMB, 200 μL) (Beyotime Co. Ltd., Shanghai, China) solution was added to the well for 30 min in the dark, and the reaction was terminated by 50 μL 2 mol·L⁻¹ H₂SO₄. The OD values were read at 450 nm by the spectrophotometer (Dy nex Technologies

Inc., VA, USA).

Indirect competitive ELISA (icELISA) was conducted following the method described by Mita *et al.*²⁰ with some modifications. The rSCP or epitope peptides as the inhibitors were diluted with CBS solution and the pooled sensitized BN rats serum was diluted with 2% skim milk (50 μ L, 1:100 dilution), then both were pre-incubated at 4 °C overnight in equal volume. Meanwhile, the original rSCP was diluted to 25 μ L mL⁻¹ with CBS solution, 100 μ L of which was placed in a 96-well plate at 4 °C overnight. After washing, the 96-well plate was incubated with 350 μ L 5% skimmed milk and then the inhibitor mixture was added. The other procedure was the same as that of the ELISA described above. The inhibition rates were calculated as follows:

$$\text{inhibition rate (\%)} = \frac{(\text{OD}_{\text{positive control}} - \text{OD}_{\text{experimental group}})}{\text{O. D.}_{\text{positive control}} \times 100\%}$$

(1)

Epitope mapping and amino acid multiple sequence alignment

The crystal structure of SCP from *Doryteuthis pealeii* (PDB code 2ccm) was used as the template for homology-based modeling. The positions of the identified epitopes were mapped into the 3D structure of SCP with PyMOL software (<http://www.pymol.org/>, DeLano Scientific, CA, USA). To study the homology of amino acid sequences among different mollusks and crustaceans, the AA-sequence alignment was carried out by comparing the AA-sequence of SCP with representative shellfish SCPs through DNAMAN software (Lynnon Corporation, Vaudreuil, Canada). All sequence databases were obtained from the National Centre for Biotechnology Information (NCBI).

Denaturation of the SCP

The purified rSCP concentration was adjusted to 0.25 mg mL⁻¹ and mixed with denaturants (sodium dodecyl sulfate (SDS), Dithiothreitol (DTT)) diluted with 0.01 mol·L⁻¹ Tris–HCl (pH 7.8) in different concentration gradients, the total reaction system was 100 μ L and stored at 4 °C for 15 h. We also conducted a Maillard reaction to explore the allergic effects of protein binding to reducing sugars, in which 0.25 mg mL⁻¹ nSCP mixed with D(+)-galactose (1:0, 1:3, 1:6, 1:9 and 1:12, w:w) at 100 °C for 60 min. The changes in IgE-binding activity were detected by direct ELISA after denaturation.

Statistical analysis

Statistical analysis was performed with a one-way ANOVA analysis of variance using Origin 8.0 (OriginLab Corp., MA, USA). Significance analysis was carried out using SPSS 25.0 software. Differences were considered to be significant when $P \leq 0.05$. All experiments were performed in triplicate and expressed as mean $\pm$ SD.

RESULTS

Purification and identification of the rSCP

Oyster rSCP was successfully expressed in *E.coli* BL21 (DE3) (Fig. 1(A), lanes 1–6). The *E. coli* was induced at 15 °C for 16 h and 37 °C for 4 h respectively. As the SDS-PAGE analysis showed, a prominent 21 kDa protein band was observed in the soluble fraction of IPTG-induced bacteria (Fig. 1(A), lanes 1–6), and it is obvious that the induction effect of 15 °C for 16 h is better than that of 37 °C for 4 h. The optimal protein expression condition was therefore cultured at 15 °C for 16 h in this experiment. It is reported that one obvious band with a molecular weight of 40 kDa in the precipitate salted out by 70–90% ammonium sulfate is AK^{9, 23} (Fig. 1(B), lane 1), and we also obtained the same result. The molecular weight of purified nSCP protein is about 20 kDa (Fig. 1(B), lane 2), slightly lower than that of rSCP (Fig. 1(B), lane 3).

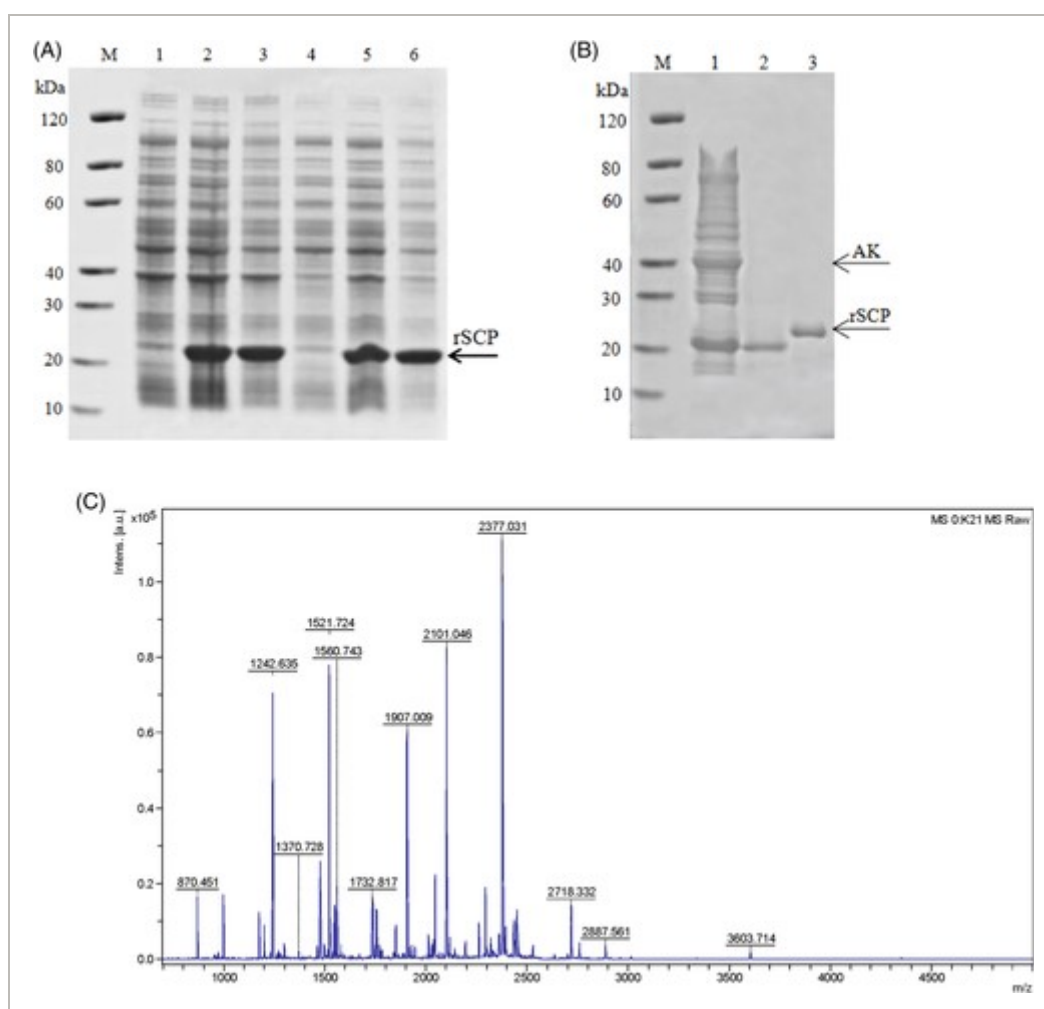


Figure 1

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Expression, purification, and identification of oyster (*Crassostrea gigas*) recombinant sarcoplasmic calcium-binding protein (rSCP). (A) SDS-PAGE analysis of rSCP (21 kDa) expression in BL21 (DE3). Lane M: Protein marker. Lane 1: Uninduced whole cell. Lane 2: The whole cells were induced at 15 °C for 16 h. Lane 3: The whole cells were induced at

37 °C for 4 h. Lane 4: Uninduced cell lysate supernatant. Lane 5: The supernatant of cell lysate induced at 15 °C for 16 h. Lane 6: The supernatant of cell lysate induced at 37 °C for 4 h. (B) SDS-PAGE analysis of purified natural SCP (nSCP) and rSCP. Lane M: Protein marker. Lane 1: Oyster crude extract. Lane 2: purified nSCP/ Lane 3: purified rSCP. (C) Mass spectrometry of rSCP.

After SDS-PAGE, the purified rSCP protein band was excised and digested in-gel with trypsin. The resulting peptide mixtures were analyzed by MALDI-TOF-MS. Figure 1(C) suggests that the excised protein band showed multiple peaks between 800 Da and 4000 Da. The prominent peak, the signal at 2377 Da, was compared with data in the NCBI database using the Mascot search tool and the Mowse value was observed. The protein with the highest score was found to be SCP from *C. gigas* (XP_011436345.1), which had a score of 212. Based on these results, it can be confirmed that the 21 kDa purified protein was the oyster SCP.

Stability of the rSCP

To evaluate the physicochemical properties of rSCP, we performed an *in vitro* stability analysis. The rSCP was still stable after incubation below 70 °C, but the protein bands gradually degraded when the temperature was higher than 70 °C (Fig. 2(A)). In the pH stability analysis, as illustrated in Fig. 2(B), rSCP was degraded when exposed to strong acid, but it was only slightly degraded in alkaline conditions.

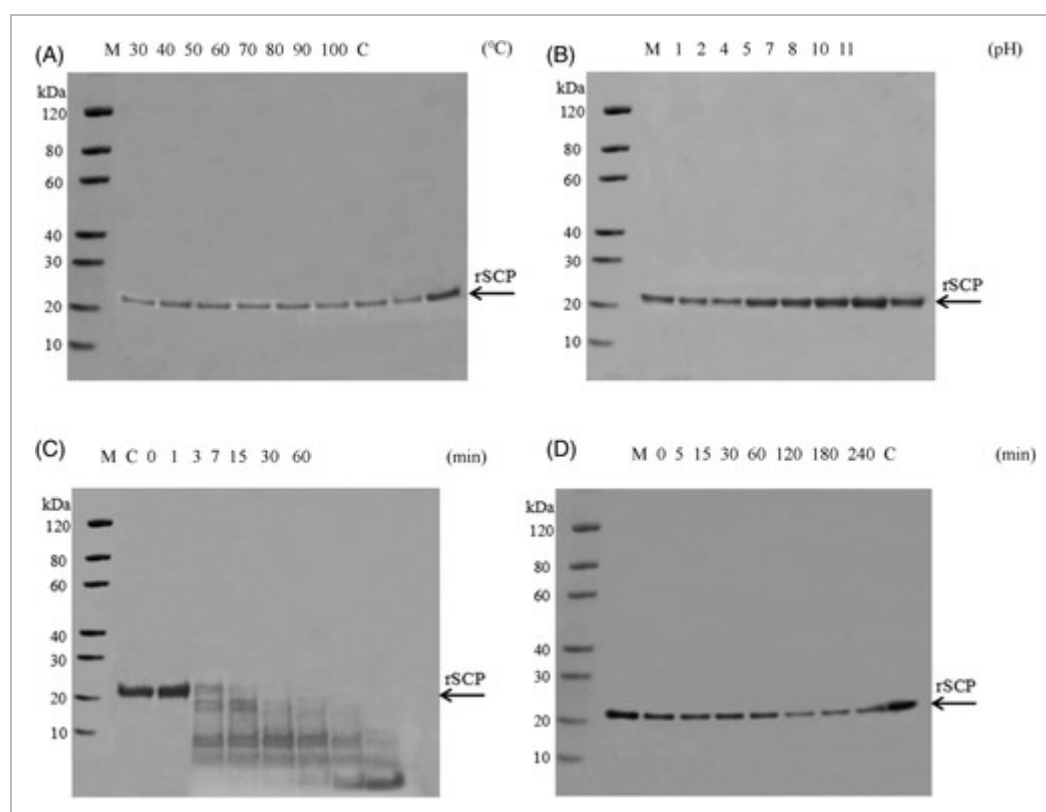


Figure 2

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Analysis of the stability of rSCP by SDS-PAGE. The heat-treated rSCP (A). The acid-alkali treated rSCP (B). The SGF-treated

rSCP (C). The SIF-treated rSCP (D).

After treatments with SGF at different times, the rSCP band gradually disappeared from 1 min, and almost entirely disappeared by 15 min. Meanwhile, the initial rSCP bands were digested into small fragments (Fig. 2(C)). After treatment with SIF, compared with the control group, the protein bands were gradually degraded over time, but no small molecular bands were observed (Fig. 2(D)).

Secondary structure of the rSCP

To observe the effects of different treatment methods on the rSCP secondary structure, structural variations were investigated through CD spectra. The CD spectrum of rSCP had double negative peaks at 222 nm and 208 nm, and a positive peak at 196 nm; the molar ellipticity (θ) 222/208 ratio was greater than 1. These were the features of a typical α -helical protein conformation. When temperature increased, the negative bands at 221 nm decreased, especially between 70 °C and 100 °C, which means that the percentage of α -helix decreased. Indeed, we found that the content of α -helix varied from 34.6% to 22.4% when the temperature ranged from 0 to 100 °C (Fig. 3(A)). All these results indicated that the α -helix was destroyed and the conformation of rSCP was partially damaged during heat treatment.

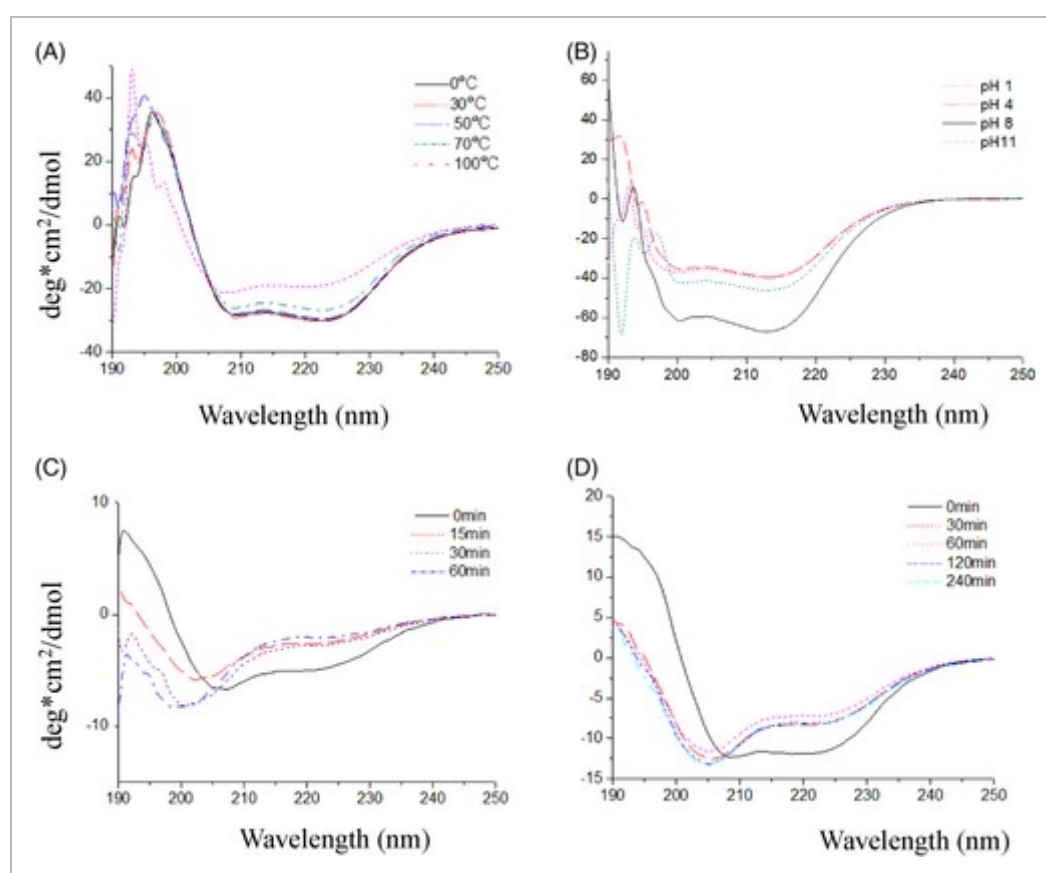


Figure 3

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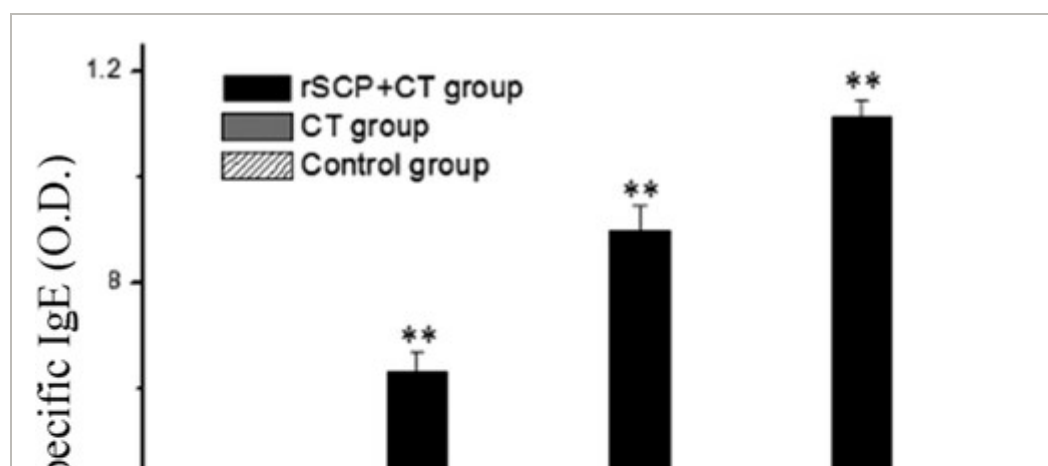
Effects of different conditions on the secondary structure of rSCP. The heat-treated rSCP at different temperatures (A). The acid-alkali treated rSCP at different pH (B); The SGF-treated rSCP at different time (C); The SIF-treated rSCP at different time (D).

As illustrated in Fig. 3(B), compared with the control (pH 8), whether exposed to acid or alkali, the α -helix structure of rSCP was decreased, but the destruction effect of acid on the α -helix was stronger than that of alkali, the content of the α -helix at pH 8 was 79.40%, whereas those at pH 1 and pH 11 were 50.5% and 60.0%, respectively, which were consistent with the corresponding SDS results (Fig. 2(B)).

The secondary structure of the protein was analyzed immediately after SGF or SIF digestion. Unlike pH and heat treatments, the secondary structure of protein digested *in vitro* (by enzyme) changed significantly. In the process of SGF digestion (Fig. 3(C)), when the time ranged from 0 min to 60 min, the negative band of the spectral line at 221 nm was reduced; meanwhile, the signal minimum was shifted to a lower wavelength, indicating that the secondary structures varied drastically. In the process of SIF digestion (Fig. 3(D)), compared with treatment 0 min, after trypsin digestion for 30–240 min, the peak position and peak shape of rSCP had been changed consistently. It can be inferred that the α -helix content was reduced. At the same time, we found that the secondary structure was 13.0% α -helix and 35.0% random coils at 0 min. The α -helix (7.5%) was slightly decreased after pepsin digestion treatment for 4 h, whereas random coils (35.3%) did not change significantly.

Evaluation of animal sensitization effect

After several weeks of regular intraperitoneal injections combined with gavage, BN rats had obvious symptoms of allergic reactions during the sensitization period, showing an increase of constant scratching of the nose, ears, around the head and abdomen, swelling around the eyes or mouth, and reduced activity. After being stimulated by high-concentration protein, the rats even showed symptoms such as shaking and convulsions. During the sensitization period, blood was taken once a week to determine rSCP-specific IgE serum levels. The rSCP + CT group rats had significantly higher rSCP-specific IgE release levels than the CT group and control group ($P < 0.01$) (Fig. 4).



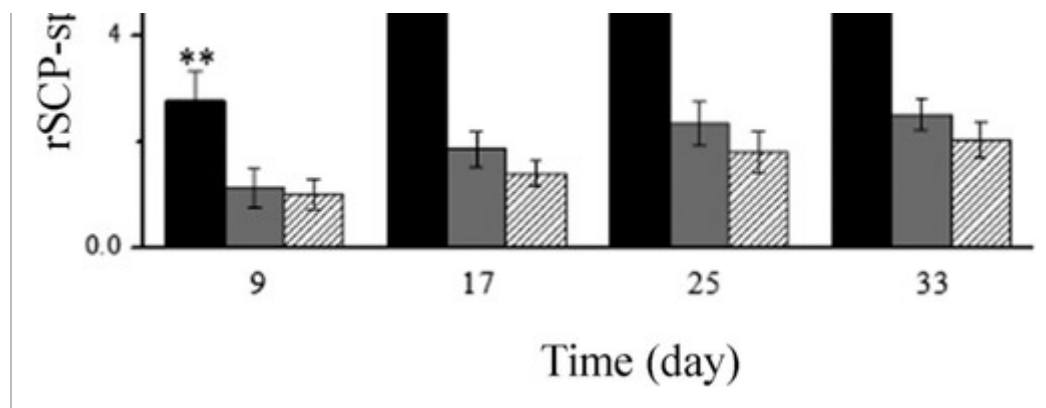
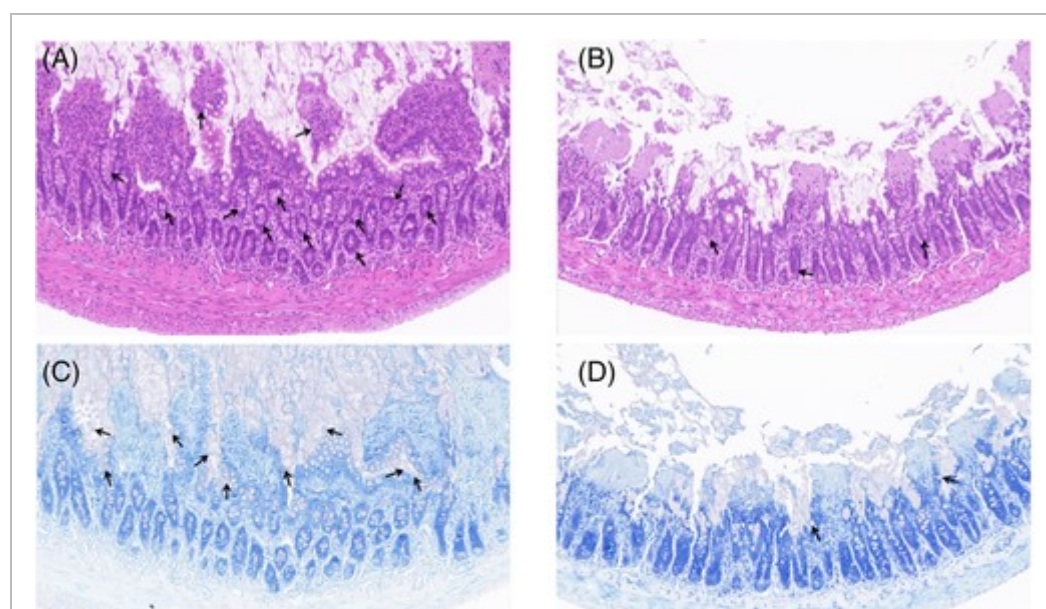


Figure 4

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Determination of serum rSCP-specific IgE activity in BN rats during sensitization. Significant differences $^{**}P < 0.01$ versus control group.

Exposure to food allergens can cause a range of clinical symptoms, ranging from mild to life-threatening allergies, and infiltrate the tissues, including the skin, liver, and gastrointestinal and respiratory tracts.²⁴ We observed the damage to various organs in rats before and after sensitization. Hematoxylin–eosin staining of intestine and liver were carried out in the sensitization group. We found that a large number of intestinal villi were broken, and fell off. In addition, lamina propria swelled, intestinal mucosa became thick and irregular, and were infiltrated by a large number of inflammatory cells. On the other hand, the control group had no abnormal symptoms. The degranulation of small intestinal mast cells was also analyzed by TB staining. The jejunal mast cells in the lamina propria aggregated and proliferated, and the degranulation was serious, the cell membrane was ruptured, and the shape was irregular compared with the control group. Obviously, the central hepatic vein and hepatic sinuses were significantly expanded and filled with blood, and some hepatocyte cords were broken and atrophied, but the morphology of hepatocytes in the control group was almost intact (Fig. 5).



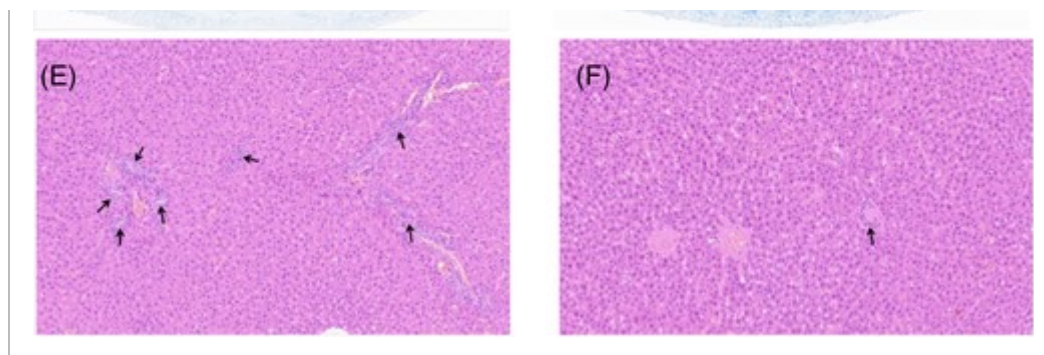


Figure 5

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Results of organ HE and TB staining. Small intestinal tissue was stained with HE and TB in the sensitization group (A, C) and in the control group (B, D). Liver was stained with HE in the sensitization group (E) and in the control group (F). All sections were magnified 20 times for observation.

Prediction of linear epitopes in rSCP

To predict the epitopes of SCP, we used bioinformatics tools to select potential epitopes based on hydrophilicity, accessibility, flexibility, and antigenicity. Hydrophilic amino acids of proteins are generally located on the surface of proteins, whereas hydrophobic amino acids are embedded in proteins. The binding sites with antibodies are therefore generally hydrophilic amino acid residues. Flexibility refers to the degree to which amino acid residues are prone to twist and fold. The β -turns and random coils are mostly exposed on the surface of protein, so they are more likely to form antigen epitopes. Accessibility and antigenicity refers to the ability of an antigen indirectly or directly to contact and bind to its specific antibody. Using the DNASTar Protean system, we chose amino acid sequence regions that have good hydrophobicity and antigenic index, high accessibility and flexibility combined with the secondary structure to predict the SCP epitopes (Fig. 6). Nevertheless, to make predicted results more accurate, we also made use of four other immunoinformatics tools to analyze the possible epitope regions comprehensively (Table 1). Through the five immunoinformatics tools, the possible epitope regions were determined based on the overlapping region that given by at least four immunoinformatics tools. Finally, seven peptides from 4 to 13 residues in length were determined, named peptide 1 (P1) to peptide 7 (P7). They will be the potential IgE-binding epitopes of SCP and be synthesized on the grounds of amino acid sequence (Table 2).

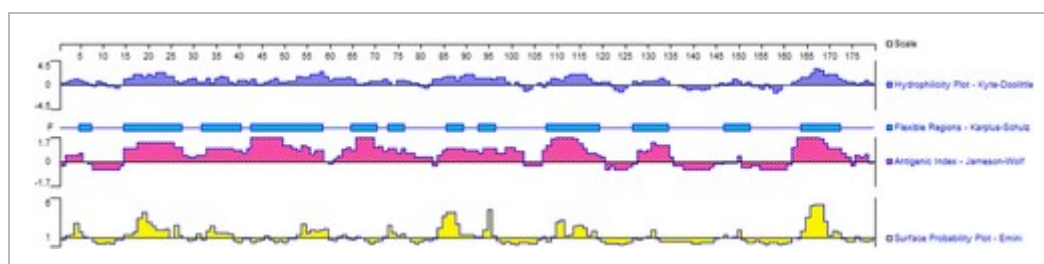


Figure 6

Analysis results of the whole amino acid sequence of SCP by DNASTar Protean system.

Table 1. Predicted results of the five immunoinformatics tools

Tools	Amino acid regions
DNASTar Protean	5–6, 17–27, 33–38, 53–59, 68–76, 86–90, 93–96, 109–120, 130–131, 164–171.
ABCPred Server	71–83, 7–22, 44–59, 152–169, 34–49, 133–148, 60–75, 159–174, 91–106, 19–34, 127–142, 27–42, 102–117, 142–157, 78–93, 112–127, 85–100.
BepiPred 1.0 Server	15–30, 45–58, 66–73, 83–91, 111–118, 129–135, 146–152, 163–171.
IEDB Analysis Resource	6–10, 20–26, 37–77, 86–95, 109–118, 128–134, 143–150, 164–175.
Immunomedicine Group	4–25, 81–89, 100–106, 111–120, 122–129, 139–152, 154–174.

Table 2. Predicted IgE-binding epitopes by combined immunoinformatics methods

Name	Amino acid sequence	Location	Length of sequence
Peptide 1 (P1)	LDADKDNKITPED	15–27	13
Peptide 2 (P2)	FDNTKW	53–58	6
Peptide 3 (P3)	KGPGVSMTKD	66–75	10
Peptide 4 (P4)	QKDK	86–89	4
Peptide 5 (P5)	ENMDRPIQEQ	109–118	10
Peptide 6 (P6)	FTAAYGQPTVQ	142–152	11
Peptide 7 (P7)	DDDQSK	164–169	6

icELISA for validating linear epitope peptides and localization

Inhibition experiments were conducted with selected peptides and original rSCP as inhibitors to inhibit the binding between original rSCP and serum IgE. To discover which of the seven predicted peptides were immunodominant epitopes, these synthesized peptides were probed with a pool of sera from ten randomly selected BN rats. Using the pooled sera,

five peptides showed greater inhibition of IgE binding by icELISA ($P < 0.05$) (Fig. 7(A)), implying that P1, P2, P4, P5, and P7 contained the immunodominant epitopes with positions at AA₁₅₋₂₇, AA₅₃₋₅₈, AA₈₆₋₈₉, AA₁₀₉₋₁₁₈, AA₁₆₄₋₁₆₉, respectively.

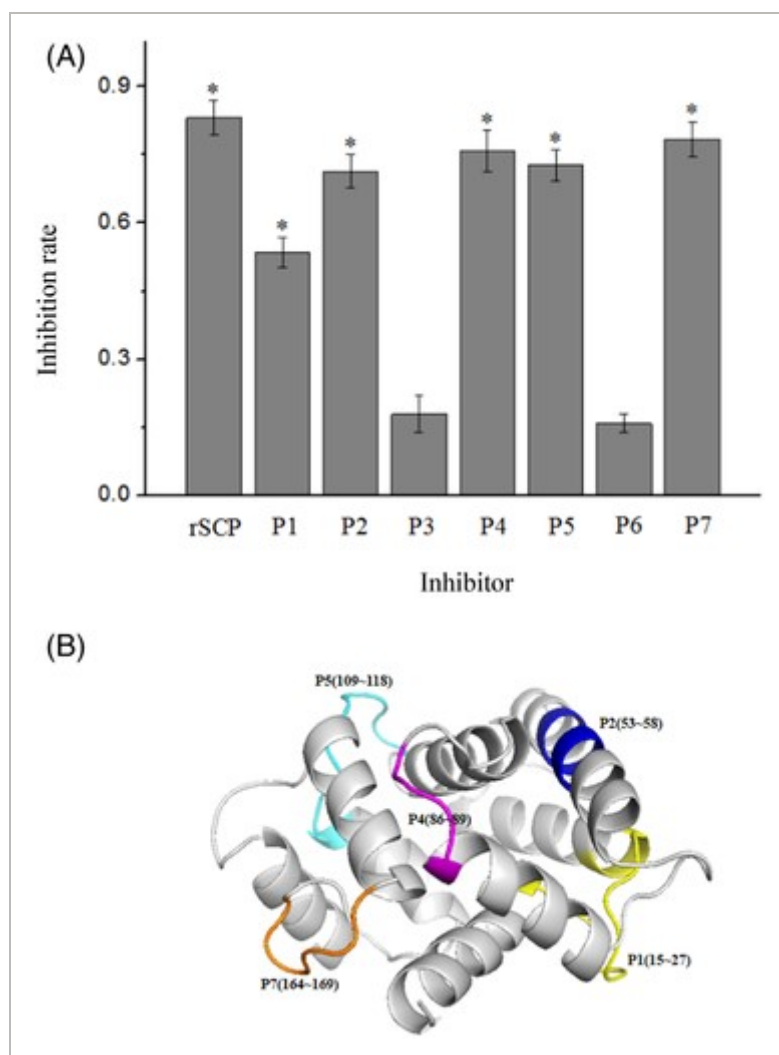


Figure 7

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Identification and labeling of rSCP linear epitopes. The icELISA assay by the serum pool of sensitized BN rats ($P < 0.05$) (A). Labeling of identified linear epitopes (B). Five immunodominant epitopes (P1, P2, P4, P5, and P7) were labeled with different colors.

We therefore successfully identified five linear epitope peptides of oyster SCP from seven predicted peptides, and the five epitope peptides were labeled on the protein three-dimensional model from *Doryteuthis pealeii* (Fig. 7(B)), which consists of six α -helixes, several β -turns and random coils to form an approximate spherical structure.

Multiple sequence alignment analysis

The amino acid sequence of SCPs from different shellfish species including oysters, clams, scallops, mussels, shrimps, and crabs are presented in Fig. 8. Homology analysis shows that,

when compared with SCPs in the mollusk group, the amino acid sequence of oyster SCP shared 63.5% identity with the mollusks (*C. angulata*, *Meretrix lusoria*, *Mizuhopecten yessoensis*, *Ruditapes philippinarum*, and *Mytilus coruscus*), of which the *C. angulata* SCP (21 kDa) was the most similar. However, the amino acid sequence of SCP exhibited only 13.8–16.9% identity compared with those from six selected crustaceans. As we illustrated, the homology between oyster SCP and crustacean SCPs was very low. We also found that the five identified linear epitopes were evenly distributed throughout the molecule, and overlapped with the linear epitopes of SCPs from *C. angulata* and *Penaeus vannamei*.

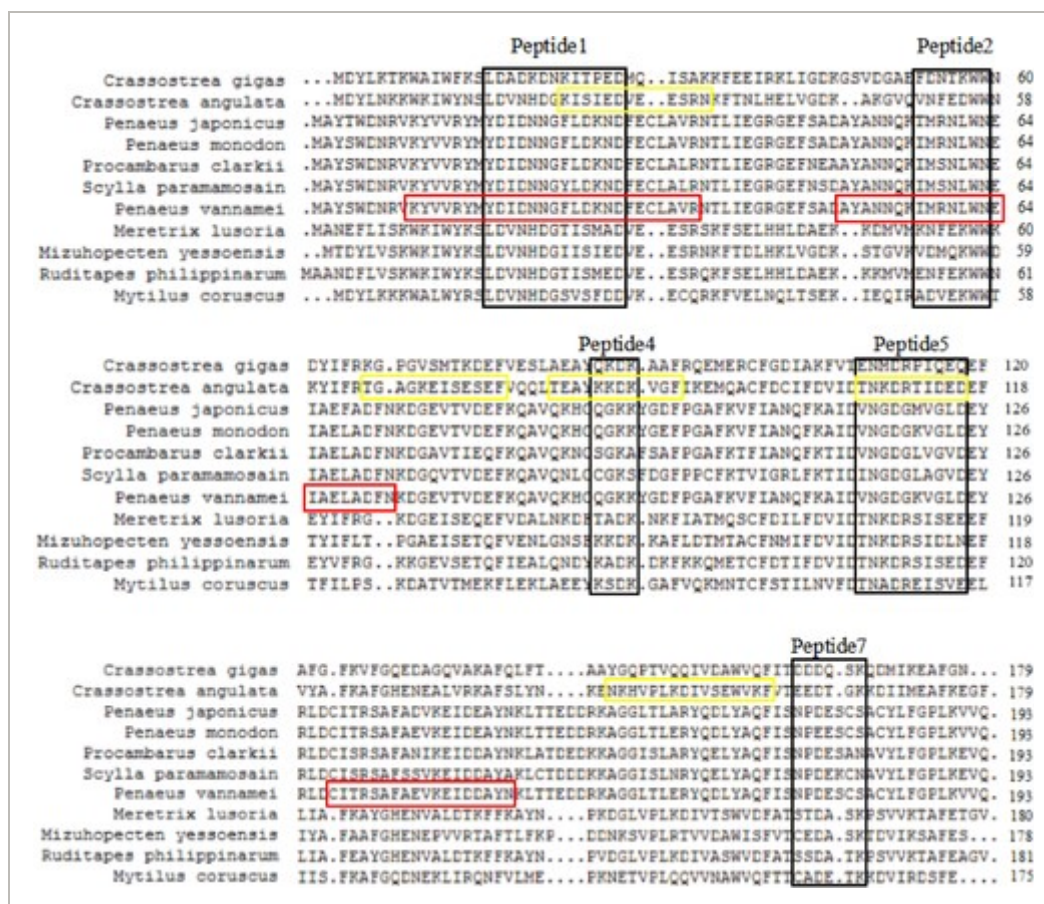


Figure 8

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Amino acid sequence alignments of SCP from 11 representative shellfish. The GenBank numbers were *C. gigas* (XP_011433684.1), *C. angulata* (QIJ32297.1), *P. japonicus* (BAL72726.1), *P. monodon* (BAL72725.1), *P. clarkii* (ABB58783.1), *S. paramamosain* (AFJ80778.1), *P. vannamei* (ACM89179.1), *M. lusoria* (BAA25310.1), *M. yessoensis* (BAA89420.1), *R. philippinarum* (BAA75223.1), *M. coruscus* (CAC5401785.1). Sequences of the 11 SCPs that correspond to the five major IgE-binding epitope regions of *C. gigas* SCP are shown in black boxes. Sequences marked with yellow and red boxes represent IgE-binding epitopes proposed for *C. angulata* and *P. vannamei*, respectively.

IgE reactivity of the rSCP after denaturation treatments

Denaturation affects the protein conformation, which in turn affects the IgE binding ability. Protein denaturation refers to the changes of conformation due to the addition of chemical

reagents or exposure to high temperature, or X-ray, etc., resulting in changes to its physical and chemical properties and loss of biological activity. Sodium dodecyl sulfate (SDS) can assemble closely with protein, and destroy the hydrophobicity and conformation of protein. Dithiothreitol (DTT) as a reducing agent can be used to prevent the formation of intramolecular or intermolecular disulfide bonds between cysteines in protein.

After SDS treatments, the binding activity of rSCP to IgE decreased significantly (Fig. 9(A)). Compared with the control, the IgE binding ability of the protein decreased by 77.7% when SDS concentration was $0.5 \text{ mmol}\cdot\text{L}^{-1}$. With the increase in SDS concentration, the IgE binding ability of the protein remained almost unchanged, which was only 13.4% of the original protein. The amino acid sequence of SCP contains cysteine, so it is very likely to form an intermolecular disulfide bond. After adding $0.05 \text{ mol}\cdot\text{L}^{-1}$ of DTT, the ability of rSCP to bind IgE was reduced to 92.9% of the original protein compared with an untreated control (Fig. 9(B)). In the DTT concentration range of $0.1 \text{ mol}\cdot\text{L}^{-1}$ to $0.16 \text{ mol}\cdot\text{L}^{-1}$, the rSCP IgE binding ability changed significantly ($P < 0.05$).

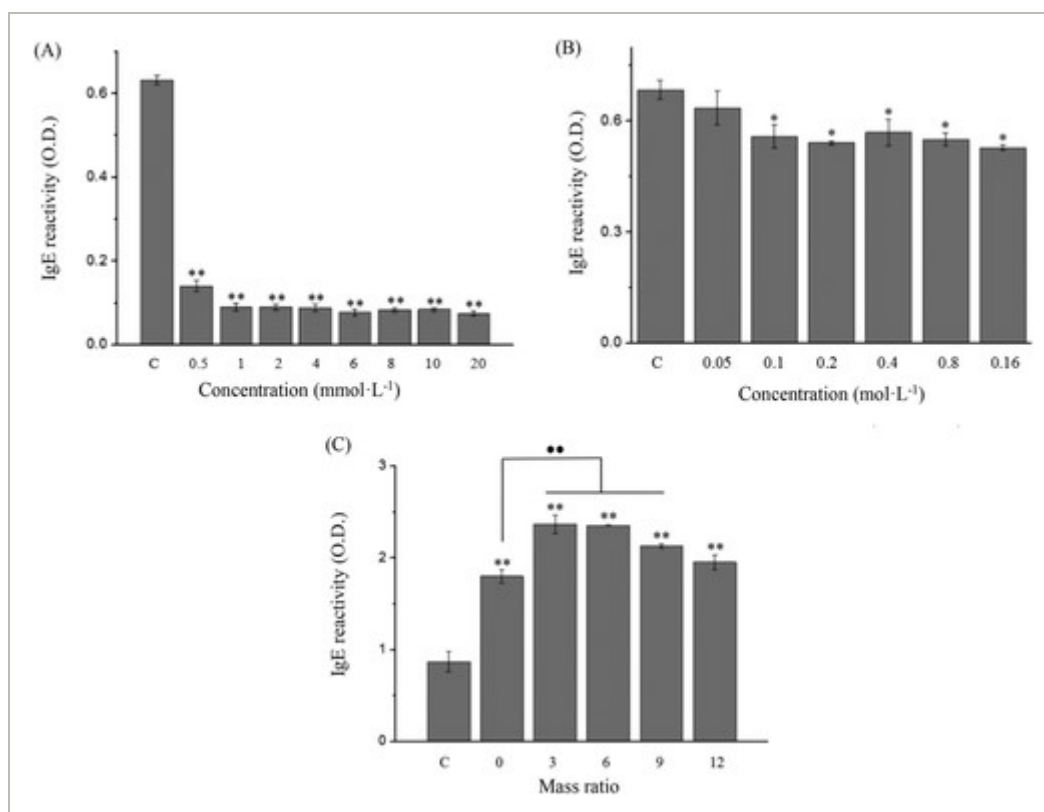


Figure 9

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IgE reactivity of the denatured SCP using ELISA. Denaturation of rSCP with different concentrations of SDS (A) and DTT (B). Maillard reaction of nSCP with different mass ratio of D(+)-galactose (C). Significance was marked with * ($P < 0.05$), ** ($P < 0.01$) versus control, '●●' ($P < 0.01$) the experimental group with sugar versus experimental group without sugar.

The Maillard reaction is a non-enzymatic browning reaction between the carbonyl group of reducing sugar and the free amino group of protein or amino acid. In comparison with the

original control group, the IgE reactivity of nSCP increased by 1.07 times after heating without reducing sugars. Interestingly, the IgE binding ability of all protein samples increased significantly when the sugar ratio rose after the Maillard reaction ($P < 0.01$) (Fig. 9(C)).

DISCUSSION

Food allergies affect 3.5 ~ 4.0% of the global population. Most food allergies are type I allergic reactions mediated by immunoglobulin E (IgE), which can specifically bind to allergens. Food allergy symptoms can lead to health risks, such as urticaria, asthma, diarrhea, and life-threatening allergic reactions.²⁵ Shellfish belongs to the eight categories of allergic food mentioned in the introduction, and it accounts for more than 90% of all food allergy cases. Today there is clear evidence that there are several proteins involved in the allergenicity and cross-reactivity of shellfish, as described in previous research.⁴⁻¹³ Oyster is one of the typical representatives of shellfish; its proteins are also involved in the immune-mediated type I allergy.²⁶ Previous experiments in our laboratory had verified that *C. gigas* tropomyosin (*Cra g 1*) after heat treatment produced higher IgE reactivity than unheated *Cra g 1*. Five major linear epitopes were distributed evenly on the surface of homology-based *Cra g 1* model and hydrophilic residues appeared to be the most important for IgE binding. Moreover, owing to alterations of the secondary structure and the hydrophobic surface of the protein during digestive processing, the antigenicity of acid-induced *Cra g 1* reduced in SGF whereas it increased significantly in SIF. We successfully designed four oyster tropomyosin hypoallergens on grounds of the identified epitopes, which had obvious reduction in IgE reactivity of sera from oyster-allergic patients and *Cra g 1*-sensitized BN rats. In addition, the four hypoallergens had significant decrease in degranulation and secretion of allergic mediators including histamine, IL-4, IL-6 and TNF- α and were able to attenuate Fc ϵ RI-mediated signaling cascades in RBL-2H3 cells.^{22, 27-29} Till now, the allergen *Cra g 1* has been well studied, while the study of SCP is scarce as another important allergen in *C. gigas*. To our knowledge, SCP as a new shrimp allergen had been confirmed, Ayuso *et al.*¹⁹ proved that shrimp SCP could be recognized by more than 50% shellfish allergy patients' sera, and confirmed that the rSCP also had strong binding activity. Recombinant allergens are now considered to be the basis of new diagnostic methods and strategies for allergy-specific immunotherapy.³⁰ We are therefore committed to the characterization and epitope identification of oyster rSCP.

Most food allergens have similar physical and chemical properties, such as relative stability to heat and acid-alkali, and may remain allergenic after digestion.⁸ This might be because they are mainly glycoproteins, and the sugar chains on the surface of proteins cross-link with proteins to protect them. The thermal stability of rSCP was similar to oyster TM: both of them were stable below 70 °C and began to disappear gradually above 70 °C (Fig. 2(A)). Moreover, the oyster rSCP was degraded in a strong acid-alkali environment, especially after acid treatment (Fig. 2(B)), implying that we might choose high temperature and acid

treatments to reduce allergic reactions during oyster processing. Pepsin is the only proteolytic enzyme in the stomach, it prefers to cleave peptide bonds after Phe or Tyr residues, and other hydrophobic amino acids.³¹ Because SCP contains 40.8% hydrophobic amino acids, pepsin could significantly hydrolyze oyster rSCP (Fig. 2(C)), demonstrating that it has weak resistance to SGF. This phenomenon was also observed by Han *et al.*,³² who suggested that oyster (*C. angulata*) SCP produced several small molecular fragments after pepsin digestion. Trypsin mainly cleaves peptide bonds following Lys or Arg residues at the P1 position with higher specificity;³³ however, after being digested with trypsin for 4 h, one obvious band around 21 kDa still existed, illustrating that rSCP displayed a strong resistance to SIF (Fig. 2(D)).

In this study, seven epitope peptides were predicted by comprehensive bioinformatics software, while five linear peptides (AA₁₅₋₂₇, AA₅₃₋₅₈, AA₈₆₋₈₉, AA₁₀₉₋₁₁₈, AA₁₆₄₋₁₆₉) were identified that had significant IgE binding activity, with the inhibition rate from 53.6% to 78.4% (Fig. 7(A)). As Pacific oyster SCP crystal structure has not been characterized, we constructed its tertiary structure through molecular modeling. Remarkably, the immunodominant epitopes were almost located on the outer surface of the molecule (Fig. 7(B)), and as we expected, there were four epitopes in the random coil regions, which may easily to twist, fold and bind specific IgE. In previous study, Ayuso *et al.*³⁴ had revealed three linear epitopes in *L. Vannamei* SCP: AA₁₀₋₃₆, AA₄₉₋₇₂ and AA₁₃₀₋₁₄₇, in which two epitopes partially overlapped with oyster SCP epitopes (Fig. 8). Moreover, Han *et al.*³⁵ had revealed five linear epitopes in *C. angulata* SCP: AA₂₂₋₃₃, AA₆₄₋₇₅, AA₈₀₋₉₀, AA₁₀₇₋₁₁₆ and AA₁₄₄₋₁₅₉; three of them overlapped with oyster SCP epitopes. This provides a molecular basis for us to study the cross reaction between mollusks and crustaceans. Amino acid composition was also analyzed in the five major epitopes. We observed that three of the five dominant epitopes contained hydrophobic amino acids, accounting for 23.1% of the amino acids in the epitopes, while the proportion of hydrophobic amino acids was 40.8% in the whole protein. Fifteen amino acids were found existing in these epitopes with the absence of valine (V), cysteine (C) and histidine (H). We found that alanine(A), phenylalanine (F), lysine (K), aspartic acid (D), glutamic acid (E) and glutamine (Q) were relatively abundant in the whole protein. Moreover, negatively and positively charged amino acids accounted for 18.4% and 13.4% of SCP respectively. It might therefore be deduced that negatively charged amino acids (D, E), positively charged amino acids (K, R) and polar, uncharged amino acids (Q, G, etc.) are easily to form IgE-binding regions in oyster SCP.

It has been reported that, under different physiological and experimental conditions, some allergens may undergo conformational changes that cause the exposure of different combinations of epitopes recognized by the IgE antibodies of allergic subjects.⁶ Generally, antigenic epitopes include linear epitopes and conformational epitopes according to different structure. To further verify whether conformational epitopes play an important role in immunoreactivity, we conducted protein denaturation experiments. Surprisingly, after adding 0.5 mmol·L⁻¹ SDS, the conformation of the protein was destroyed, and the

binding ability of rSCP to serum IgE decreased significantly by 77.7% (Fig. 9(A)), suggesting that rSCP was depolymerized from dimer to monomer during SDS treatment and conformational epitopes play a major role in SCP. The disulfide bond is the S—S covalent bond formed by the oxidation of sulfhydryl (-SH) at two different cysteine sites in proteins or peptides. The oyster SCP contains a cysteine; hence, it may form intermolecular disulfide bonds. In evidence, DTT had significant effect on the IgE binding activity of rSCP with the increase of its concentration (Fig. 9(B)), indicating that DTT destroyed the conformation of rSCP.

We found in previous experiments that oyster nSCP is a glycoprotein, and its surface polysaccharide content measured by phenol-sulfuric acid method is 7.89% (data not shown). As oyster nSCP contains reducing sugars, heat treatment may cause protein modifications by Maillard reaction to produce polymers, leading to previously inward-oriented hydrophobic residues to turn outward, exposing more antigenic epitopes to the molecular surface (Fig. 9(C)). Meanwhile, nSCP was also modified with reducing sugars by the Maillard reaction at elevated temperatures. In this process, nSCP combined with reducing sugars through covalent bonds to form polymers and expose new epitopes. As a result, we confirmed that heat-treated nSCP strongly increased IgE reactivity compared with the unheated form due to conformational changes induced by the Maillard reaction, which is consistent with previous observations.^{24, 36}

CONCLUSIONS

Pacific oyster rSCP was produced by a cDNA cloning technique. Through *in vitro* characterization, rSCP showed strong physicochemical stability. In addition, BN rats had a strong sensitization to rSCP after intragastric and intraperitoneal injection. As far as we are aware, five linear immunodominant epitopes of rSCP were identified by icELISA for the first time. Furthermore, oyster SCP is mainly composed of conformational IgE epitopes. Therefore, in order to understand the immune response mechanism of allergic patients to oyster SCP and to offer available preventive measures and treatment programs, it is necessary to study the conformational epitopes of oyster SCP in further research.

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