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IgE epitope analysis of sarcoplasmic-calcium-binding protein, a heat-resistant allergen in *Crassostrea angulata*[†]

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Sarcoplasmic-calcium-binding protein (SCP) has been investigated as a novel allergen in *Crassostrea angulata*. Nevertheless, knowledge of its effector-cell-based allergic relevance and epitopes is limited. In this study, the heat-resistant allergen SCP was able to induce significant upregulation of CD63 and CD203c ($p < 0.05$), which showed obvious allergenicity in a basophil activation test. Furthermore, immunoinformatic tools, a one-bead-one-compound peptide library, and phage display technology were combined to analyze the allergenic epitopes of SCP. Five linear epitopes named L-SCP-1 (AA_{22–33}), L-SCP-2 (AA_{64–75}), L-SCP-3 (AA_{80–90}), L-SCP-4 (AA_{107–116}), and L-SCP-5 (AA_{144–159}) were verified using serological tests. Additionally, two conformational epitopes (C-SCP-1 and C-SCP-2) were determined, and C-SCP-1 was located at one of the calcium-binding sites (AA_{106–117}). Moreover, SCP showed weaker typical α -helical features and higher hydrophobicity after Ca²⁺ depletion, which reduced its IgE-binding capacity. Overall, these epitope data could enhance our understanding of oyster allergens, which could be used to develop hypoallergenic shellfish products.

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Introduction

Per annum molluscan shellfish production and consumption have been increasing.¹ Although these products are important sources of dietary nutrients, they are also common causes of type I food allergies in coastal populations.^{2,3} Unlike most other food allergies, seafood allergy persists for life in up to 90% of patients, and can occur due to skin contact or inhalation of aerosolized proteins generated during cooking or processing.⁴ It was reported that the prevalence of molluscan allergy in the pediatric population of the United States was as high as 0.15%.⁵ An international survey based on a questionnaire administered to 17 280 adults in the age group of 20–44 years from 15 countries showed that 2.3% of allergy symptoms were related to oysters.⁶ However, the allergens in oysters listed in the World Health Organization/International Union of

Immunological Societies (WHO/IUIS) database are knowledge-deficient and worth exploring (<http://www.allergen.org>).

Sarcoplasmic-calcium-binding protein (SCP) has been well established as an important myosinogen allergen in crustaceans,^{7–11} and elicits 29–50% positive IgE-binding among shrimp-allergic patients.⁴ It was reported that common co-sensitization to tropomyosin (a major allergen in a number of mollusc species) with SCP reached up to 31%.¹² SCP is considered to be a very stable allergen. Liu *et al.*¹³ observed three heat/digestion-stable peptides in the epitope regions of *Scy p* 4, which retain their allergenicity after digestion. In our previous research, *Crassostrea angulata* SCP was identified, and recombinant SCP (rSCP) was acquired in high yield.¹⁴ In type I food allergies, a protein immunogen cross-links IgEs, leading to the degranulation of effector cells (basophil/mast cells), resulting in symptoms that range from mild irritation to anaphylaxis.¹⁵ Nonetheless, an allergic assessment of IgE reactivity to rSCP based on effector cells was limited. Thus, a more extensive study of oyster SCP should be conducted.

Type I food allergies and cross-reactivity have been determined by epitopes located on the allergens that can be recognized by specific antibodies.¹⁶ Epitope mapping methods have also been widely investigated. Based on computer technology and the improvement of biological databases, immunoinformatics has become a useful method to predict epitopes from allergens.¹⁷ Seven allergenic epitopes of *Rap v* 2 and eight

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shrimp tropomyosin linear epitopes were determined using immunoinformatic tools.^{18,19} Furthermore, as a relatively novel approach for epitope determination, a one-bead-one-compound (OBOC) library was successfully used for identifying epitopes of shrimp tropomyosin,²⁰ and phage display technology has been applied in the identification of B cell epitopes such as the peanut allergen *Ara h 1*,²¹ grass pollen *Phl p 5*,²² and myosinogen allergens in crab.²³ Identification of epitopes of food allergens is crucial, but currently, tropomyosin is the only epitope-identified allergen in oysters (*Crassostrea gigas*).¹⁷ Due to the lack of information on IgE epitopes in Portuguese oyster SCP, the identification of epitopes in antibody-antigen interactions is an indispensable step for the in-depth analysis of oyster allergy and the rational design of hypoallergenic food.

The epitope is an essential factor in allergic responses to allergens; additionally, it has been demonstrated that the structural features of allergens can provide important insight into their biological functions, and may affect their ability to induce an IgE response.²⁴ SCP belongs to a family of Ca^{2+} -binding proteins that are structurally characterized by the presence of EF-hand motifs (helix-loop-helix motifs). It is worth mentioning that the IgE-binding capacity of some EF-hand Ca^{2+} -binding allergens, such as parvalbumin,²⁵ *Bet v 4*,²⁶ and *Scy p 4*,²⁷ is remarkably reduced with Ca^{2+} -depletion. No information is available regarding the relationships between IgE reactivity and structure for molluscan SCP. Therefore, research into the effect of Ca^{2+} -depletion on the SCP structure and its IgE-binding capacity is important to elucidate the relevant EF-hand family allergens.

Under these circumstances, the allergenicity of rSCP was measured using the basophil activation test (BAT). Moreover, the IgE epitopes were obtained using immunoinformatic tools, an OBOC peptide library, and a phage display random peptide library. Subsequently, the mimotopes were comprehensively analyzed, synthesized, and identified by dot blot and inhibition enzyme linked immunosorbent assay (iELISA). Additionally, circular dichroism (CD) spectroscopy, hydrophobicity measurements, and an immunoblot assay were performed to determine the effect of Ca^{2+} -depletion on the structure and IgE-binding capacity of rSCP.

Materials and methods

Materials

Horseradish peroxidase-labeled goat anti-human IgE antibody and goat anti-rabbit IgG antibody were purchased from Southern Biotech (Birmingham, Alabama, USA). TentaGel S NH₂ beads, anti-human IgE (ϵ -chain specific) antibody, and 8-anilino-1-naphthalenesulfonic acid (ANS) were purchased from Sigma Chemical Co. (Saint Louis, Missouri, USA). 5-Bromo-4-chloro-3-indolyl phosphate (BCIP)/nitroblue tetrazolium chloride (NBT) substrate color development kit was obtained from Solarbio Co., Ltd (Beijing, China). The Ph.D.-12 Phage Display Random Peptide Library and *E. coli* ER2738

were purchased from New England Bio-Laboratories (Beverly, Massachusetts, USA). All other chemical reagents were analytically pure.

Human blood and serum

Whole blood and serum samples from 19 oyster-sensitized individuals and 2 non-atopic individuals were collected for the present study (Table 1). All serological procedures were performed in accordance with the Women and Children's Hospital Affiliated to Xiamen University and the Second Affiliated Hospital of Xiamen Medical College, and the experiments were approved by the Human Ethics Committee of No. KY-2019-014 and 2020018. Signed informed consent was obtained from all individuals. Oyster-specific IgE levels greater than or equal to $0.35 \text{ kU}_A \text{ L}^{-1}$ as determined using ImmunoCAP from Phadia AB (Rapskatan, Uppsala, Sweden) were defined as positive. All serum samples were stored at -80°C until further use.

Thermal stability analysis of *C. angulata* myosinogen protein and cell lysate in recombinant protein

The myosinogen protein and cell lysate of the recombinant protein in *C. angulata* were diluted to a concentration of 1 mg mL^{-1} and heated for 1 min, 3 min, or 5 min at 100°C and then analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. The SCP polyclonal antibody was prepared as the primary antibody ($1:10\,000$ dilution) in our laboratory as previously described.¹⁴

Table 1 Specific IgE levels and symptoms of the oyster-sensitized individuals

Blood sample no.	Age (years)	Sex	Specific ^a IgE ($\text{kU}_A \text{ L}^{-1}$)	Symptoms
1	46	M	73.9	Chronic urticaria
2	34	M	34.7	Cough
3	28	F	35.4	Anaphylactic rhinitis
4	23	F	69.9	Sleep apnea
5	25	M	77.2	Atopic dermatitis
6	28	M	29.8	Chronic urticaria
7	49	M	68.8	Atopic dermatitis
8	12	M	33.1	Atopic dermatitis
9	35	F	>100.0	Urticaria
10	25	F	57.8	Anaphylactic rhinitis
11	20	F	3.5	Cough
12	22	F	>100.0	Allergic purpura
13	23	F	25.7	Chronic urticaria
14	49	F	23.5	Atopic dermatitis
15	5	M	>100.0	Bronchitis
16	15	F	17.1	Cough
17	29	F	>100.0	Edema
18	4	F	20.9	Acute tonsillitis
19	39	F	61.3	Atopic dermatitis
20 ^b	24	M	0.2	— ^c
21 ^b	23	F	0.1	— ^c

^a Serum sample with a specific IgE $\geq 0.35 \text{ kU}_A \text{ L}^{-1}$ is defined as positive. ^b Nonallergic individual. ^c No symptoms in the experiment. M, male; F, female.

IgE-immunoblot and BAT analysis of rSCP

SCP was expressed and purified according to the methods described by Han *et al.*¹⁴ The serum samples (numbers 1–7, 20 and 21) verified by dot blot¹⁰ were used to further detect the effect of the allergen on the patients' effector cells using BAT. An rSCP concentration of 50 $\mu\text{g mL}^{-1}$ was used, and the level of CD63/CD203c upregulation was analyzed using flow cytometry on a Guava instrument (Millipore, Massachusetts, USA) as described previously.³

Epitope prediction of SCP using immunoinformatic tools

The sequence of SCP was analyzed using five immunoinformatic tools, including ABCpred, BepiPred 1.0 Server, NetMHC II 2.3 Server, Immunomedicine Group, and DNASTar Protean-based computational approaches. The epitopes were predicted primarily based on the hydrophilicity, flexibility, accessibility, and antigenicity of the amino acid sequences.²⁸ The surface conservation was calculated using the ConSurf server (<https://consurf.tau.ac.il/>).²⁹

Screening of SCP epitopes using the OBOC peptide library

An OBOC linear peptide library was prepared using TentaGel S NH₂ beads as the solid support as previously described,²⁰ with some modifications. In brief, nineteen Fmoc-protected eukaryotic amino acids (all but cysteine) were then added separately to each of the bead resin aliquots. A threefold excess of amino acids, diisopropylcarbodiimide (DIC) and *N*-hydro-benzotriazole (HOBt) were mixed gently at room temperature for 1 h to initiate the coupling reaction adequately. The completion of the coupling was confirmed by the ninhydrin test. After the beads from the 19 tubes were drained in a vacuum centrifuge, the beads were then collected and washed with *N,N*-dimethylformamide (DMF) and methanol. Fmoc-deprotection was achieved with 50% diethylamine (in DMF). The beads were evenly distributed into nineteen aliquots for another cycle of coupling, and the above operation was repeated eleven times so that peptide library twelve amino acids in length were synthesized. We then adopted the approach of OBOC library screening described by Leung *et al.*²⁰ The colorless or yellow beads that turned blue (false positive) were first removed using the BCIP/NBT substrate color development kit, and the same procedure used in the first stage was repeated, but with incubation in pooled human serum (numbers 8–19, 1 : 3 dilution). True positive beads (dark blue) were collected and determined *via* Edman sequencing as described in ref. 30 using a PPSQ-51A protein sequencer (SHIMADZU, Kyoto, Japan).

Phage display random peptide library for the screening of SCP epitopes

The antigenic epitopes were screened using the Ph.D.-12 Phage Display Random Peptide Library technique. Biopanning, colony picking, isolation, and amplification of the phages were performed as described by the manufacturer. Briefly, the ELISA plates were coated with 100 μL goat anti-human IgE (ϵ -chain specific) and left overnight at 4 °C to

capture IgE. After 2 h of blocking with BSA, the oyster-sensitized individual sera samples (numbers 1–5) were incubated and 250 μL ($\sim 2 \times 10^9$ phages) of the phage peptide library was coated and left overnight at 4 °C, respectively. The specific binding phages were eluted, neutralized, and then amplified in *E. coli* ER2738. The titers of the bound phages were determined after the third round selection; positive phage clones were entrusted to TSINGKE Biological Technology (Xiamen, Fujian, China) for sequencing.³¹ Mimotope peptides were aligned to the SCP linear sequence using the software DNAMAN and mapped on the three-dimensional (3D) structure surface using EpiSearch (<http://curie.utmb.edu/episearch.html>). The 3D structure was modeled using SWISS-MODEL (<http://swissmodel.expasy.org/>), and the PDB file was viewed using PyMol.

Peptide synthesis and verification of SCP linear mimotope peptides

The results from the immunoinformatic tools, phage display random peptide library, and OBOC were combined, and the mimotopes were synthesized by Cellmano Biotech Limited (Hefei, Anhui, China) and stored at -20 °C until use.

Dot blot was performed to analyze the IgE-binding capacity of mimotope peptides as described previously,³² and quantification of the dots was performed using the software ImageJ.³³

To analyze the relevance of the IgE-binding capacity of the individual synthetic peptides (P1–P5), an iELISA was performed using the pooled serum samples (number 8–19, dilution 1 : 3) of oyster-sensitized individuals according to the method described by Karmakar *et al.*³⁴

Sequence-based comparison analysis among shellfish SCPs

The amino acid sequences of the epitope-identified shellfish SCPs were aligned *via* ClustalW (<http://clustalw.expasy.org/>), the calcium-binding sites of the SCPs were predicted using ExPASy peptide cutter (<https://www.expasy.org/>), and disulfide bond sites were predicted using Cyscon (<http://sjtu.edu.cn/bioinf/Cyscon/>).

Analysis of the effects of Ca²⁺ depletion on the structure and IgE-binding capacity of rSCP

The secondary structure of the rSCP was measured in the presence or absence of 5 mM EDTA using CD spectroscopy (Applied Photophysics Ltd, Surrey, UK) according to the methods previously described by Raith *et al.*³⁵ All samples were homogeneously mixed and adjusted to a concentration of 0.2 mg mL^{-1} .

The surface hydrophobicity of rSCP was determined in the presence or absence of 5 mM EDTA using ANS as the fluorescence probe *via* previously described methods.¹⁴

The dot blot technique was used to analyze the effect of Ca²⁺-depletion on the IgE-binding capacity of rSCP using the oyster-sensitized individuals' serum pool (number 8–19, dilution 1 : 3), as previously described.¹⁰

Statistical analysis

Data from the studies are presented as the mean $\pm$ standard deviation (SD). A one-way repeated-measures analysis of variance (ANOVA) with Tukey test was used to compare quantitative outcomes, and $p < 0.05$ was considered to be statistically significant. All data were repeated three times independently.

Results

Thermal stability and allergenicity analysis of *C. angulata* SCP

In the thermal stability assay, after being heated for 1 min, 3 min, or 5 min at 100 °C, both the *C. angulata* myosinogen protein and cell lysate of the recombinant protein retained strong SCP bands as revealed by SDS-PAGE, while the other protein bands were basically unrecognized by western blot using SCP polyclonal antibody (Fig. 1A and B).

The rSCP showed a specific IgE reaction to the serum samples from the oyster-sensitized individuals and a negative reaction to that of nonallergic individuals in the dot blot test, as listed in Table 1 (Fig. 1C). BAT was used to further explore the apparent correlation *via* the reactivity of basophils from the above volunteer donors. Fig. 1D indicates that, compared to that of the negative controls, rSCP stimulation induced a significantly higher stimulation index of CD63 ($p < 0.05$) and CD203c ($p < 0.05$) on the surfaces of sensitized individuals' basophils.

Subsequently, *C. angulata* SCP was submitted to the WHO/IUIS allergen nomenclature sub-committee (<http://www.allergen.org/viewallergen.php?aid=1056>), and was named *Cra a 4*.

Prediction of allergenic epitopes in *Cra a 4*

Immunoinformatic tools were used to analyze the allergenic epitopes of *Cra a 4*. The majority of the sequences were covered by hydrophilic regions; thus, the protein indicated high hydrophilicity, as shown in Fig. 2A. The stretched, flexible, and easily exposed regions were predicted using surface accessibility and flexibility analysis (Fig. 2A). As shown in Fig. 2B, the antigenic indexes were calculated to show the potential allergenicity of the protein regions. In addition, the twenty-three predicted antigenic epitopes are presented in Table S1.†

The evolutionary conservation of the amino acid positions was estimated based on phylogenetic homologous sequences *via* ConSurf, and the conserved feature result reveals the degree of homology. The higher conservation amino acids located in the amino acid ranges AA_{11–20}, AA_{121–130}, and AA_{161–170} were distributed on the surface of *Cra a 4* sequence (Fig. 2C).

Construction of the OBOC peptide library and epitope screening in *Cra a 4*

An OBOC linear library with 12 amino acids was screened using the pooled serum samples from the oyster-sensitized individuals. In the selection process, the positive beads showed a dark blue color (Fig. 3A), and were picked out one by

one using pipette tips under an inverted microscope (Fig. 3B). After N-terminal sequencing, the mimotope sequences (PEWWKKEWAVVV, NKVRPKPEPPMY, YEKTKPKEIPEL, AYNNSQYSYKVN, and YPVKSLFEWNNEN) were aligned with the primary sequence of *Cra a 4*. The sequence alignment indicated that three of the five mimotopes matched more than three amino acid residues in the *Cra a 4* sequence, including YEKTKPKEIPEL, NKVRPKPEPPMY, and PEWWKKEWAVVV (Fig. 3C). The position of the three linear mimotopes is shown on the 3D structure of *Cra a 4* (Fig. 3D).

Determination of the linear and conformational epitopes in *Cra a 4* using biopanning phages

After determining the titration of the phages (Fig. S1A†), twenty plaques on the third round plate were randomly selected for extraction of their single-stranded DNA, which was sequenced after verification using agarose gel electrophoresis (Fig. S1B†). The gene sequences were then translated into the amino acid sequences, and four mimotopes matched a segment of more than three amino acids in the *Cra a 4* sequence, including YNHFGQPYHLDV, SLYRDWAGADHL, HFVKTPARWAWG, and SENRKVPLKELR (Fig. 4A). Additionally, the linear mimotopes were mapped on the 3D structure of *Cra a 4*, and disulfide bonds were displayed using a cyan sphere (Fig. 4B). As shown in Fig. 4C, two mimotopes (C-SCP-1: G₄₈, D₉₉, I₁₀₁, D₁₀₃, I₁₀₅, D₁₁₀, R₁₁₁, D₁₁₄, D₁₁₆, A₁₂₄, A₁₃₆, W₁₅₆; C-SCP-2: D₁₁₄, R₁₃₄, A₁₃₆, S₁₃₈, L₁₃₉, N₁₄₁, N₁₄₄, L₁₄₉, D₁₅₁, V₁₅₃, S₁₅₄, V₁₅₇, F₁₅₉) were mapped to the modeled conformational structure of *Cra a 4* (template PDB: 1q80.1.A) with the key amino acids I₁₀₅ and D₁₁₄, respectively. Thus, based on the biopanning results, four linear epitopes and two conformational epitopes of *Cra a 4* were identified.

Verification of linear mimotope peptides in *Cra a 4*

Based on the above three methods (immunoinformatic tools, a phage library, and an OBOC library), five mimotopes (P1–P5, Table 2) were synthesized for further verification. The mimotope peptides and positive control recombinant *Cra a 4* (r*Cra a 4*) presented a specific IgE-binding capacity with the serum of the 12 oyster-sensitized individuals by dot blot, whereas BSA did not react (Fig. 5A). Additionally, the quantification of the IgE-binding capacity in the dot blot results was analyzed. P1 and P3 showed slightly lower mean values than r*Cra a 4*, P2, P4, and P5 (Fig. 5B).

Moreover, to investigate the relevance of the IgE-binding capacities of the individual synthetic peptides, an iELISA assay was performed *via* competitive IgE interaction between r*Cra a 4* and the five mimotope peptides from the oyster-sensitized individual serum pool. In Fig. 5C, the IC₅₀ values of the inhibition of the individual synthetic peptides (P1–P5) toward r*Cra a 4* were 71.5 ng mL^{−1}, 28.7 ng mL^{−1}, 60.0 ng mL^{−1}, 56.0 ng mL^{−1} and 35.9 ng mL^{−1}, respectively. The IC₅₀ of r*Cra a 4* inhibition toward itself was 33.2 ng mL^{−1}, and no inhibitory activity was observed with the negative control BSA. In addition, when P4 and P5 were used as inhibitors of r*Cra a 4*, the inhibition rate was over 80% at the highest inhibitor con-

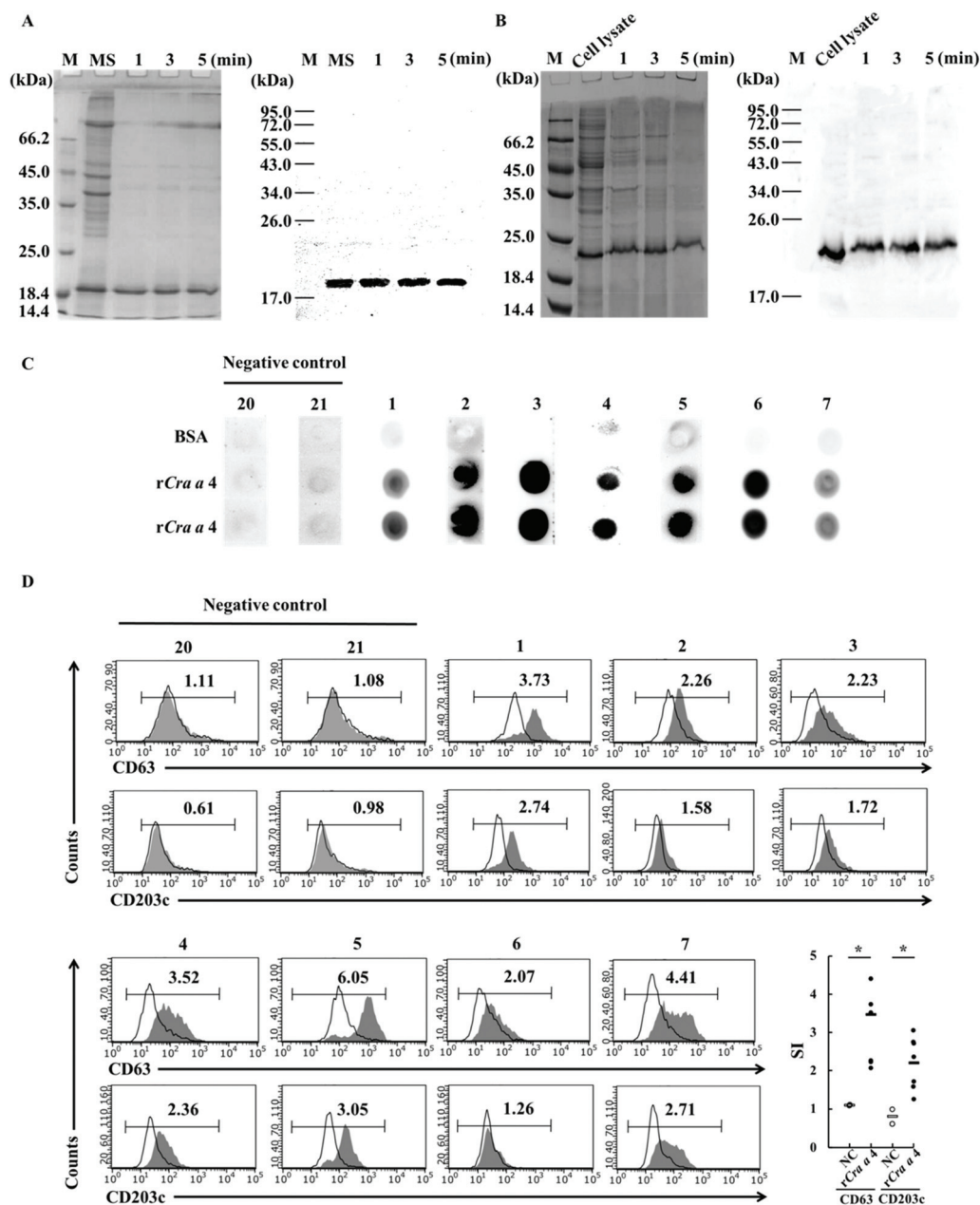


Fig. 1 Thermal stability and BAT analysis of *Cra a 4*. (A) Thermal stability analysis of *C. angulata* myosinogen with a *Cra a 4* polyclonal antibody. Lane M, protein marker; MS, myosinogen. (B) Thermal stability analysis of cell lysate with a *Cra a 4* polyclonal antibody. Lane M, protein marker. (C) IgE-immunoblot assay of r*Cra a 4*. Numbers 1–7 are the serum samples of oyster-sensitized individuals. Numbers 20–21 are the serum samples of non-atopic individuals. BSA was used as the negative control. (D) BAT analysis of r*Cra a 4* from oyster-sensitized patients (numbers 1–7). The black wire-frames without a fill color and the gray-filled regions represent the cell populations stimulated by PBS and r*Cra a 4*, respectively; NC: the serum samples of the two non-atopic individuals were used as the negative control. The horizontal line represents the average values of SI upon PBS or r*Cra a 4* stimulation. *: $p < 0.05$ compared to the negative control.

centration. The five identified linear epitopes of *Cra a 4* are listed in Table 2, and were named L-SCP-1 (P1), L-SCP-2 (P2), L-SCP-3 (P3), L-SCP-4 (P4), and L-SCP-5 (P5).

Epitope and sequence alignment analysis among shellfish SCPs

The IgE epitopes were analyzed comprehensively among the epitope-identified shellfish SCPs in Fig. 6A. The amino acid

sequence alignment of *Litopenaeus vannamei* (Genebank accession number: ACM89179.1), *Penaeus monodon* (Genebank accession number: BAL72725.1), *Scylla paramamosain* (Genebank accession number: JQ860424.1), and *C. angulata* (Genebank accession number: MN956521) was analyzed (Fig. 5A); the identical residues and strongly similar residues are represented by red backgrounds and red text, respectively.

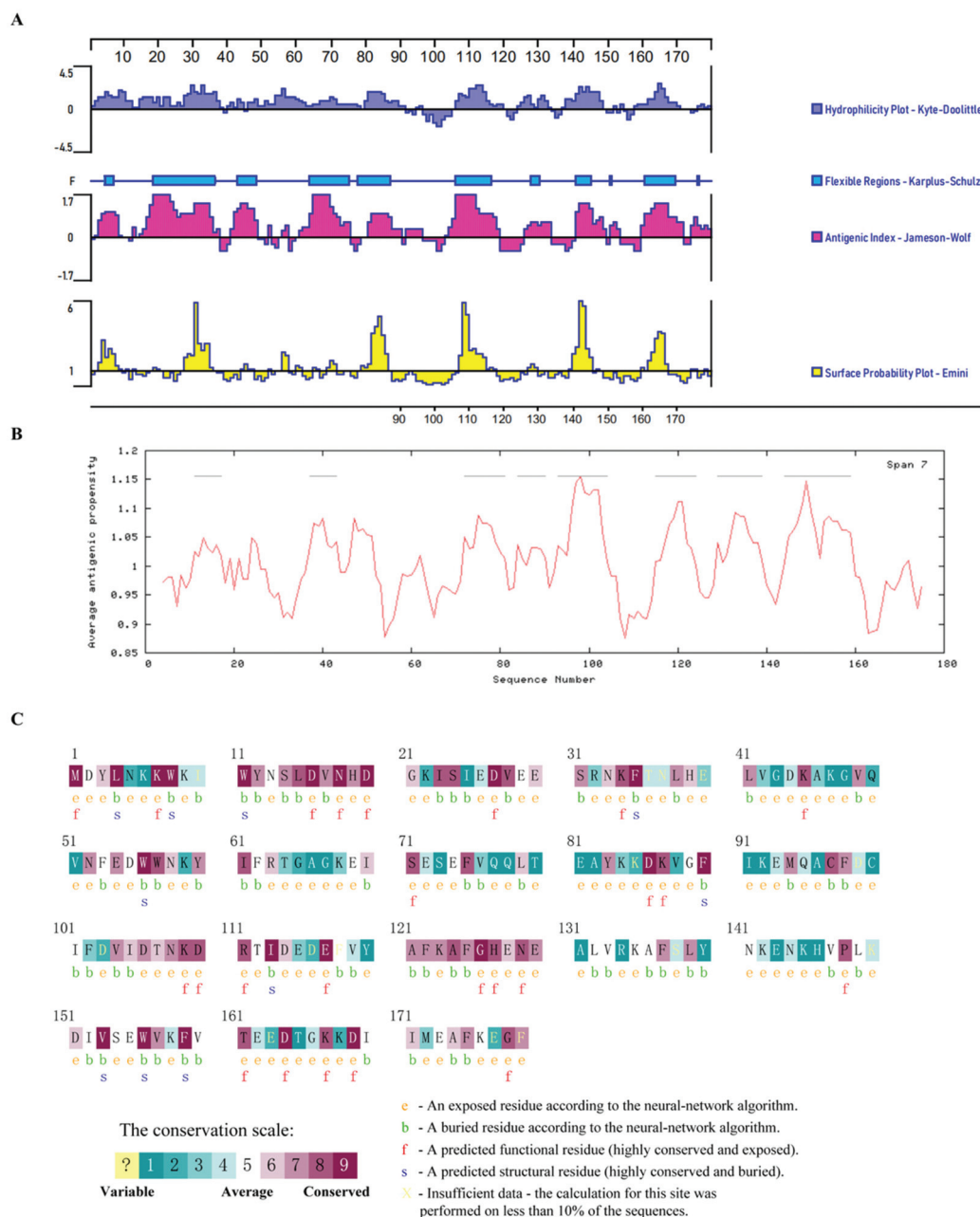


Fig. 2 Prediction of allergenic epitopes in *Cra a 4* using immunoinformatic tools. (A) Prediction of allergenic epitopes in *Cra a 4* using the DNASTAR Protean system. (B) Prediction of allergenic epitopes in *Cra a 4* using Immunomedicine Group. (C) Sequence conservation of *Cra a 4* determined using the ConSurf web server. A score of one to four represents variable, a score of five to six is intermediate, and a score of seven to nine indicates conserved. The most well-conserved residues are presented in dark magenta and the most variable residues are shown in dark cyan.

The amino acid sequences of the SCPs were 10.95%, 16.58%, and 17.44% identical to the *Cra a 4* sequence. Furthermore, the linear epitopes of *L. vannamei* and *Cra a 4*, and the conformational epitopes of *Scy p 4* and *Cra a 4* were marked, the disulfide bonds were predicted, and the calcium-binding sites of the four shellfish species were located on the sequences. It is worth noting that, although the amino acid sequence homology of the SCPs was not very high, the calcium-binding sites were conserved.

Ca²⁺ dependence of the structure and IgE-binding capacity of r*Cra a 4*

In the Ca²⁺-depletion assay, calcium-binding ability was used as an indicator to evaluate the associated structural and IgE-binding capacity changes of the r*Cra a 4*. CD spectroscopic analysis showed that the peaks of r*Cra a 4* were not obvious after the addition of 5 mM EDTA; the spectrum exhibited a weaker typical α -helical feature at approximately 210 nm and

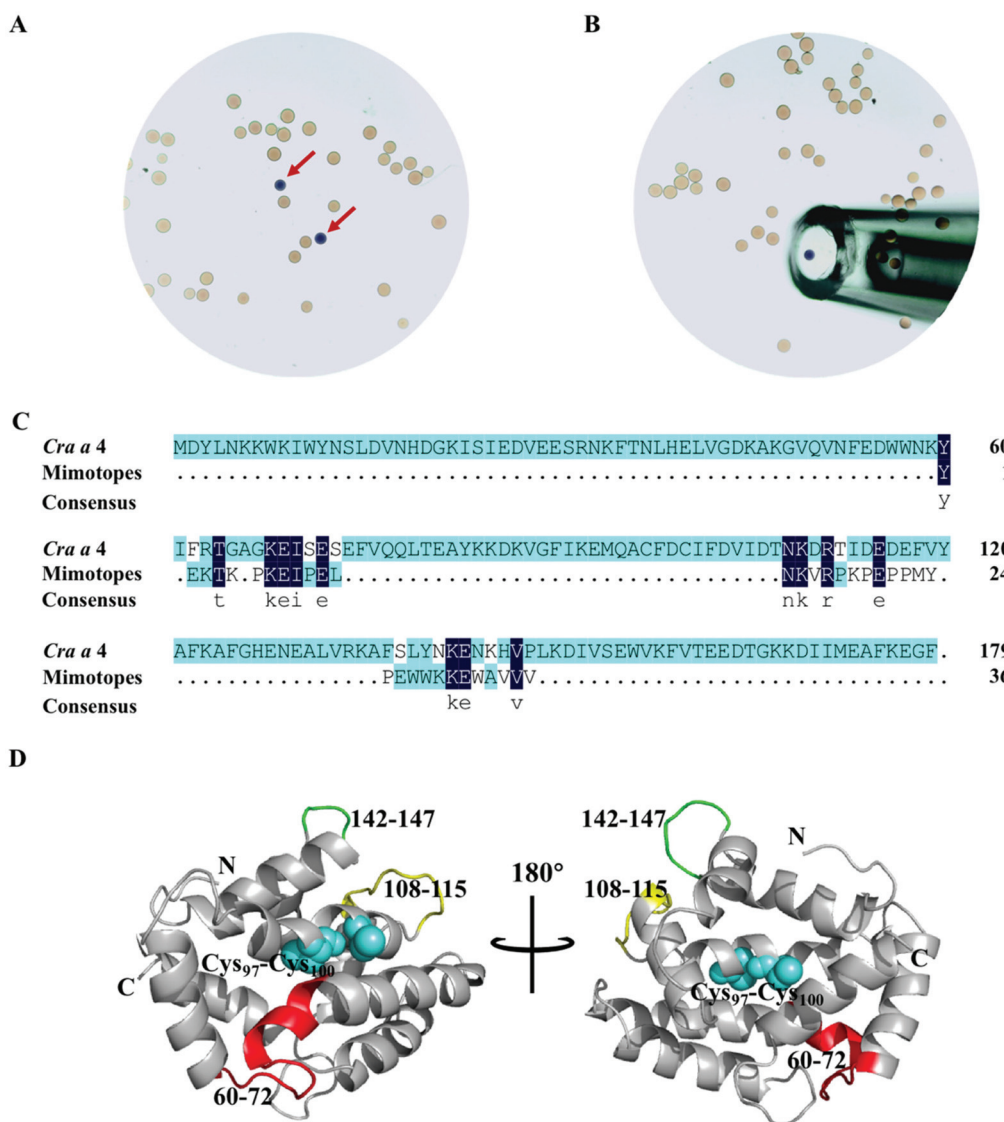


Fig. 3 The IgE mimotopes of *Cra a 4* obtained using the OBOC library. (A) Beads transferred to a Petri dish under an inverted microscope. Positive beads stained in blue are indicated with red arrows. (B) Positive beads were selected with a pipette tip under the microscope. (C) Alignment of the amino acid sequence and linear mimotopes of *Cra a 4*. Matching amino acid sequences and linear epitopes are marked in dark blue. (D) Positions of the IgE linear mimotopes on the 3D structure of *Cra a 4*. IgE linear mimotopes: AA_{60–72}, AA_{108–115}, AA_{142–147}; disulfide bonds: cyan spheres.

220 nm (Fig. 6B). In the surface hydrophobicity results, r*Cra a 4* showed higher hydrophobicity *via* the absorbance of 410 nm to 600 nm in the presence of 5 mM EDTA (Fig. 6C). According to the dot blot analysis, r*Cra a 4* exhibited a specific IgE-binding capacity with the serum pool of the oyster-sensitized individuals, whereas BSA did not react. In contrast, r*Cra a 4* showed a weak IgE-binding capacity in the presence of 5 mM EDTA (Fig. 6D).

Discussion

Molluscan shellfish is a common cause of type I allergic reactions, especially in coastal countries.³ In addition to the major myofibrillar allergen tropomyosin, SCP in myosinogen is con-

sidered to be a very stable allergen among shellfish allergens, and the cross-reactivity of SCP between different molluscan and crustacean species has been explored.^{10,11,14} We have previously shown that *Cra a 4* and its recombinant form demonstrate specific recognition by IgE from oyster-allergic patients.¹⁴ Patent protection for the preparation methods and application of the recombinant protein has been applied for in China under patent application number 201910818141.7. It is known that the degranulation of effector cells such as basophil cells results in the anaphylaxis symptoms in type I food allergies.¹⁵ Since the knowledge of oyster SCP is currently limited, there is a need to further detect the effects of the allergen on patients' effector cells and identify the IgE epitopes to provide a basis for developing food processing methods that can reduce or eliminate the allergenicity of the allergen.

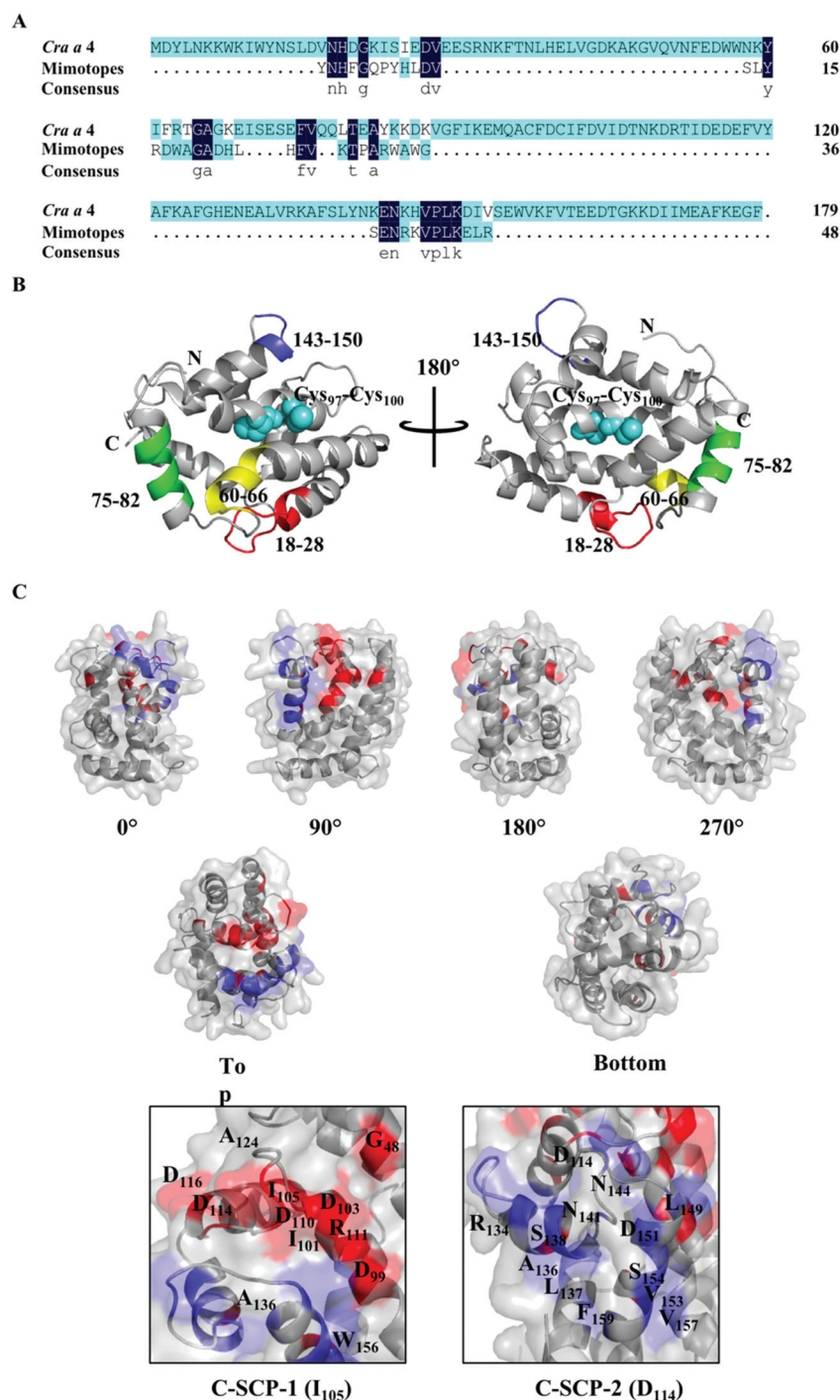


Fig. 4 IgE mimotope mapping of *Cra a 4* using biopanning. (A) Alignment of the amino acid sequence and linear mimotopes of *Cra a 4*. Matching amino acid sequences and linear epitopes are marked in dark blue. (B) Position of the IgE linear epitopes and disulfide bonds on the 3D structure of *Cra a 4*. IgE linear mimotopes: AA_{18–28}, AA_{60–66}, AA_{75–82}, AA_{143–150}; disulfide bonds: cyan spheres. (C) Distribution of the IgE conformational mimotopes in the 3D structure of *Cra a 4*. Upper panel: a molecular surface representation; lower panel: amino acids representing two different potential IgE-binding areas of *Cra a 4*. C-SCP-1 (red), G₄₈, D₉₉, I₁₀₁, D₁₀₃, I₁₀₅, D₁₁₀, R₁₁₁, D₁₁₄, D₁₁₆, A₁₂₄, A₁₃₆, W₁₅₆; C-SCP-2 (blue), D₁₁₄, R₁₃₄, A₁₃₆, S₁₃₈, L₁₃₉, N₁₄₁, N₁₄₄, L₁₄₉, D₁₅₁, V₁₅₃, S₁₅₄, V₁₅₇, F₁₅₉. The key amino acids are I₁₀₅ and D₁₁₄, respectively.

BAT is a useful diagnostic test for food allergens that can expeditiously protect patients from the risk of inducing a severe allergic reaction, especially when skin tests and specific IgE tests are inconclusive.²⁸ Thus, *in vitro* BAT was used to

further confirm the IgE reactivity in effector cells in our present study. r*Cra a 4* induced significant upregulation of CD63/CD203c, and showed obvious allergenicity, in agreement with the obtained specific IgE values; these results were

Table 2 Information relating to the synthetic peptides and verified linear epitopes of *Cra a 4*

Peptide no.	Sequence	Position	Purity (%)	Molecular weight (Da)	Verified linear epitope no.
P1	KISIEDVEESRN	22–33	96.6	1418.50	L-SCP-1
P2	TGAGKEISESEF	64–75	98.6	1254.30	L-SCP-2
P3	TEAYKKDKVGF	80–90	98.5	1285.44	L-SCP-3
P4	TNKDRTIDED	107–116	98.2	1206.21	L-SCP-4
P5	NKHVPLKDIVEWVKF	144–159	97.5	1939.25	L-SCP-5

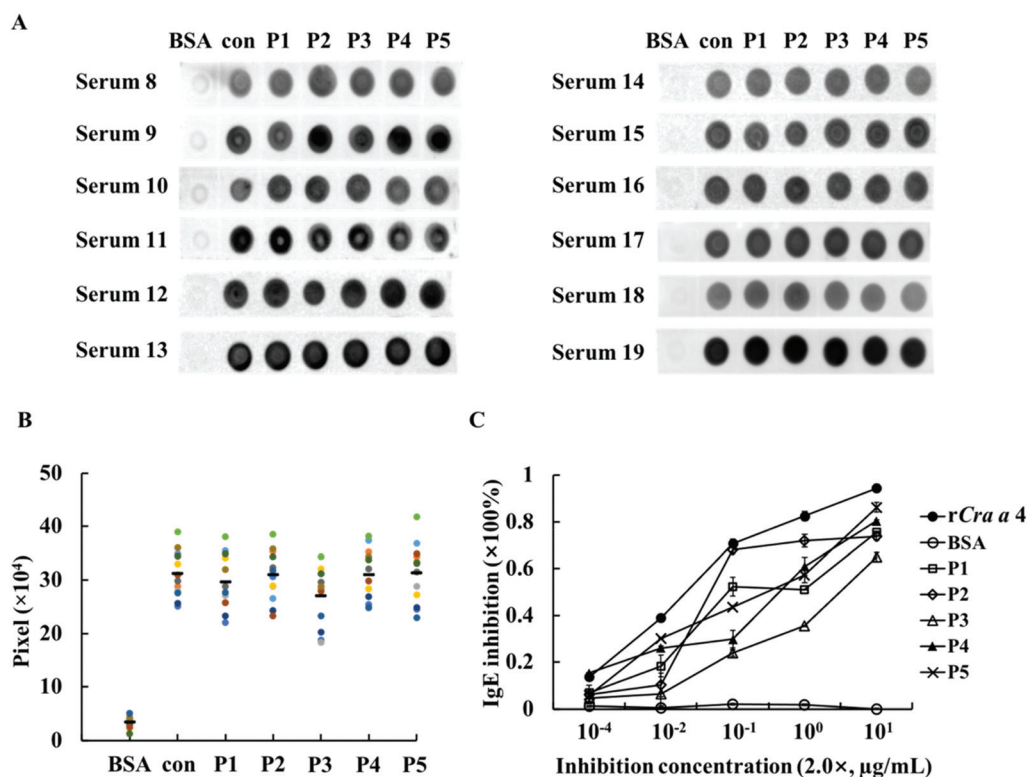


Fig. 5 IgE-binding capacity of the *Cra a 4* mimotope peptides. (A) IgE-immunoblot assay of five mimotope peptides (P1–P5). Serum 8–19 are the serum samples of oyster-sensitized individuals. BSA was used as the negative control; *rCra a 4* was used as the positive control. (B) Intensity of the dots shown on the nitrocellulose membranes in A. Quantification of the grayscale dots was conducted using the software ImageJ. The horizontal lines represent the average values of the dot pixels. BSA was used as the negative control; *rCra a 4* was used as the positive control. (C) Inhibition ELISA assay of the five mimotope peptides. *rCra a 4* was in the solid phase; the IgE-binding of the serum pool (numbers 8–19, 1 : 3 dilution) was determined using the different mimotope peptides (P1–P5). BSA was used as the negative control; *rCra a 4* was used as the positive control. All data are presented as mean $\pm$ SD ($n = 3$).

similar to previous studies of immunological reactivity to crustacean SCPs.^{7,10,11}

With the development of computer technology and bioinformatics, researchers have developed new types of software to predict antigen epitopes.³⁶ In this study, the *Cra a 4* sequence was analyzed using five immunoinformatic tools to improve the prediction accuracy, and twenty-three mimotopes were predicted based on hydrophilicity, flexibility, and accessibility, which provided a data basis for subsequent epitope verification. Additionally, OBOC peptide libraries^{37,38} and phage display technology^{39,40} are powerful tools to identify ligand and receptor interactions. As a result of the OBOC library screening, three linear mimotopes, YEKTKPKEIPEL,

NKVRPKPEPPMY, and PEWWKKEWAVV, were obtained using the serum pool of the oyster-sensitized individuals. Based on the location of the amino acids and whether they are contiguous in a protein primary sequence, epitopes are typically categorized as either linear or conformational. Phage-display libraries allow the selection of mimotope peptides regardless of whether the epitope is linear or conformational.⁴¹ Hereby, four linear mimotopes (YNHFGQPYHLDV, SLYRDWAGADHL, HFVKTPARWAWG, and SENRKVPLKELR) and two conformational mimotopes (C-SCP-1: G₄₈, D₉₉, I₁₀₁, D₁₀₃, I₁₀₅, D₁₁₀, R₁₁₁, D₁₁₄, D₁₁₆, A₁₂₄, A₁₃₆, W₁₅₆; C-SCP-2: D₁₁₄, R₁₃₄, A₁₃₆, S₁₃₈, L₁₃₉, N₁₄₁, N₁₄₄, L₁₄₉, D₁₅₁, V₁₅₃, S₁₅₄, V₁₅₇, F₁₅₉) were obtained from the serum of the individuals who were confirmed to be

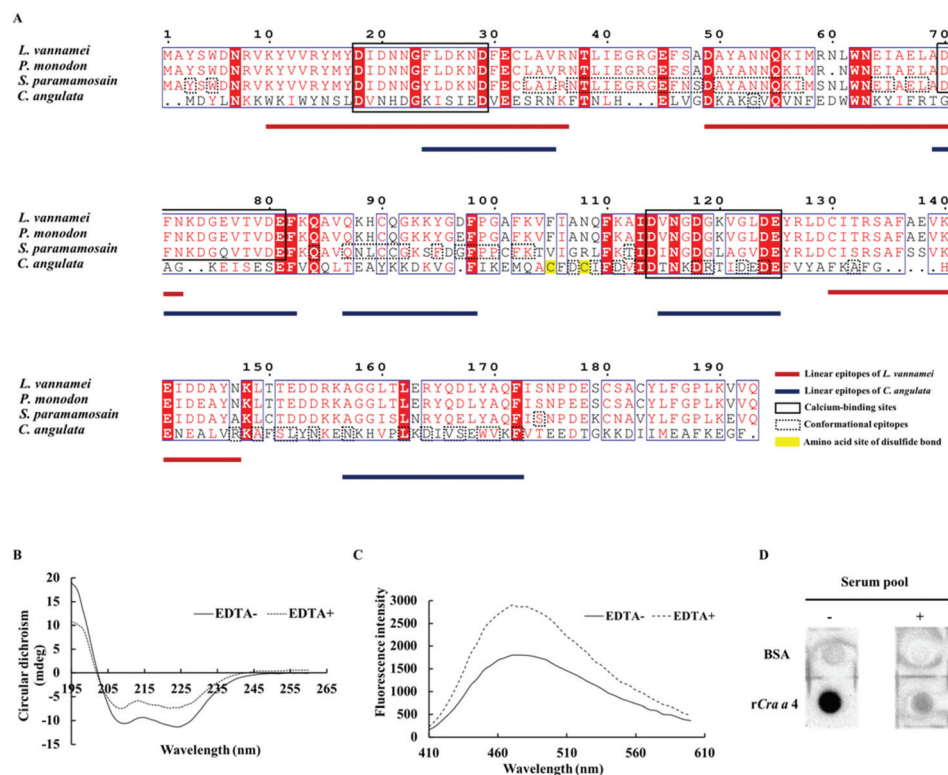


Fig. 6 Comprehensive analysis of the IgE mimotopes and calcium-binding sites in *Cra a 4*. (A) Amino acid sequence alignment of the epitope-identified shellfish SCPs. The sequences are the following: *L. vannamei* (Genebank accession number: ACM89179.1), *P. monodon* (Genebank accession number: BAL72725.1), *S. paramamosain* (Genebank accession number: JQ860424.1), and *C. angulata* (Genebank accession number: MN956521). The color represents residue similarity: red background, identical; red text, strongly similar. Linear epitopes of *L. vannamei* are marked with red lines; linear epitopes of *C. angulata* are marked with dark blue lines; calcium-binding sites of the four shellfish species are marked by black boxes; conformational epitopes of *Scy p 4* and *Cra a 4* are marked by black dotted boxes; amino acid sites of the disulfide bonds are highlighted in yellow. (B) Secondary structural analysis of *rCra a 4* after the addition of EDTA. (C) Surface hydrophobicity analysis of *rCra a 4* after the addition of EDTA. (D) IgE-immunoblot assay of *rCra a 4* after the addition of EDTA. The serum pool (1 : 3 dilution) is the primary antibody including serum sample numbers 8–19.

allergic by BAT. At present, allergenic epitope screening is typically detected using a single method. Three linear epitopes of *L. vannamei* SCP were identified using synthetic overlapping peptides spanning,⁴² and six conformational epitopes of *Scy p 4* were measured using biopanning;²³ the absence of conformational epitopes in *L. vannamei* SCP and linear epitopes in *Scy p 4* may have been due to the use of a small number of serum samples or a single screening pattern. Herein, five linear mimotopes and two conformational mimotopes were obtained using the above three methods. We found that the two mimotopes (AA_{60–66} and AA_{143–150}) determined by biopanning were consistent with the mimotopes (AA_{60–72} and AA_{142–147}) obtained by OBOC screening, and three mimotopes (AA_{18–28}, AA_{60–66}, and AA_{143–150}) screened using the above methods were similar to the reported linear epitopes in *L. vannamei* SCP (AA_{10–36}, AA_{49–72}, and AA_{130–147}),⁴² indicating that the compound methods are more reliable and more accurate than a single method. Additionally, the cross-reactivity between the two species that was explored¹⁴ may relate to the conserved epitopes. Due to the fact that the serum of different sensitized individuals was used in the phage display library

and OBOC library experiments, the mimotopes are complementary, which could allow the completion of the epitope information of *Cra a 4* synthetically by further combination with immunoinformatic tools.

Three traditional and recent methods were integrated to determine the epitopes of *Cra a 4* in this study to improve the reliability. The five mimotopes were synthesized and displayed strong IgE-binding capacity with the serum of 12 oyster-sensitized individuals by dot blot. Furthermore, competitive-binding methods, such as an iELISA assay, have already been shown to be a rapid and efficient means of epitope identification.³⁴ Similarly, the immunoreactivity of P1–P5 was identified *via* an iELISA. In the results, L-SCP-1 (AA_{22–33}), L-SCP-2 (AA_{64–75}), L-SCP-3 (AA_{80–90}), L-SCP-4 (AA_{107–116}), and L-SCP-5 (AA_{144–159}) showed strong IgE-binding capacity with slight differences; these regions may be crucial epitopes of *Cra a 4*. However, future studies should verify the conformational IgE epitopes of *Cra a 4* to advance our knowledge of the allergen epitopes in oyster.

EF-hand Ca²⁺-binding allergens have been shown to contain conformational IgE epitopes associated with the Ca²⁺

chelation.⁴³ In this study, the sequence alignment analysis of epitope-identified shellfish SCPs showed high sequence similarity in the EF-hand Ca^{2+} -binding sites, whereas the homology of the overall sequences was not high. In addition, we found that C-SCP-1 with the key amino acid D₁₁₄ was located in the calcium-binding sites AA_{106–117}. It was demonstrated that the secondary structure of shrimp SCP was changed and the IgE reactivity was considerably reduced by 38–83% in the presence of EGTA.⁴³ When the calcium-chelating agent EDTA was added, the peaks of *Scy p 4* were not obvious in the CD spectrum and the fluorescence peak increased by nearly 10-fold.²⁷ Similarly, the secondary structure and hydrophobicity of r*Cra a 4* changed after Ca^{2+} -depletion, which led to an obvious reduction of the IgE-binding capacity. These facts may demonstrate the potential of the Ca^{2+} -binding sites to create conformational IgE epitopes and cause cross-reactivity between allergenic Ca^{2+} -binding proteins. Moreover, the mutagenesis of critical amino acids in epitopes has been carried out to regulate the allergen allergenicity,^{44,45} and site-directed mutations were used to replace key amino acids in the conformational epitopes and calcium-binding sites of *Scy p 4*, which effectively reduced the immunoreactivity.²⁷ Thus, the key amino acids I₁₀₅ and D₁₁₄ located in the conformational epitope and calcium-binding site should be further researched, as they may provide evidence to design mutated hypoallergenic forms for reducing the immunoreactivity of various allergens *via* molecular modification.

In the present study, we investigated the allergenicity of the heat-resistant protein *Cra a 4*. Five linear IgE epitopes (L-SCP-1, L-SCP-2, L-SCP-3, L-SCP-4, and L-SCP-5) and two conformational IgE epitopes (C-SCP-1 and C-SCP-2) in *Cra a 4* were obtained synthetically using three methods. The five linear epitopes exhibited strong IgE-binding capacity and may act as crucial epitopes. In addition, C-SCP-1 is located at one of the calcium-binding sites (AA_{106–117}), which affected the allergen sensitivity. Collectively, the combined epitope screening regimen might provide a more reliable basis for obtaining allergenic epitopes, and the reduced sensitization *via* Ca^{2+} depletion could provide the foundation for the effective development of hypoallergenic products.

Abbreviations

AIT	Allergen immunotherapy
ANOVA	One-way repeated-measures analysis of variance
ANS	8-Anilino-1-naphthalenesulfonic acid
bp	Base pair
BSA	Bovine serum albumin
CD	Circular dichroism
DIC	Diisopropylcarbodiimide
DMF	<i>N,N</i> -Dimethylformamide
HOBt	<i>N</i> -Hydro-benzotriazole
iELISA	Inhibition enzyme-linked immune sorbent assay
SCP	Sarcoplasmic-calcium-binding protein
SD	Standard deviation

OBOC	One-bead-one-compound
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
WHO/IUIS	World Health Organization/International Union of Immunological Societies
3D structure	Three-dimensional structure

Author contributions

Tian-Jiao Han: Conceptualization, methodology, validation, investigation, formal analysis, writing – original draft, Visualization. Fei Huan: Investigation, data curation. Meng Liu: Conceptualization, methodology. Meng-Si Li: Methodology. Yang Yang: Investigation. Gui-Xia Chen: Resources. Dong Lai: Resources. Min-Jie Cao: Supervision, writing – review & editing. Guang-Ming Liu: Writing – review & editing, supervision, project administration, funding acquisition.

Conflicts of interest

The authors declare no competing financial interests.

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1 Supporting information

2 Figure legends

3 Figure S1. The biopanning process of *Cra a 4* mimotopes by phage display technology

4 (A) Titration of the eluted and amplified phages.

5 (B) Agarose gel electrophoresis of phage DNA. Lane M: DNA marker; Lane 1-20, DNA phages.

6 Table S1. Location of the allergenic epitopes of *Cra a 4* predicted by immunoinformatic tools.

Tools	Amino acid regions
ABCpred	7-91, 93-178
BepiPred 1.0 Server	22-33, 44-48, 66-72, 81-85, 107-115, 144-147
NetMHC II 2.3 Server	58-73
Immunomedicine Group	11-17, 37-43, 72-81, 84-90, 93-104, 115-124, 129-139, 144-159
DNASTar Protean	19-36, 64-75, 80-87, 106-116, 141-145, 161-169

7

8

9 **Figure S1**

