

Linear Epitopes Play an Important Role in the Immunoglobulin G (IgG)/Immunoglobulin E (IgE)-Binding Capacity of Scy p 4

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ABSTRACT: Sarcoplasmic calcium-binding protein is a stable allergen in *Scylla paramamosain* and named Scy p 4. To explore the importance of linear epitopes in the immunoglobulin G (IgG)/immunoglobulin E (IgE)-binding capacity of Scy p 4, chemical denaturants were used to destroy the structure. Scy p 4 was reduced with dithiothreitol and subsequently alkylated with iodoacetamide (IAA). Furthermore, the structural analysis indicated that IAA–Scy p 4 was an unstructured protein. The inhibition enzyme-linked immunosorbent assay showed that IAA–Scy p 4 could inhibit the binding of Scy p 4 to sensitize serum, with inhibition rates reached 55%. Moreover, the linear mimotopes of Scy p 4 were predicted *in silico*. Three linear epitopes were verified by serological tests and named L-Scy p 4-1 (AA_{76–91}), L-Scy p 4-2 (AA_{111–125}), and L-Scy p 4-3 (AA_{137–146}). Overall, these data provide an understanding of the relationship between the structure and allergenicity about Scy p 4, and the identified linear epitopes can be used for diagnosis and food processing of shellfish allergy.

KEYWORDS: denaturation treatment, disulfide bonds, IgG/IgE-binding capacity, linear epitopes, sarcoplasmic calcium-binding protein

INTRODUCTION

Food allergies are defined as adverse immune responses to food proteins that result in typical clinical symptoms involving the dermatologic, respiratory, gastrointestinal, cardiovascular, and/or neurologic systems.¹ Eight foods cause 90% of food-allergic reactions,² with shellfish being one of them. Similar to peanuts, shellfish account for a high proportion of food-induced allergic reactions, affecting nearly 42% adults and 12% children.³ Allergens have been studied extensively in shellfish, which include tropomyosin, arginine kinase, myosin light chain, triosephosphate isomerase, filamin C, and sarcoplasmic calcium-binding protein (SCP).

SCP is a heat-resistant protein found in shellfish.^{4,5} It has been identified as an allergen in crustaceans and mollusks. In crustaceans, SCPs demonstrate significant amino acid sequence homology that varied between 78 and 98%, whereas the homology between crustacean and mollusk SCPs is only 15–21%.^{6–8} SCP was first identified as an allergen in *Penaeus monodon* (Pen m 4).⁹ Hu et al.⁵ purified and confirmed SCP from *Scylla paramamosain* and subsequently was named Scy p 4 by the World Health Organization and International Union of Immunological Societies. SCP elicits 29–50% positive immunoglobulin E (IgE)-binding among shrimp-allergic patients.¹⁰ In shrimp, SCP was recognized with particular intensity in the serum of 59.6% of subjects (31/52), and SCP had higher intensity than tropomyosin in several cases.⁷ Hence, allergic reactions caused by SCP should not be ignored.

Previous studies have found that the enzyme cross-linking reaction and Maillard reaction could reduce the immunoreactivity of Scy p 4, which is due to Scy p 4 being modified to change its structure.⁵ Besides, the immunoglobulin G (IgG)/

IgE-binding capacity of food allergens was reduced by high-pressure, ultrasonic, and irradiation treatment as a result of the secondary structure being changed and non-covalent bonds being destroyed.¹¹ The structure and activity of allergen are closely related.¹² Chemical denaturants were used to study the conformation–activity relation of protein.^{13,14} However, the effects of chemical denaturants on the IgG/IgE-binding capacity of Scy p 4 was rarely studied.

SCP belongs to the EF-hand calcium-binding protein family.¹⁵ The conformation, stability, and binding of the IgE antibody of EF-hand-type allergens largely depend upon the binding of allergens to calcium ions.¹⁶ In addition, disulfide bonds have been reported to play a critical role in stabilizing the allergen structure.¹⁷ Mei et al.¹⁸ destroyed the disulfide bonds of arginine kinase, which significantly reduced the immunoreactivity of arginine kinase. There were seven cysteines in the amino acid sequence of Scy p 4. Whether there were disulfide bonds in Scy p 4 and the effects of disulfide bonds on the structure and IgG/IgE-binding capacity of Scy p 4 should be further investigated.

There are many forms of food allergy, of which the most common are IgE-mediated.¹⁹ Epitopes are related to the allergenicity of allergens, and linear and conformational IgE epitopes are generally believed to exist in food allergens.²⁰

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Linear epitopes were a sequence of contiguous amino acids that are recognized by the IgE antibody and the dependence of conformational epitopes upon the tertiary structure.²¹ Linear epitopes can be identified by immune-screening assays of allergen-derived overlapping of synthetic peptides with the serum of sensitized individuals.²² Further, the ongoing progress in bioinformatics largely contributed to the understanding of the epitopes of allergenicity. In recent years, bioinformatics combined with immune screening has become a common way to predict the epitopes of allergens.²³ However, identification of linear epitopes in Scy p 4 has not been reported. Identifying the linear epitopes and perfecting the epitope map of Scy p 4 were an essential step for in-depth analysis of shellfish allergy.

This study was designed to analyze the importance of linear epitopes on Scy p 4 IgG/IgE-binding capacity and identify the linear epitopes of Scy p 4. For these purposes, chemical denaturants were used to destroy the tertiary structure of Scy p 4, and the effects of different forces on the immune-binding activity of Scy p 4 were analyzed by dot blotting. To further analyze the effect of disulfide bonds on the immune-binding capacity of Scy p 4, secondary structures and surface hydrophobicities were analyzed by circular dichroism (CD) and fluorescent 8-anilino-1-naphthalenesulfonic acid (ANS) probes. Moreover, the IgE-binding capacity was analyzed by the inhibition enzyme-linked immunosorbent assay (iELISA) with serum from crab-sensitized individuals. Furthermore, the linear mimotopes were predicted by immunoinformatic tools. Subsequently, the mimotopes were comprehensively analyzed, synthesized, and identified by inhibition dot blotting and iELISA.

MATERIALS AND METHODS

Materials. The rabbit anti-Scy p 4 polyclonal antibody was prepared in our laboratory as previously described.⁵ BL21(DE3)-pET-22b-Scy p 4 was prepared in our previous studies.²⁴ Goat anti-rabbit IgG-horseradish peroxidase (HRP) antibody, goat anti-human IgE-HRP antibody, iodoacetamide (IAA) and ANS were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). (2S,3S)-1,4-bis(sulfanyl)butane-2,3-diol (DTT), urea, guanidine hydrochloride (GdnHCl) and ethylenediaminetetraacetic acid (EDTA) were purchased from Biotech (Shanghai, China). All other reagents were of analytical grade.

Serum Samples. Serum was collected from different crab-sensitized individuals and non-allergic individuals at the Women and Children's Hospital Affiliated to Xiamen University (human ethical approval KY-2019-014, Xiamen, China) and The Second Affiliated Hospital of Xiamen Medical College (human ethical approval 2020018, Xiamen, China). The specificities of the IgE levels to crab were assessed *in vitro* using ImmunoCAP (Phadia AB, Rapsgatan, Uppsala, Sweden); samples with values of ≥ 0.35 kUA/L were stored at -20 °C until use. Table 1 showed the individual sensitivity profiles and clinical symptoms that were recorded in the present study. Pooled serum was prepared by mixing equal amounts of serum samples (Table 1).

Preparation, Chemical Denaturation, and Dot Blotting Analysis of Scy p 4. Scy p 4 was expressed in *Escherichia coli* and purified as described elsewhere.²⁴ Scy p 4 was destroyed by chemical denaturation treatment as described according to Yang et al.,²⁵ which revealed some minor changes. Scy p 4 was mixed with different concentrations of DTT, urea, GdnHCl, or EDTA. Then, all of the mixtures were incubated at 4 °C overnight. Denaturant-treated Scy p 4 was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Denaturant-treated Scy p 4 was detected by dot blotting based on a previously described protocol.²⁶ Briefly, denaturant-treated Scy p 4 was incubated with the rabbit anti-Scy p 4 polyclonal antibody (dilution of $1:10^5$) or the serum pool of crab-sensitized individuals (mixed serum of S1–S10 in Table 1; dilution of 1:3).

Table 1. Specific IgE Levels and Symptoms of the Crab-Sensitized Individuals

serum number	age (years)	sex ^a	sIgE crab (f23) by ImmunoCAP (kUA/L) ^b	symptoms
S1	46	M	15.3	pruritus and nausea
S2	12	F	14.4	nausea and emesis
S3	35	M	6.4	allergic purpura
S4	21	F	58.4	allergic eczema
S5	6	F	24.5	allergic eczema and itchy skin
S6	20	F	22.5	chronic urticaria
S7	7	M	51.5	pruritus and nausea
S8	9	M	40.2	atopic dermatitis
S9	29	F	32.5	anaphylactic rhinitis
S10	23	F	17.0	pruritus
C1	26	M	0.2	c
C2	14	M	0.2	c

^aM, male; F, female. ^bA serum with specific IgE of <0.35 kUA/L is defined as negative. ^cNo symptoms during the experiment. The people were non-allergic individuals.

Reduction and Alkylation Treatment and IgG/IgE-Binding Capacity Analysis of IAA–Scy p 4. The disulfide bonds of Scy p 4 were destroyed by alkylation following DTT and IAA treatment, as described according to Tscheppe et al.²⁷ IAA–Scy p 4 was analyzed by SDS–PAGE and western blotting using the rabbit anti-Scy p 4 polyclonal antibody (dilution of $1:10^4$).

Enzyme-linked immunosorbent assay (ELISA) was conducted according to the method described by Huang et al.,²⁸ with some modification. IAA–Scy p 4 (or Scy p 4) was incubated with the rabbit anti-Scy p 4 polyclonal antibody (dilution of $1:10^4$) or the serum pool of crab-sensitized individuals.

iELISA was conducted according to the method described by Sun et al.,²⁹ with slight modification. The inhibitor (Scy p 4 or IAA–Scy p 4) was diluted to different concentrations and then pre-incubated with the rabbit anti-Scy p 4 polyclonal antibody (dilution of $1:10^5$) or the serum pool of crab-sensitized individuals. The inhibition rates were calculated as follows:

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

When the inhibition rates were 50%, the corresponding concentration of the inhibitor was called the half-maximal inhibitory concentration (IC_{50}).

Secondary Structure and Hydrophobicity Analysis of IAA–Scy p 4. The secondary structure of IAA–Scy p 4 was determined from the CD spectroscopy (Applied Photophysics, Ltd., Surrey, U.K.) at 25 °C. Scy p 4 or IAA–Scy p 4 was prepared at a concentration of 0.30 mg/mL in 20 mM phosphate-buffered saline (PBS, pH 8.0).

The surface hydrophobicity of IAA–Scy p 4 was detected using the ANS fluorescent probe method.³⁰ The sample was diluted with PBS to 0.30 mg/mL. The fluorescence value was measured at an excitation wavelength of 375 nm, and the emission wavelength range was 400–600 nm.

Prediction and Identification of Linear Epitopes. The sequence of Scy p 4 was analyzed using five immunoinformatic tools, including ABCpred-, BepiPred 2.0-, Immunomedicine Group-, DNASTar Protean-, and BCPred Predictions-based computational approaches. The predicted peptides were synthesized by GenScript Biotech Corp. (Nanjing, Jiangsu, China).

Inhibition dot blotting was performed to analyze the IgE-binding capacity of synthetic peptides as described by Zhang et al.³¹ The inhibitor (Scy p 4 or synthetic peptide) was diluted to different concentrations and then pre-incubated with serum samples from 10 crab-sensitized individuals (serum of S1–S10 in Table 1). The quantification of the dots was performed with ImageJ software.

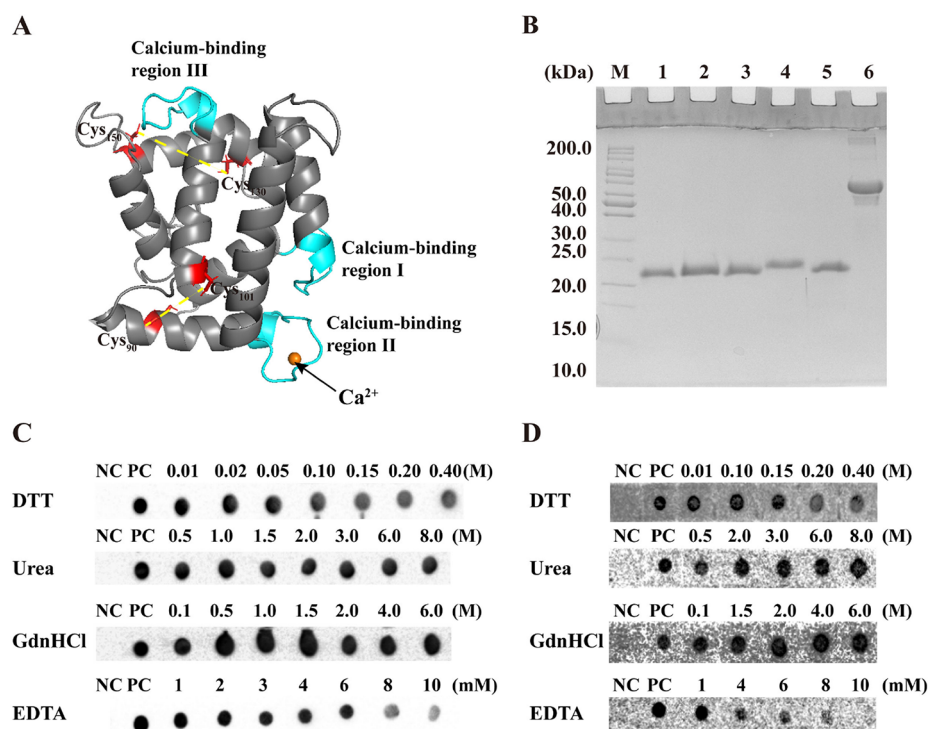


Figure 1. IgG/IgE-binding capacity analysis of Scy p 4 with denaturant treatment. (A) Position of disulfide bonds and calcium-binding sites on the 3D structure of Scy p 4. Cysteines were colored in red; the yellow dotted line represented the disulfide bonds; and light blue represented calcium-binding sites in Scy p 4. (B) SDS-PAGE analysis of denaturant-treated Scy p 4. Lane M, protein marker; lane 1, Scy p 4; lane 2, Scy p 4 with 0.40 M DTT treatment; lane 3, Scy p 4 with 8.0 M urea treatment; lane 4, Scy p 4 with 6.0 M GdnHCl treatment; lane 5, Scy p 4 with 10 mM EDTA treatment; and lane 6, BSA. (C) Dot blotting analysis of the IgG-binding capacity of denaturant-treated Scy p 4, with the rabbit anti-Scy p 4 polyclonal antibody dilution of 1:10⁴. NC, BSA; PC, Scy p 4. (D) Dot blotting analysis of the IgE-binding capacity of denaturant-treated Scy p 4 by the serum pool of crab-sensitized individuals (mixed serum of S1–S10 in Table 1; dilution of 1:5). NC, BSA; PC, Scy p 4.

Synthetic peptides were detected with iELISA. The inhibitor (Scy p 4, synthetic mimotope peptides, or peptide mix) were pre-incubated with the serum pool of crab-sensitized individuals (mixed serum of S1–S10 in Table 1; dilution of 1:3).

Structure Prediction and Linear Epitope Comparison among Shellfish SCPs. The three-dimensional (3D) structure was modeled using I-TASSER (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>), and the Protein Data Bank (PDB) file was viewed with PyMol software. Disulfide bonds and calcium-binding sites of Scy p 4 were predicted using Cyscon (<http://www.csbio.sjtu.edu.cn/bioinf/Cyscon/>) and ExPASy peptide cutter (<https://www.expasy.org/>), respectively. Amino acid frequency of Scy p 4 and linear epitopes was measured by BioEdit. The amino acid sequences of shellfish SCPs were aligned using ClustalW (<https://www.expasy.org/resources/clustalw/>), and linear epitopes of shellfish SCPs were identified.

Statistical Analysis. Data are presented as the mean $\pm$ standard deviation (SD). Significant differences were calculated using a one-way analysis of variance with Tukey's test. $p < 0.05$ was regarded as statistically significant.

RESULTS

Effects of Chemical Denaturant on the IgG/IgE-Binding Capacity of Scy p 4. According to structure prediction, Scy p 4 had six helix–loop–helix motifs (namely, EF-hand domains), forming three calcium-binding sites (Figure 1A). Besides, there were two pairs of disulfide bonds (Cys₉₀–Cys₁₀₁ and Cys₁₃₀–Cys₁₅₀). The formation of purified Scy p 4 and denaturant-treated Scy p 4 was from SDS-PAGE. To prevent incomplete denaturation, the maximum concentration of the denaturant was selected in SDS-PAGE. The amount of denaturing agent was 0.40 M DTT, 8.0 M urea, 6.0 M GdnHCl, and 10 mM EDTA, separately. In comparison to Scy p 4,

denaturant-treated Scy p 4 had approximately the same molecular weights (Figure 1B).

To investigate the IgG-binding capacity of Scy p 4 under different denaturation conditions. The chemical denaturant (DTT, urea, GdnHCl, or EDTA) was used. The result of dot blotting (Figure 1C) revealed that DTT and EDTA influenced the IgG-binding capacity of Scy p 4 to different degrees. Scy p 4 reactivity was reduced by 0.10 M DTT, and the reactivity of Scy p 4 was significantly reduced when the EDTA concentration reached 8 mM. In the urea/GdnHCl-treated samples, there was no significant change in the IgG-binding capacity of Scy p 4.

To explore the IgE-binding capacity of Scy p 4, the denatured proteins were probed with the serum pool of crab-sensitized individuals (Figure 1D). There was a slight change in the IgE-binding activity of Scy p 4 after 0.20 M DTT (and above) treatment. Moreover, the IgE-binding capacity of Scy p 4 treated with 4 mM EDTA was significantly lower than non-denatured Scy p 4. Nevertheless, even when the concentration of GdnHCl reached 6.0 M and urea reached 8.0 M, the IgE-binding capacity of the samples was unchanged. Among the four treatments, the IgE-binding capacity of Scy p 4 was reduced by high-concentration DTT and EDTA.

Construction and Characterization of IAA–Scy p 4. Cystine linkages in proteins are often opened with reducing agents (such as DTT), often to eliminate disulfide linkages from complicating analysis of the digest. After reduction, the sulfhydryls usually reacted with IAA or another electrophile to prevent reformation of disulfide linkages in a random manner. To explore the importance of disulfide bonds, Scy p 4 was treated with DTT and IAA to generate IAA–Scy p 4 (Figure

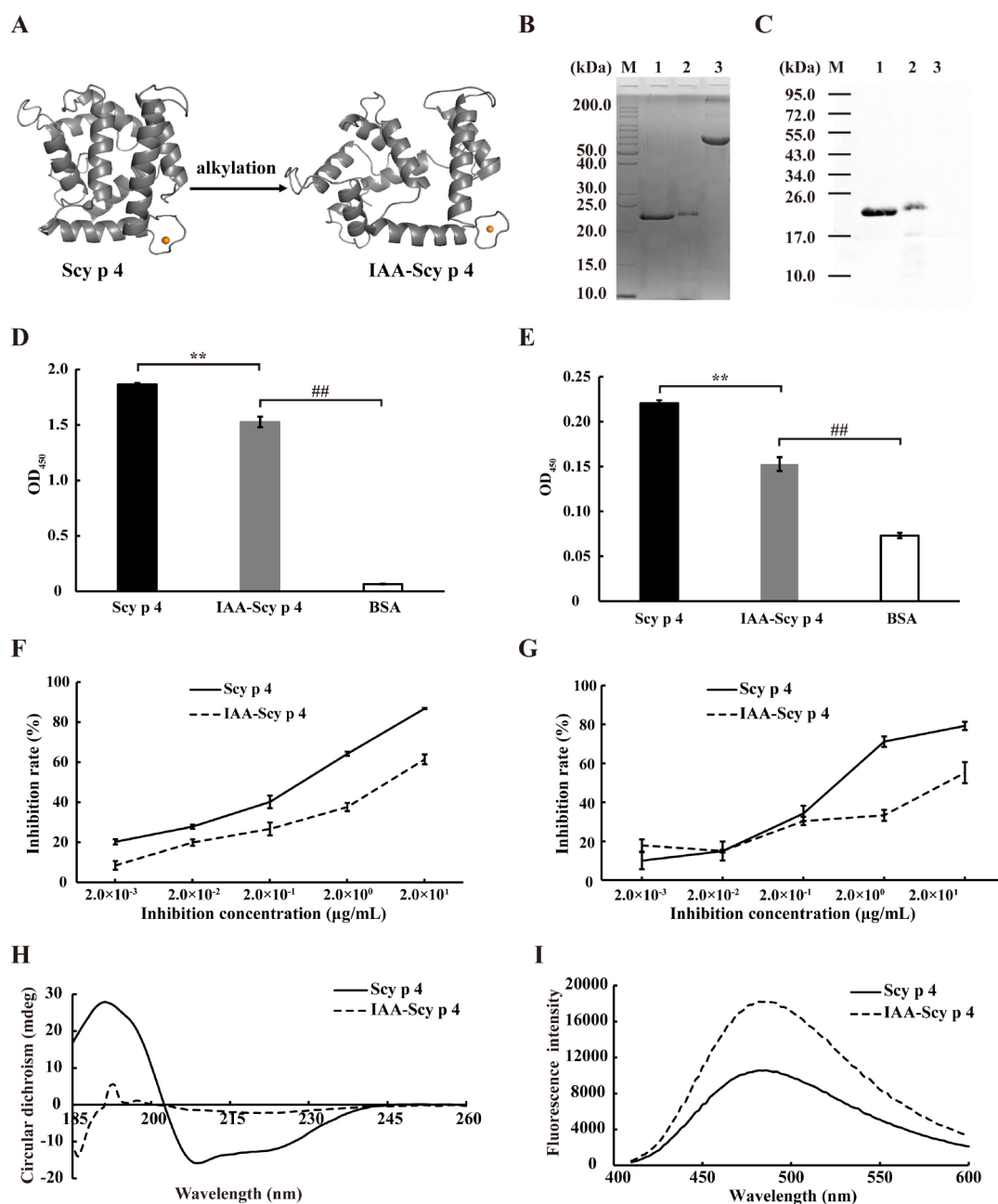


Figure 2. Preparation and characterization of IAA–Scy p 4. (A) Structural representations of Scy p 4 and IAA–Scy p 4. (B) SDS–PAGE analysis of IAA–Scy p 4. Lane M, protein marker; lane 1, Scy p 4; lane 2, IAA–Scy p 4; and lane 3, BSA. (C) Western blotting analysis of IAA–Scy p 4 by rabbit anti-Scy p 4 polyclonal antibody (dilution of $1:10^4$). Lane M, protein marker; lane 1, Scy p 4; lane 2, IAA–Scy p 4; and lane 3, BSA. (D) ELISA by rabbit anti-Scy p 4 polyclonal antibody (dilution of $1:10^4$). (***) $p < 0.01$, versus the Scy p 4 group. (##) $p < 0.01$, versus the BSA group. (E) ELISA by the serum pool of crab-sensitized individuals (mixed serum of S1–S10 in Table 1; dilution of 1:5). (***) $p < 0.01$, versus the Scy p 4 group. (##) $p < 0.01$, versus the BSA group. (F) iELISA by rabbit anti-Scy p 4 polyclonal antibody (dilution of $1:10^5$). (G) iELISA by the serum pool of crab-sensitized individuals (mixed serum of S1–S10 in Table 1). (H) Secondary structure analysis of IAA–Scy p 4 determined by CD spectroscopy. (I) Hydrophobicity analysis of IAA–Scy p 4 determined by the ANS probe.

2A). Reduction and alkylation of Scy p 4 was confirmed by SDS–PAGE. Scy p 4 and IAA–Scy p 4 had single bands around 22 kDa in SDS–PAGE (Figure 2B). As shown in Figure 2C, Scy p 4 and IAA–Scy p 4 could bind to a specific IgG antibody.

The ELISA results revealed that Scy p 4 and IAA–Scy p 4 had different IgG/IgE-binding capacity. As shown in Figure 2D, the ability of IAA–Scy p 4 to bind to specific rabbit anti-Scy p 4 polyclonal antibody was significantly reduced, but it retained part of the IgG-binding capacity. This was consistent with the

specific IgE-binding capacity determined using the serum pool of crab-sensitized individuals (Figure 2E).

To further verify the allergenicity of IAA–Scy p 4, iELISA was performed on the basis of the IgG/IgE competitive interactions between IAA–Scy p 4 and Scy p 4. In terms of IgG-binding capacity to Scy p 4 (Figure 2F), the IC₅₀ values of Scy p 4 and IAA–Scy p 4 were 0.13 and 3.53 μg/mL, respectively. Meanwhile, the results show that IAA–Scy p 4 could effectively inhibit the binding of Scy p 4 to the specific antibody, and the inhibition rate reached 61%.

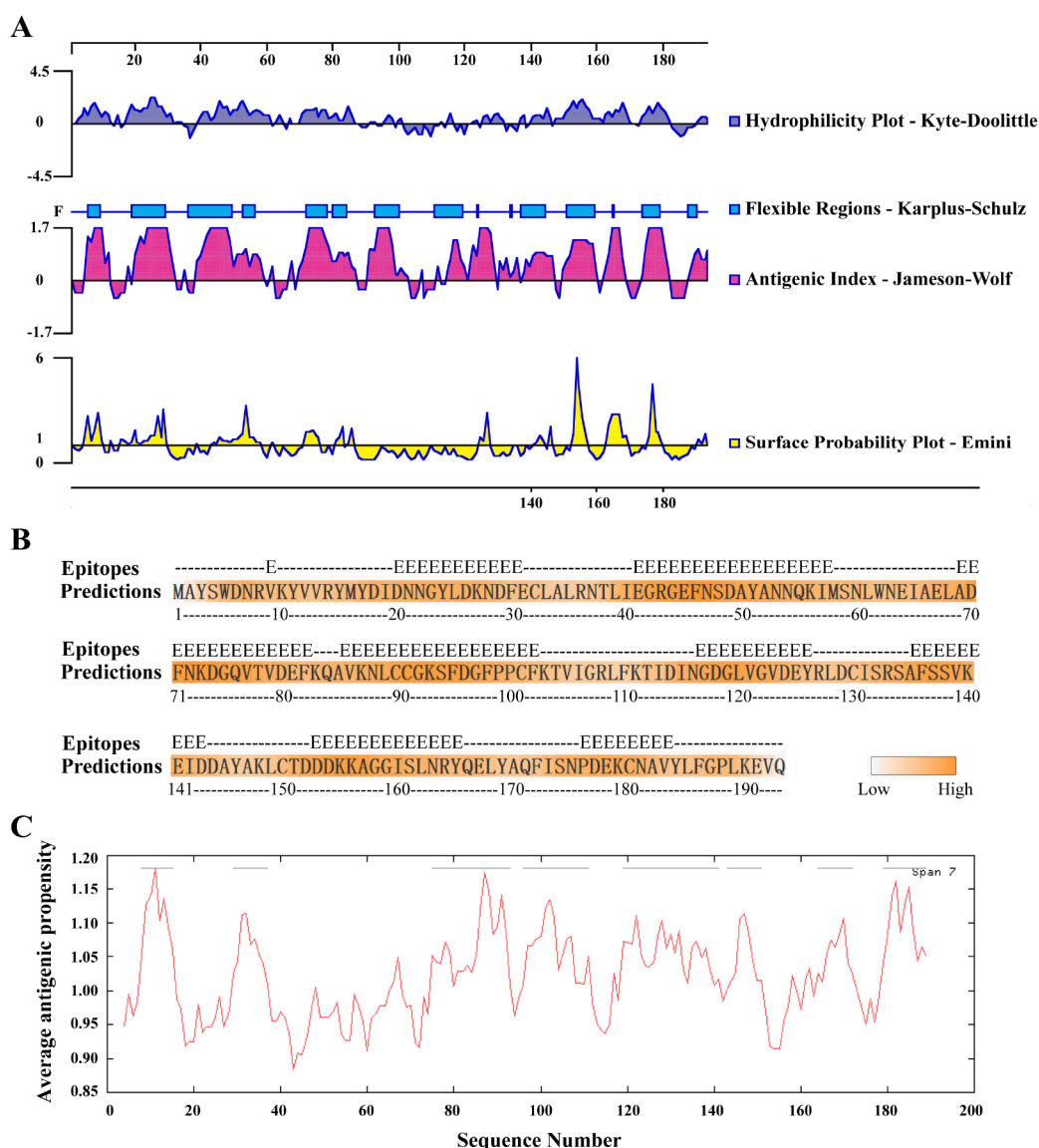


Figure 3. Prediction of linear mimotopes of Scy p 4 using immunoinformatic tools. (A) Prediction of Scy p 4 linear mimotopes using the DNA Star Protean system. (B) Prediction of Scy p 4 linear mimotopes using BepiPred 2.0. (C) Prediction of Scy p 4 linear mimotopes by the Immunomedicine Group.

Similarly, as shown in Figure 2G, the IgE-binding capacity of IAA–Scy p 4 were further confirmed. With regard to the IgE-binding capacity to Scy p 4, the IC_{50} values of Scy p 4 and IAA–Scy p 4 were 0.26 and 13.07 $\mu\text{g/mL}$, respectively. The inhibition rate of IAA–Scy p 4 was up to 55% at various protein concentrations.

To further analyze the influence of structural variations of IAA–Scy p 4, the secondary structure and surface hydrophobicity were investigated via CD spectroscopy and the ANS fluorescent probe. As shown in Figure 2H, in comparison to Scy p 4, the curves of IAA–Scy p 4 tended to be flat, which confirmed that they were alkylated. For the surface hydrophobicity (Figure 2I), the fluorescence absorption peak of IAA–Scy p 4 exhibited enhanced absorptions, and the fluorescence absorption value of IAA–Scy p 4 increased approximately 1-fold at 486 nm.

Prediction of Linear Mimotopes in Scy p 4. Immunoinformatic tools were used to analyze the linear mimotopes of Scy p 4. Therefore, we used immunoinformatics tools, such as

ABCpred, BepiPred 2.0, Immunomedicine Group, DNASTar Protean, and BCPred Predictions to analyze linear mimotopes. As shown in Figure 3A, a great mass of Scy p 4 sequences was covered by hydrophilic regions, indicating that Scy p 4 had high hydrophilicity. Besides, flexible regions and surface accessibility were analyzed for the flexible and easily exposed regions (Figure 3A). Moreover, by means of the antigenic indexes, the potential allergenicity of the protein regions was calculated (panels B and C of Figure 3). Finally, the overlap of the three immunoinformatic software prediction results was selected as the potential linear mimotopes. These peptides were named P1–P6 and synthesized for further verification (Table 2).

Verification of Linear Mimotope Peptides. In inhibition dot blotting, the IgE-binding capacity of the mimotope peptides was quantified as a measure of their respective capacity to inhibit IgE-binding to whole allergen protein (Scy p 4). To verify the IgE-binding capacity of the mimotope peptides, the inhibition dot blotting assay was executed on the basis of the IgE competitive interaction between the mimotope peptides and

Table 2. Information Relating to the Synthetic Peptides of Scy p 4 Using Five Immunoinformatic Tools

number	amino acid sequence	position	purity by LC-MS/MS (%)
peptide 1 (P1)	EGRGEFNSDAYA	41–52	98.9
peptide 2 (P2)	QVTVDEFKQAVKNLCC	76–91	96.9
peptide 3 (P3)	KTIDINGDGLVGVDE	111–125	95.0
peptide 4 (P4)	AFSSVKEIDD	137–146	99.3
peptide 5 (P5)	TDDDKKAGGI	151–160	98.7
peptide 6 (P6)	SNPDEKCNAYYL	174–185	95.0

whole allergen protein (Scy p 4) with the serum from crab-sensitized individuals.

In comparison to the PC group (without inhibitor), strong inhibition was evident with the Scy p 4 group (the inhibitor was Scy p 4), and the experimental group (the inhibitors were P1–P6) presented different degrees of inhibition among the 10 sera of crab-sensitized individuals (Figure 4A). Furthermore, the quantification of the IgE-binding capacity in inhibition dot blotting was analyzed (Figure 4B). P2–P4 were critical to IgE binding; this is due to their inhibition of the reactions between Scy p 4 and sera from 10 crab-sensitized individuals. In contrast, P1, P5, and P6 had no significant inhibition activity.

In the result of iELISA (Figure 4C), when the concentration of the mixed peptide was increased, the inhibition rate was gradually upward and the highest inhibition rate was 51%, while the IC_{50} of Scy p 4 and mixed peptide inhibition to Scy p 4 was 0.18 and 6.74 $\mu\text{g/mL}$, respectively. Hence, three linear epitopes of Scy p 4 were identified.

Mapping and Amino Acid Frequency Analysis of Linear Epitopes and Comprehensive Analysis among

Shellfish. The three identified linear epitopes of Scy p 4 were named L-Scy p 4-1 (AA_{76–91}), L-Scy p 4-2 (AA_{111–125}), and L-Scy p 4-3 (AA_{137–146}). On the basis of its amino acid sequence, the 3D structure of Scy p 4 was modeled by homology modeling. The linear epitopes of Scy p 4 were mapped on the 3D model, and the result showed that three epitopes were distributed on the outer surface of Scy p 4, which may easily bind to specific IgE (Figure 5A).

After identification of the linear epitopes, we tried to further calculate each amino acid frequency in linear epitopes using BioEdit software. As shown in Figure 5B, amino acids Cys, Asp, Glu, Gly, Ile, Lys, Gln, Thr, and Val in Scy p 4 performed higher frequency occurrence in linear epitopes. The linear epitopes were analyzed comprehensively among the epitope-identified SCPs in shellfish (Figure 5C).

The amino acid sequence homology of *P. monodon* (GenBank accession number BAL72725.1), *Litopenaeus vannamei* (GenBank accession number ACM89179.1), and *S. paramamosain* (GenBank accession number JQ860424.1) was analyzed, and the results showed the amino acid sequences of the SCPs were 79.27 and 77.20% to Scy p 4. In Figure 5C, the identical residues and strongly similar residues were represented by red backgrounds and red text, respectively. It is worth noting that the epitope region of L-Scy p 4-3 overlaps with the identified linear epitope region in *L. vannamei*. Furthermore, L-Scy p 4-1 and L-Scy p 4-2 were located in two calcium-binding sites of SCPs. No linear epitope was identified in *P. monodon*.

DISCUSSION

Shellfish allergy was a long-lasting disorder typically affecting adults.¹⁵ Among shellfish allergens, SCP is considered to be a stable allergen. Despite SCP having been identified in different

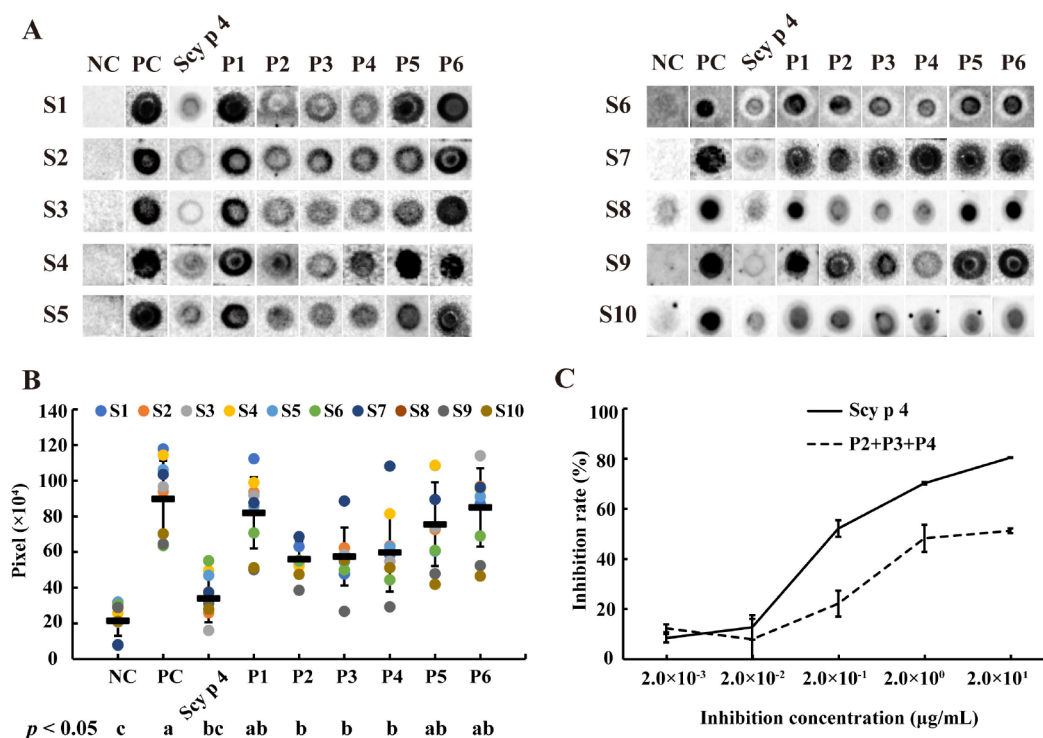


Figure 4. Immunoscreening of linear peptides. (A) IgE-binding capacity analysis of synthetic peptides by inhibition dot blotting, with Scy p 4 in the solid phase. S1–S10, sera from crab-sensitized individuals of Table 1 used to determine Scy p 4 or the different peptides (P1–P6); NC, BSA; PC, without inhibitor. (B) Intensity of the dots. The quantification of grayscale dots was analyzed using ImageJ software. (C) iELISA by the serum pool of crab-sensitized individuals (mixed serum of S1–S10 in Table 1; dilution of 1:3).

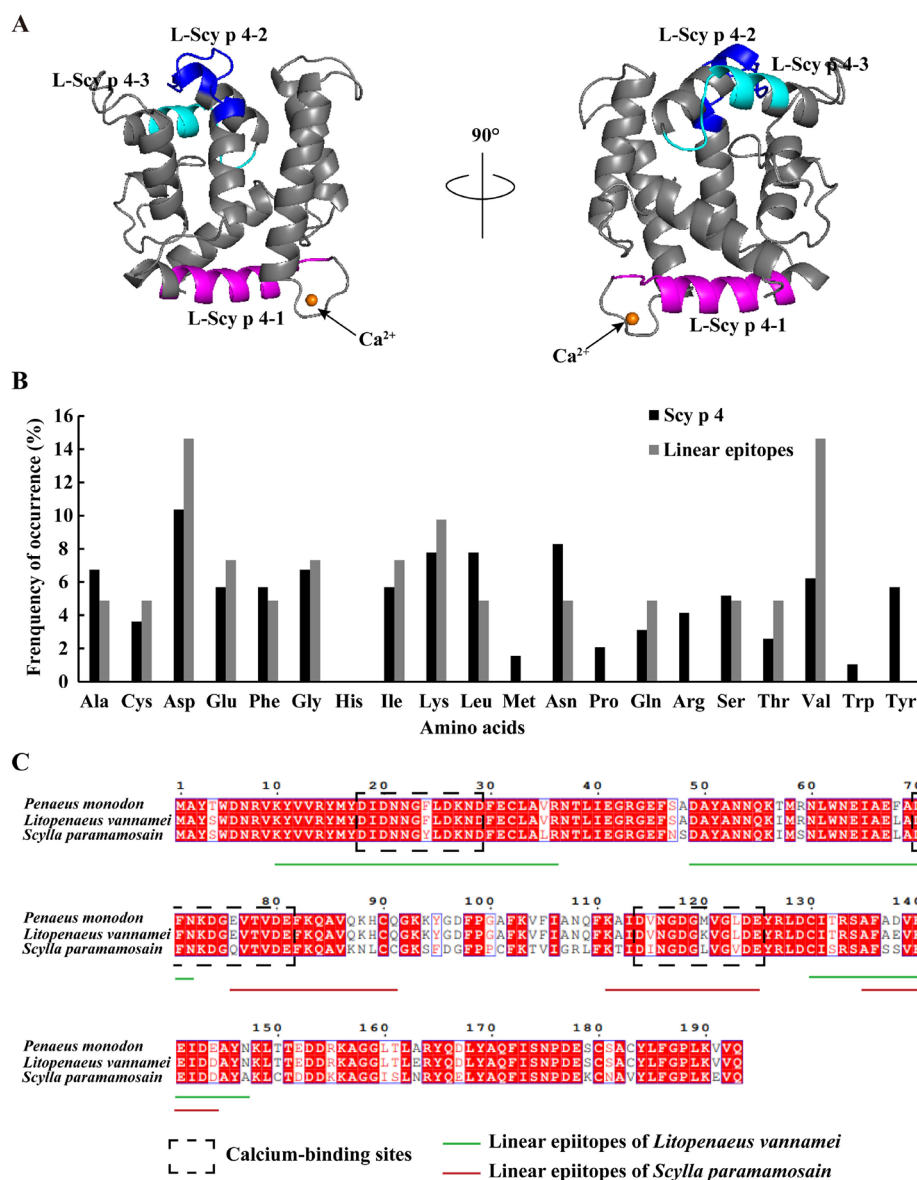


Figure 5. Mapping and comprehensive analysis of linear epitopes. (A) Position of linear epitopes on the 3D structure of Scy p 4. Linear epitopes: L-Scy p 4-1, L-Scy p 4-2, and L-Scy p 4-3. (B) Amino acid frequency of Scy p 4 whole protein and linear epitopes was measured. (C) Amino acid sequence alignment of the epitope-identified shellfish SCPs. The sequences were the following: *L. vannamei* (GenBank accession number ACM89179.1), *P. monodon* (GenBank accession number BAL72725.1), and *S. paramamosain* (GenBank accession number JQ860424.1). The color represents residue similarity: red background, identical; red text, strongly similar. Linear epitopes of *L. vannamei* were marked with green lines; linear epitopes of *S. paramamosain* were marked with red lines; and calcium-binding sites of the three shellfish species are marked by black dotted boxes.

species, information on the factors influencing the IgG/IgE-binding capacity of SCP and the epitopes involved in such allergic reactions was limited. Thus far, epitopes of SCP have only been identified in *L. vannamei*,³² *P. monodon*,³³ *Crassostrea angulata*,⁴ and *S. paramamosain*.⁵ In Scy p 4, only conformational epitopes were identified, whereas linear epitopes are important to food allergens mainly because the immune system encounters them only after they have been partially denatured and digested by the human gastrointestinal tract.³⁴ The perfection of IgE epitopes may contribute to the understanding of allergens and may provide aid to diagnosis and immunotherapy.

SCPs were members of the EF-hand protein family, and they are characterized by the presence of consecutive EF-hand motifs, which are able to chelate calcium ions.³⁵ The depletion of calcium ions in EF-hand family protein will lead to the rearrangement of its tertiary structure.³⁶ Therefore, EDTA was

selected to study the effects of structural changes on the immunobinding capacity of Scy p 4. Raith et al.¹⁶ discovered that the destruction of the calcium-binding sites will affect the structure of Phl p 7 (pollen allergen). Our previous study found that the allergenicity of Scy p 4 was decreased by mutation of the calcium-binding sites. Through the calcium ion chelation experiment, our previous conclusion was verified.

Through the 3D model, it is found that Scy p 4 had a certain spatial structure, and its structure was mainly α -helical and random coils. Chemical bonds were essential for maintaining the structure of protein. DTT was a disulfide-bond-reducing agent, used to investigate the relationships between the bioactivity and disulfide bonds in protein.³⁷ According to this study, DTT reduced the IgE-binding capacity of Scy p 4, suggesting the presence of disulfide bonds in Scy p 4, and it could affect the IgE-binding capacity of Scy p 4. Meanwhile, urea and GdnHCl

destroy the hydrogen-bond network of water and protein molecules, thus affecting the stability and activity of proteins in solution.³⁸ In this study, these two factors had no significant effect on allergenicity of Scy p 4. In addition, the immunobinding capacity of Scy p 4 cannot be completely disabled, even at the maximum concentrations of DTT used. These findings lend further support to the notion that there were disulfide bonds in Scy p 4, and the influence of the structure on the IgG/IgE-binding capacity of Scy p 4 was limited.

Many of the food allergens are protein-containing intramolecular disulfide bonds that may be important to their allergenicity.³⁹ Eichhorn et al.⁴⁰ destroyed the structural integrity of Pru p 3 by site-directed mutation of the disulfide bond, resulting in reduced antigenicity. Herein, it was found that the damage of the disulfide bonds resulted in the transformation of Scy p 4 into unstructured protein, which reduced the immunobinding capacity. Therefore, we hypothesized that the damage of disulfide bonds resulted in the allergenicity of Scy p 4 to be altered. Interestingly, like the damage of the other chemical denaturants, the structure of non-reversible damage by DTT and IAA did not eliminate Scy p 4 allergenicity and only partially destroyed Scy p 4 activity. The loss of the tertiary structure in food allergens leads to the destruction of conformational epitopes.⁴¹ Linear epitopes are independent of the spatial structure and consist only of spatially continuous amino acids.²² Sircar et al.⁴² suggested that the binding site of the IgE antibody on denatured Rhi o 1 was linear after irreversible degeneration of Rhi o 1. As a result, it can be inferred that the existence of linear epitopes was one of the reasons why the IgE-binding capacity of denaturant-treated Scy p 4 was not lost.

Linear epitopes play a vital role in food allergens because the immune system encounters them after they have been partially denatured and digested by the human gastrointestinal tract.³⁴ Therefore, the linear epitopes of food allergens have garnered attention. The epitopes were predicted primarily based on the hydrophilicity, flexibility, accessibility, and antigenicity of the amino acid sequences.⁴³ Immunoinformatics has been shown to be an effective method for predicting allergen epitopes in linear epitope mapping.⁴⁴ In this study, six mimotope peptides were predicted by immunoinformatic software, while three linear mimotope peptides (AA_{76–91}, AA_{111–125}, and AA_{137–146}) of them were identified to have significant IgE-binding capacity. Interestingly, complete IgE inhibition (80%, as displayed by autoinhibitor Scy p 4) was not observed, even when these three peptides were simultaneously used (P2 + P3 + P4) as inhibitors. The mixed peptides collectively displayed a maximum IgE-binding inhibition to Scy p 4 of up to 51%. Hence, L-Scy p 4-1, L-Scy p 4-2, and L-Scy p 4-3 were unable to capture the full repertoire of Scy p 4-specific IgE antibodies of sera of sensitized individuals. Consequently, IgE-binding to Scy p 4 was only partially inhibited. This partial inhibition can be interpreted as the existence of other linear IgE epitopes in addition to these three linear epitopes. It also suggested the likely presence of particular IgE antibodies in sera of sensitized individuals specifically directed toward the topological conformations on the native folds of Scy p 4.

Ayuso et al.³² reported SCP (in *L. vannamei*) contains three linear epitopes, in which one epitope partially overlapped with Scy p 4 epitopes (L-Scy p 4-3). In previous laboratory reports, it was found that Scy p 4 had heat-resistant and digestible epitopes, and L-Scy p 4-3 was located in the region of heat-resistant and digestible epitopes.⁴⁵ Besides, L-Scy p 4-1 and L-Scy p 4-2 were located in the calcium-binding sites of SCP, and the calcium-

binding region plays an important role in the allergenicity of SCP.²⁴ Consequently, we suspect that L-Scy p 4-1, L-Scy p 4-2, and L-Scy p 4-3 were important linear epitope regions. Liu et al.⁴⁶ found that the hypo-IgE-binding capacity in processed shrimp may be due to the modification of Lys, Arg, and Cys residues in antigen epitopes. In this study, amino acids Lys and Cys in Scy p 4 performed higher frequency occurrence in linear epitopes, which can provide a basis for reducing the IgE-binding capacity in food processing. However, key amino acids in Scy p 4 epitopes must be further researched, which are expected to reduce allergenicity.

In this study, the influence of a linear epitope on the IgG/IgE-binding capacity of Scy p 4 was studied by chemical denaturation. Then, the disulfide bonds were damaged by reduction and alkylation, and the importance of disulfide bonds to the IgG/IgE-binding capacity of Scy p 4 was further investigated. Finally, three linear epitopes (L-Scy p 4-1, L-Scy p 4-2, and L-Scy p 4-3) of Scy p 4 were analyzed by bioinformatic software combined with serology. In conclusion, the importance of a linear epitope for Scy p 4 was confirmed, improving Scy p 4 epitope information and amino acid distribution of linear epitopes to be widely used in the diagnosis and food processing of shellfish allergens.

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ABBREVIATIONS USED

AA, amino acid; ANS, 8-anilino-1-naphthalenesulfonic acid; BSA, bovine serum albumin; CD, circular dichroism; DTT, (2S,3S)-1,4-bis(sulfanyl)butane-2,3-diol; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; GdnHCl, guanidine hydrochloride; HRP, horseradish peroxidase; IAA, iodoacetamide; IC₅₀, half-maximal inhibitory concentration; iELISA, inhibition enzyme-linked immunosorbent assay; Ig, immunoglobulin; PBS, phosphate-buffered saline; SCP, sarcoplasmic calcium-binding protein; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; 3D, three-dimensional

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