

Site-Directed Mutations of Calcium-Binding Sites Contribute to Reducing the Immunoreactivity of the EF-Hand Sarcoplasmic Calcium-Binding Protein in *Scylla paramamosain*

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Cite This: *J. Agric. Food Chem.* 2021, 69, 428–436



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ABSTRACT: In order to reduce the immunoreactivity of sarcoplasmic calcium-binding protein (SCP), site-directed mutations were used to replace key amino acids in the conformational epitopes and calcium-binding sites. The mutant SCPs (mSCPs) were expressed in *Escherichia coli*, and their immunoreactivities were analyzed using iELISA and basophil activation assays. Furthermore, the structural changes of mSCPs were determined from the circular dichroism spectra. The iELISA results showed that mSCPs could effectively inhibit the binding of wild-type SCP (wtSCP) to sensitive serum, with inhibition rates that reached 90%. Moreover, mSCPs could downregulate the expression levels of CD63 and CD203c on the basophil surface. Compared with wtSCP, the peak values were significantly changed, and the calcium binding ability was impaired, which explained the decline in immunoreactivities of the mSCPs. All of the data confirmed that this approach was effective in reducing the immunoreactivity of SCP and could be applied to other shellfish allergens.

KEYWORDS: calcium-binding sites, conformational epitope, food allergy, sarcoplasmic calcium-binding protein, site-directed mutagenesis

INTRODUCTION

Food allergy is a common allergic disease worldwide, and therefore, the study of food allergens has long been a hot topic among researchers. Shellfish such as shrimp and crabs and their relevant products are important food sensitizers among the eight kinds of food sensitizers defined by the Food and Agriculture Organization of the United Nations.¹ Statistics on aquatic food allergies show that the incidence of food allergies caused by fish and crustacean aquatic products is about 5% worldwide, whereas it is 10.3% in Asia.²

The food components that cause an abnormal immune response are known as food allergens. To date, some shellfish allergens have been identified, including tropomyosin,³ arginine kinase,⁴ myosin light chain,⁵ triosephosphate isomerase,⁶ filamin C,⁷ and sarcoplasmic calcium-binding protein (SCP).⁸ SCP is an EF-hand calcium-binding protein that is present in invertebrate muscles and nerve tissues and is mainly involved in muscle contraction. SCP have been identified as an allergen in Pacific whiteleg shrimp *Litopenaeus vannamei* (Lit v 4).⁹ Hu et al.¹⁰ reported that SCP purified from *Scylla paramamosain* generated an immune response in crab-sensitive patients, confirming that SCP is the allergen in *S. paramamosain*, and obtained the full length sequence of the gene. The SCP in *S. paramamosain* was named Scy p 4 by the official database of allergen nomenclature that is approved by the World Health Organization and International Union of Immunological Societies.

The specific chemical groups in allergens that determine their antigenic specificity are called epitopes.¹¹ Antigenic epitopes can be divided into linear epitopes and confor-

mational epitopes according to their structures. Studies have shown that most IgE epitopes are conformational epitopes.¹² Analysis of the IgE epitopes of an allergen is a prerequisite for understanding the mechanism of the allergic reaction. Yang et al.¹³ applied phage display technology to select the IgE epitopes of SCP in *S. paramamosain* and found six IgE conformational epitopes and key amino acids through bioinformatics software analysis, but these have not been verified. Therefore, it was necessary to further analyze the influence of the key amino acids on the conformational epitopes of SCP and on its immunoreactivity.

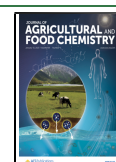
It has been demonstrated that, for the EF-hand family, the IgE reactivities of calcium-depleted forms of the proteins were significantly reduced compared with the calcium-bound proteins, which indicates that the presence of calcium affects the binding ability of the allergenic IgE.¹⁴ Previous studies had shown that mutations in the calcium-binding domain of parvalbumin (PV), which is a major fish allergen, could successfully lead to proteins with low immunoreactivities.¹⁵ And, in our earlier study, two calcium-binding sites were found in SCP through bioinformatics software analysis.¹⁰ Hence, whether the calcium-binding sites on SCP are related to its immunoreactivity was also worth investigating.

Received: September 5, 2020

Revised: October 25, 2020

Accepted: November 3, 2020

Published: December 30, 2020



Allergic reactions caused by food allergy usually persist throughout people's lives. Although fasting is effective in preventing the occurrence of allergic reactions to specific foods, strictly speaking, it is not a method that permanently eliminates the allergy. Allergen-specific immunotherapy is the way to ameliorate allergic diseases.¹⁶ However, the side effects it may cause often lead to the cessation of treatment. One way to reduce these side effects is to use genetic engineering to recombine allergen variants of low immunoreactivity, thereby reducing the binding capacity of IgE but retaining the reactivity of T cells.¹⁷ In recent years, site-directed mutation technology has been widely used for protein modification, as it can effectively change the characteristics of the target protein encoded by DNA. Freidl et al.¹⁵ used site-directed mutations to produce hypoallergenic PV mutants that could relieve the symptoms in allergic mice caused by food allergies. To date, the use of site-directed mutations to generate SCP mutants of low immunoreactivity has not been reported. However, it is expected to be applied in the prevention and control of food allergies caused by shellfish products.

The purpose of this study was to obtain recombinant mutant proteins of low immunoreactivity. Toward this end, a site-directed gene mutation technique was used to obtain a series of mutant SCPs (mSCPs). The recombinant proteins were cloned and expressed by genetic engineering. Then, the immunoreactivity could be verified using an enzyme-linked immunosorbent assay (ELISA) and basophil activation test (BAT). Furthermore, the secondary structures and surface hydrophobicities of the mSCPs were determined from the circular dichroism (CD) spectra and an ANS fluorescence probe. Finally, the low immunoreactivity mutant proteins obtained could be used to prevent and control shellfish allergy.

MATERIALS AND METHODS

Materials. Nde I and Sal I were purchased from TaKaRa (Takara Bio, Kyoto, Japan). The Ni²⁺-NTA chromatographic column was purchased from Transgen Biotech (Transgen Bio, Beijing, China). The rabbit anti-crab SCP polyclonal antibody was prepared in our laboratory as previously described.¹⁰ The goat anti-rabbit IgG-HRP antibody and goat anti-human IgE-HRP antibody were purchased from Abmart (Berkeley Heights, NJ, USA). 8-Anilino-1-naphthalene-sulfonic acid (ANS) was purchased from Sigma-Aldrich (Seelze, Germany). The enhanced chemiluminescence substrate was purchased from Pierce (Rockford, IL, USA). All other reagents were of analytical grade.

Patient Sera. Sera were collected from different crab-sensitive patients and nonallergic 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). Blood donors provided signed informed consent forms. The specificities of the IgE levels to crab were assessed *in vitro* using ImmunoCAP (Phadia AB, Rapsatan, Uppsala, Sweden): samples with values ≥ 0.35 kU/L were stored at -20 °C until use, and food allergy-free serum was used as the negative control. Table 1 shows the individual sensitivity profiles and clinical symptoms that were recorded in the present study.

Expression and Purification of the Recombinant Proteins. Wild-type SCP (wtSCP) gene encodes 193 amino acids (GenBank accession number no. AFJ80778.1), and the ends of the sequence were inserted into the restriction sites Nde I and Sal I, respectively. The cloned target gene was inserted into the pET-22b expression vector to form a recombinant plasmid. The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) competent cells.

The mSCPs genes were obtained by cloning specific primers (Table 2) using overlapping extension PCR, and the mutant

Table 1. Specific IgE Levels and Symptoms of the Crab-Sensitive Patients

no.	serum ID	age (years)	sex ^a	crab-specific IgE (kU/L) ^b	symptoms
P1	6125	15	M	4.6	dyspnea and diarrhea
P2	2543	12	F	14.4	nausea and emesis
P3	1311	35	M	6.4	allergic purpura
P4	2431	22	M	3.2	Eczema
P5	2546	6	F	24.5	allergic eczema and itchy skin
P6	4114	26	F	11.6	Eczema
P7	3625	9	F	23.5	urticaria, emesis, and diarrhea
P8	2424	11	M	5.2	Dyspnea
P9	4363	12	F	61.6	acute bronchiolitis
P10	4652	46	M	15.3	pruritus and nausea
P11	5132	8	F	78.1	acute urticaria
P12	5363	56	M	51.6	bronchopneumonia
P13	3264	27	M	29.3	itchy skin
P14	3143	21	F	58.4	allergic eczema
P15	3149	6	M	38.6	itchy skin
C1	4443	26	M	0.2	^c
C2	1336	25	M	0.1	^c
C3	2657	25	M	0.2	^c

^aM, male; F, female. ^bA serum with specific IgE <0.35 kU/L is defined as negative. ^cMeans no symptoms during the experiment. C1–3: the person was nonallergic individual.

Table 2. Oligonucleotide Primers Used in the Overlap Extension PCR

target sites	oligonucleotide sequence ^a (5'-3')
F-SCP	CATATGATGGCTTACTCTTGGGACA
R-SCP	GTCGACCTGCACCTCCTTCAGGGGT
F-S48A	CCGCGGCGAATTCAACGCAGACGCT
R-S48A	GGTTGTTGGCGTAAGCGTCTGCGTT
F-P100A	GAGCTTTGATGGTTTCCCTGCATGC
R-P100A	GACAGTCTTAAAGCATGCGAGGAAA
F-D70A	GAGATCGCCGAAGTGGCTGCATTCA
R-D70A	CTGACCGTCCTTGTGTAATGCAGCC
F-D18A/D20A	TCAGGTATATGTACGCAATCGCAAA
R-D18A/D20A	GAGGTAGCCATTGTTTGGCATTGCG

^aThe nucleotide changes are underlined. CATATG and GCTGAC are restriction sites for Nde I and Sal I enzymes, respectively.

expression strain was constructed as described above. For the method of obtaining the mutant genes, refer to Li et al.¹⁸ The expression strain was induced by adding isopropyl β -D-1-thiogalactopyranoside and allowing the reaction to proceed for 18 h at 16 °C. The expression products were purified using a Ni²⁺-NTA chromatographic column according to the method described by Xia et al.¹⁹ The recombinant proteins were analyzed by SDS-PAGE and Western blotting.

Thermal and pH Stability Analysis of the mSCPs. The thermal and pH stabilities of the mSCPs were obtained as reported by Hu et al.¹⁰ Each mSCP was diluted to a concentration of 0.25 mg/mL and then analyzed by SDS-PAGE and Western blotting. The control was a sample incubated at 4 °C or pH 7.0.

ELISA Analysis of the mSCPs. ELISA was conducted according to the method described by Morii et al.,²⁰ with some modification. The mSCPs were incubated with the serum of crab-sensitive subjects (mixed serum of numbers P1–15 in Table 1, dilution 1:5) to give the primary antibody. The goat anti-human IgE-HRP antibody (1:2 $\times$ 10³ dilution) was incubated as a secondary antibody. The specific IgG-binding activity was analyzed using the rabbit anti-crab SCP polyclonal antibody (1:10⁴ dilution) recruited as the primary

antibody. The goat anti-rabbit IgG-HRP ($1:2 \times 10^4$ dilution) antibody was used as the secondary antibody.

iELISA was conducted according to the method described by Tscheppe et al.,²¹ with some modification. The wtSCP or mSCP as the inhibitor was diluted to different concentrations and then pre-incubated with the rabbit anti-crab SCP polyclonal antibody (dilution $1:10^5$) or the serum pool of crab-sensitive patients (mixed serum of numbers P1-15 in Table 1). Primary antibodies were obtained as described previously. 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 inhibitor was called IC_{50} .

BAT of the mSCPs. BAT was performed using the sera from crab-sensitive patients (patients P11-15 in Table 1) and three nonallergic patient controls (C1-3 in Table 1), as described previously.²² An mSCP concentration of 50 $\mu\text{g/mL}$ was used, as given in the paper by Yang et al.¹³ Samples were analyzed using flow cytometry on a Guava instrument (Millipore, Massachusetts, USA). Allergen-induced CD203c and CD63 up regulation was calculated using the mean fluorescence intensities (MFIs) obtained with stimulated (MFI_{stim}) and unstimulated (MFI_{con}) cells and was expressed as the stimulation index (SI), defined as $MFI_{\text{stim}}/MFI_{\text{con}}$.²³

Secondary Structure and Hydrophobicity Analysis of the mSCPs. The secondary structure was determined from the CD spectral data (Applied Photophysics, Ltd., Surrey, UK) at 25 °C according to the method described by Raith et al.²⁴ CD spectra were recorded with or without the addition of 5 mM EDTA.

The surface hydrophobicity changes of the mSCPs were detected using an ANS fluorescent probe method,²⁵ with some modification. The sample with or without 5 mM EDTA was diluted with PBS to 0.25 mg/mL . The fluorescence value was measured at an excitation wavelength of 375 nm, and the emission wavelength range was 400 to 600 nm.

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

RESULTS

Construction and Purification of the mSCPs. With the theoretical support of published studies, we replaced the key amino acids (T_{38} , E_{41} , P_{100} , C_{90} , N_{53} , and S_{48}) in six conformational epitopes of SCP by alanine. In addition, based on the EF-hand family structure, the three aspartic acids (D_{18}/D_{20} , D_{70}) in two calcium-binding areas were mutated to alanine. In Figures 1A and S1G, the mutation positions are marked blue in the conformational epitopes and red in the calcium-binding sites. All mSCPs contained the correct sequence of expression vectors (Figure 1B). An *E. coli* expression system was used to express them and, after purification using a Ni^{2+} -NTA chromatographic column, all mSCPs had obvious single bands around 20 kDa in SDS-PAGE (Figures 1C, S1A,E). As shown in Figures 1D, S1B,F, except for D70A and D18A/D20A/D70A, wtSCP, and the mSCPs could bind to the specific IgG antibody. This is because wtSCP has a strong specific IgG response, leading to D70A and D18A/D20A/D70A did not appear bands in Western blotting analysis. However, when mutants D70A and D18A/D20A/D70A were solely examined by western blotting, obvious bands were observed (Figure S1C,D).

Thermal and pH Stability Analysis of the mSCPs. In the thermal stability analysis, both wtSCP and mSCPs (S48A, P100A, D70A, and D18A/D20A/D70A) were relatively stable (Figure 2). Compared with wtSCP (Figure 2A), SDS-PAGE

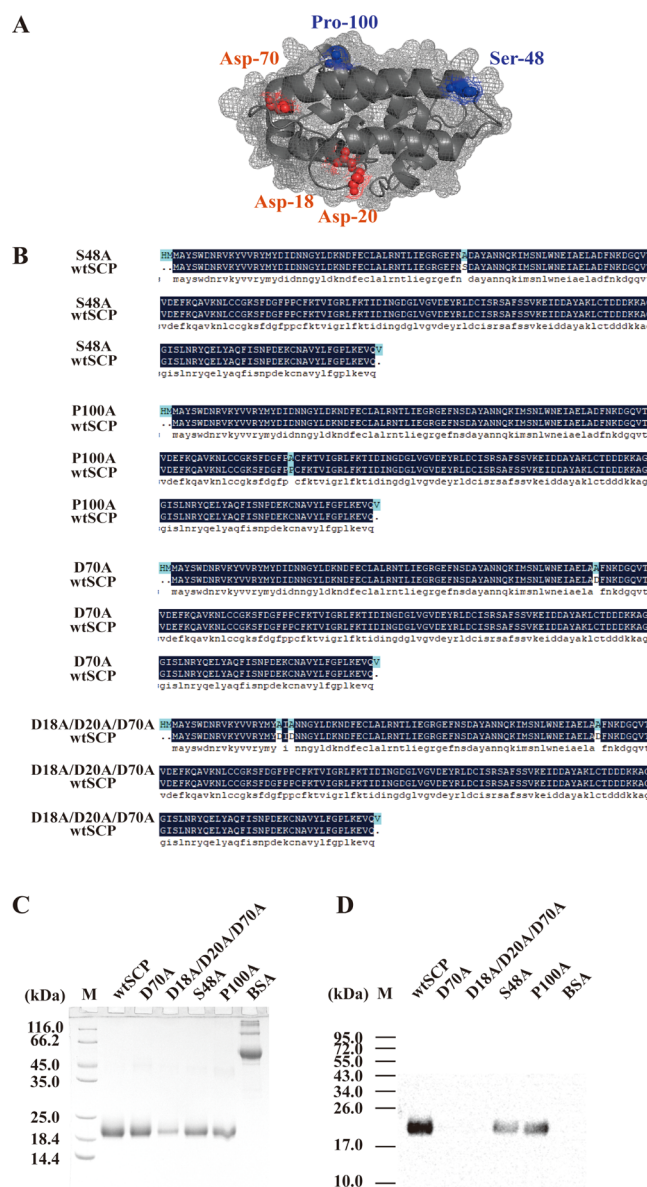


Figure 1. Construction and purification of the SCP variants. (A) Structural representations of mSCPs. Calcium mutation sites are colored in red, and conformational epitope mutations are colored in blue. (B) Amino acid sequence alignment between wtSCP and mSCPs. (C,D) SDS-PAGE and Western blotting analysis of mSCPs (primary antibody: specific SCP-polyclonal antibodies, dilution $1:5 \times 10^3$). Lane M, protein marker.

showed that the main bands of the mSCPs had not distinctly changed even at 100 °C (Figure 2C,E,G,I). In addition, apart from P100A, heating led to different degrees of thermal polymerization at 70 °C for wtSCP but at 30 °C for S48A, D70A, and D18A/D20A/D70A. Western blotting analysis indicated that after heat treatment, all SCPs (wtSCP, S48A, P100A, D70A, and D18A/D20A/D70A) could still bind to the rabbit anti-crab SCP polyclonal antibody, but the binding activity decreased with increasing temperature (Figure 2B,D,F,H,J). Therefore, wtSCP and P100A had similar thermal stabilities, whereas the other variants were more sensitive to heat.

Figure 3 displays the pH stabilities of wtSCP and the mSCPs (S48A, P100A, D70A, and D18A/D20A/D70A). The main band of wtSCP did not change in the pH range 2.0–11.0,

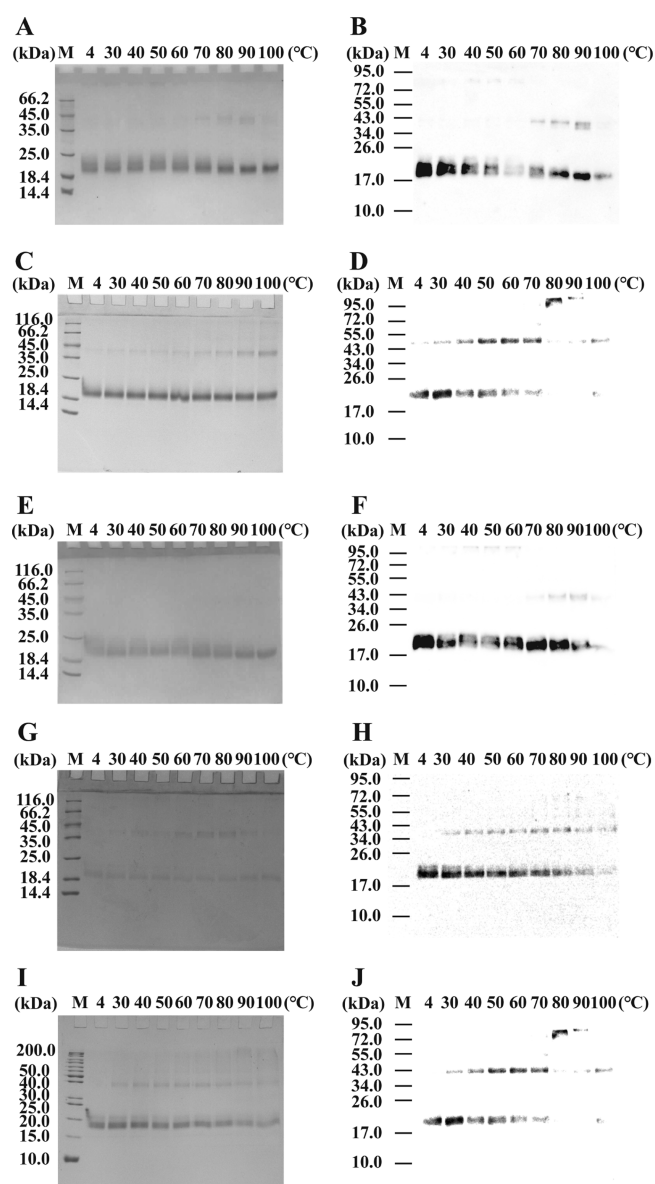


Figure 2. SDS-PAGE and Western blotting analysis of thermal stabilities of mSCPs. SDS-PAGE and Western blotting analysis of the thermal stabilities of (A,B), wtSCP; (C,D), S48A; (E,F), P100A; (G,H), D70A; (I,J), D18A/D20A/D70A. Primary antibody: specific SCP-polyclonal antibodies, dilution $1:5 \times 10^3$. Lane M, protein marker.

while it had almost disappeared at pH 1.0 (Figure 3A). Compared with wtSCP (Figure 3A), the mSCPs (S48A, P100A, D70A, and D18A/D20A/D70A) were degraded at pH 10.0–11.0 (Figure 3C,E,I). Moreover, polymers of S48A and D70A appeared at pH 2.0–5.0, and the main band of D70A had almost disappeared at pH 6.0–9.0 (Figure 3G). The results of Western blotting (Figure 3B,D,F,H,J) were consistent with the SDS-PAGE results, which proved the pH instability of D70A. Moreover, S48A and D18A/D20A/D70A were more unstable than wtSCP under acidic conditions.

IgG/IgE Binding Analysis of the mSCPs. The ELISA results revealed that wtSCP and the mSCPs (S48A, P100A, D70A, and D18A/D20A/D70A) had different immunoreactivities. As shown in Figure 4A, except for P100A that showed no significant difference, the abilities of the other variants to bind

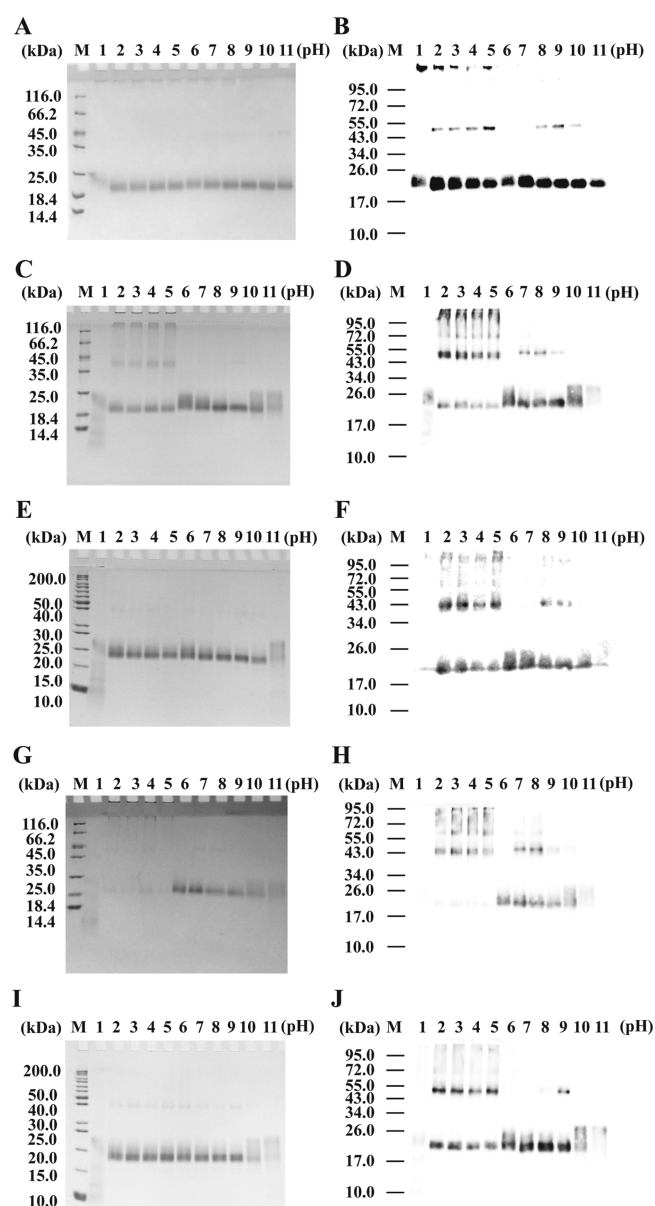


Figure 3. SDS-PAGE and Western blotting analysis of the pH stabilities of mSCPs. SDS-PAGE and Western blotting analysis of the pH stabilities of (A,B), wtSCP; (C,D), S48A; (E,F), P100A; (G,H), D70A; and (I,J), D18A/D20A/D70A. Primary antibody: specific SCP-polyclonal antibodies, dilution $1:5 \times 10^3$. Lane M, protein marker.

to the specific rabbit anti-crab SCP polyclonal antibody were significantly reduced. This was consistent with the specific IgE binding activities determined using the serum pool of crab-sensitive patients (Figure 4B). Furthermore, T38A, E41A, C90A, N53A, and D18A/D20A displayed no significant differences with wtSCP (Figure S2).

To further verify the allergenicities of the mSCPs, an iELISA assay was performed based on the IgG/IgE competitive interactions between the mSCPs and wtSCP. In terms of IgG-binding activity to wtSCP (Figure 4C), the IC_{50} values of wtSCP, S48A, and P100A were 17.9, 81.3, and 61.4 ng/mL, respectively. However, D70A and D18A/D20A/D70A revealed poor inhibition effects, as their inhibition rates were less than 20%. Similarly, as shown in Figure 4D, D70A and D18A/D20A/D70A had little or no inhibitory effect on binding of

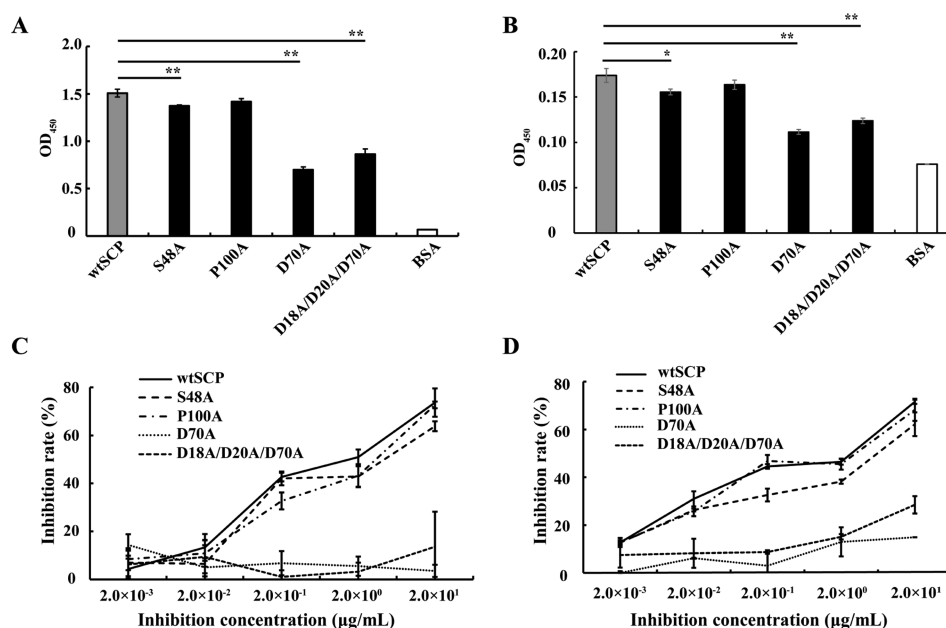


Figure 4. IgG/IgE binding activity analysis of mSCPs. (A) ELISA assay by specific SCP-polyclonal antibodies (dilution 1:10⁴). All data are presented as the mean $\pm$ SD ($n = 3$). **, $p < 0.01$, versus the wtSCP group. (B) ELISA assay by the serum pool of crab-sensitive patients (dilution 1:5). All data are presented as the mean $\pm$ SD ($n = 3$). **, $p < 0.01$, versus the wtSCP group. (C) Inhibition ELISA assay by rabbit anti-crab SCP IgG pAb (dilution 1:10⁵). (D) Inhibition ELISA assay by the serum pool of crab-sensitive patients.

wtSCP to the serum pool of crab-sensitive patients. This showed that D70A and D18A/D20A/D70A could not effectively block the specific binding between serum and wtSCP, whereas S48A and P100A could.

Immunoreactivity Analysis of the mSCPs. BAT was used to evaluate the apparent correlation by the reactivity of basophils from volunteer donors. Figure 5A indicates that, compared with the negative controls, wtSCP stimulation could induce a significantly higher SI of CD63 ($p < 0.01$) and CD203c on the surfaces of basophils ($p < 0.05$) in sensitive patients. Thus, in the presence of wtSCP, basophils were activated effectively. Nevertheless, the SI values of D70A and D18A/D20A/D70A were substantially lower (Figure 5B,C), which meant that they were associated with reduced expression levels of CD63 and CD203c. In contrast, the SI values of S48A and P100A were not significantly different from those of wtSCP (Figure 5B,C).

Secondary Structure and Hydrophobicity Analysis of the mSCPs. To analyze the differences in immunoreactivities of the mSCPs, structural variations were investigated via CD spectra and surface hydrophobicity measurements. It can be seen from Figure 6A that, compared with wtSCP, S48A and P100A had positive peaks at about 190 nm and double negative peaks at 210 and 210 nm, both of which showed typical features of α -helical proteins. However, the content of α -helices was significantly lower in D70A and D18A/D20A/D70A as the transformations in peak values in the spectra demonstrated changes in the secondary structure. For the surface hydrophobicity (Figure 6B), the fluorescence absorption peaks of S48A and P100A were not significantly different from wtSCP, and in particular, the fluorescence absorption curve of P100A almost coincided with that of wtSCP. In contrast, D70A and D18A/D20A/D70A exhibited enhanced absorptions and fluorescence peak shifts to the left: the fluorescence absorption value of D70A increased 7.2-fold at 476 nm and those of D18A/D20A/D70A increased 6.9-fold at

478 nm. D18A/D20A displayed typical features of a helical protein (Figure S3A). For the surface hydrophobicity (Figure S3B), the fluorescence absorption peaks of D18A/D20A were not significantly different.

Furthermore, calcium-binding ability was also used as an indicator to evaluate the associated structural changes of the mSCPs. When the calcium chelating agent EDTA was added (Figure 6C), the peaks of wtSCP, S48A, and P100A were not obvious in the CD spectra, which no longer exhibited typical α -helical features. However, the peaks of D70A and D18A/D20A/D70A did not change greatly after the addition of EDTA. Figure 6D shows the surface hydrophobicities; when the calcium chelating agent EDTA was added, the fluorescence peak of wtSCP increased by nearly 10-fold, and peaks because of S48A and P100A also increased significantly. For D70A and D18A/D20A/D70A, there was no significant difference between the fluorescence absorption curves recorded after the addition of EDTA and before the addition of EDTA. When EDTA was added (Figure S3C), the peaks of D18A/D20A were not obvious in the CD spectrum, which did not exhibit typical α -helical features. As shown in Figure S3D, when EDTA was added, the intensity of the fluorescence peak of D18A/D20A in the spectrum increased significantly.

DISCUSSION

Shellfish can cause a severe type I allergic reaction, which has a growing prevalence in Asian countries.²⁶ Among crab allergens, SCP is considered to be a very stable allergen, although changes in allergenicity via nonthermal processes such as enzymatic cross-linking have been reported.¹⁰ Currently, with the development of molecular biology techniques, molecular modifications are increasingly being applied to modify allergens to reduce their allergenicity.²⁷ Therefore, the immunoreactivity of SCP should be altered by substituting key amino acids by site-directed mutation. The key amino

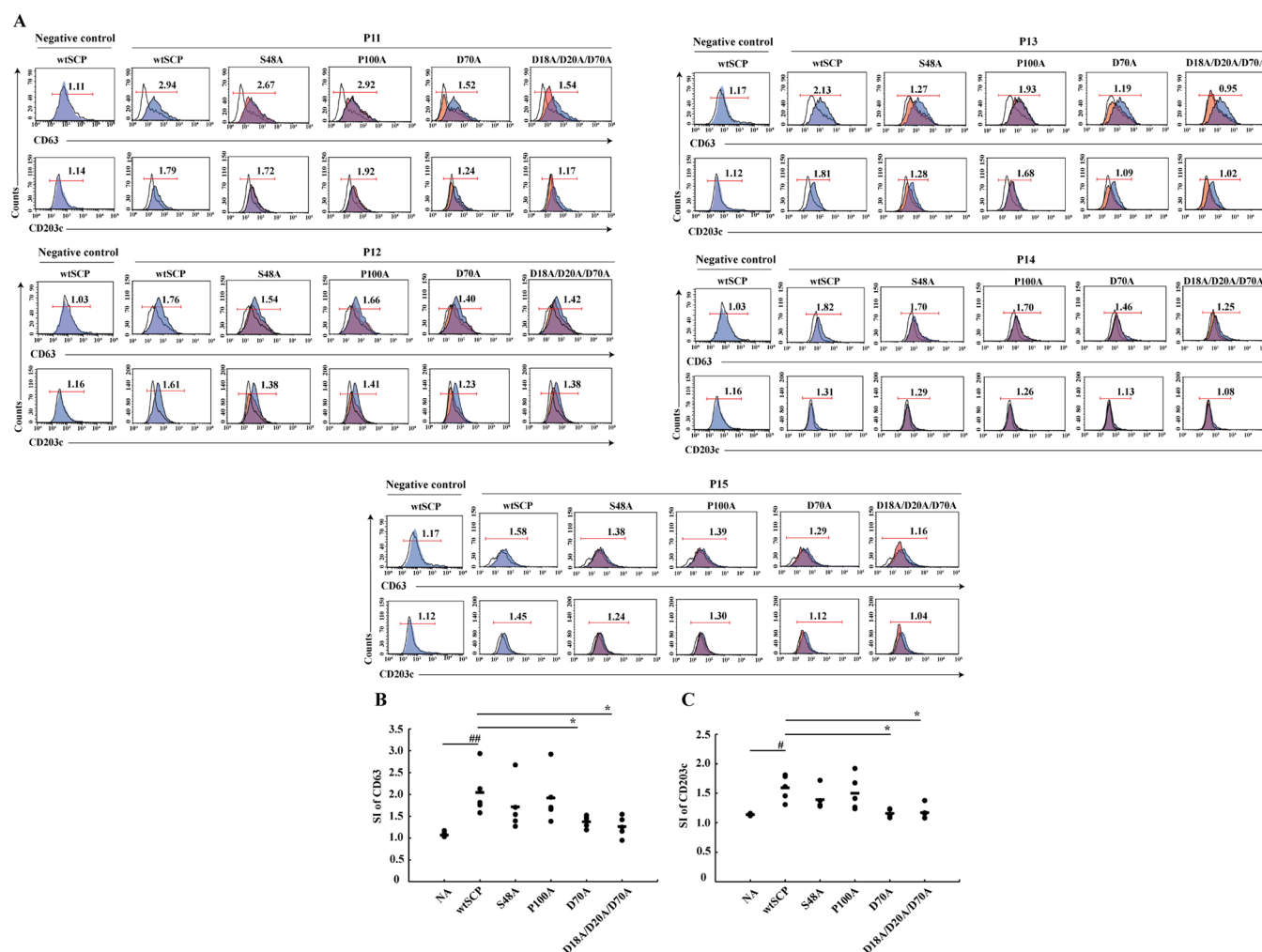


Figure 5. Immunoreactivity analysis of mSCPs from crab-sensitive patients by BAT. (A) BAT was performed to compare the immunoreactivities of wtSCP and the mSCPs from five crab-sensitive patients (P11–15). White and blue represent the cell populations stimulated by PBS and wtSCP, respectively. Red represents the cell population stimulated by mSCPs. NA represents healthy individuals, and wtSCP indicates sensitive patients. (B) Effects of wtSCP and mSCPs on the expression of CD63 of basophils. The horizontal line represents the average values of SI by PBS, wtSCP, or mSCP stimulation. Data are presented as the mean $\pm$ SD ($n = 3$). ###, $p < 0.01$ compared to the negative control. *, $p < 0.05$ compared to wtSCP. C, Effects of wtSCP and mSCPs on the expression of CD203c of basophils. Data are presented as the mean $\pm$ SD ($n = 3$). #, $p < 0.05$ compared to the negative control. *, $p < 0.05$ compared to wtSCP.

acids are most likely located at the antigenic epitopes and the calcium-binding region of SCP.

Gerald et al.²⁸ obtained hypoallergenic mutants by mutating 12 key amino acids in 8 major epitopes of Pen a 1 in shrimp. A previous study identified the antigenic epitopes of SCP from *S. paramamosain*,¹³ of which six conformational epitopes and the key amino acids including T₃₈, E₄₁, P₁₀₀, C₉₀, N₅₃, and S₄₈ were determined. Our results showed that the immunoreactivity of S48A was decreased relative to wtSCP, as this amino acid is located in the main epitope region. In addition, the thermal stability and pH stability of S48A were altered slightly compared to wtSCP. Studies have reported that high temperature largely destroys conformational epitopes of casein.²⁹ It is possible that the reduction in the binding of SCP polyclonal antibodies to heat treated wtSCP/mSCPs, and it was explained by the loss of conformational epitopes. Previous studies have reported that the production of polymers results in the loss of immunobinding activity of whey proteins.³⁰ The loss of IgG binding activity of SCP may be due to polymer production during research. Therefore,

through the analysis of thermal stability and pH stability, it was found that compared with wtSCP, the change of IgG binding activity of S48A was caused by conformational epitope mutation. These aspects together explain why the immunoreactivity of the S₄₈ mutant was lower than that of the other key amino acid mutants of the conformational epitopes.

The key amino acid of proteins in the EF-hand family is usually Asp (D).^{15,31–33} In this study, three SCP mutants were obtained by substituting Asp of the calcium-binding sites (D18A/D20A, D70A, and D18A/D20A/D70A). The changes in immunoreactivities before and after mutation were further explored.

SCP, PV, and myosin light chain are members of the EF-hand protein family,⁹ and they are characterized by the presence of consecutive EF-hand motifs (helix-loop-helix) called AB, CD, and EF.³⁴ However, only CD and EF are able to chelate metal ions, such as Ca²⁺,³⁵ although the affinity of CD toward calcium is weaker than that of EF.³⁶ Furthermore, the CD site is a 12-residue binding ring that begins with Asp/X/Asp (X represents arbitrary amino acids), and the EF site

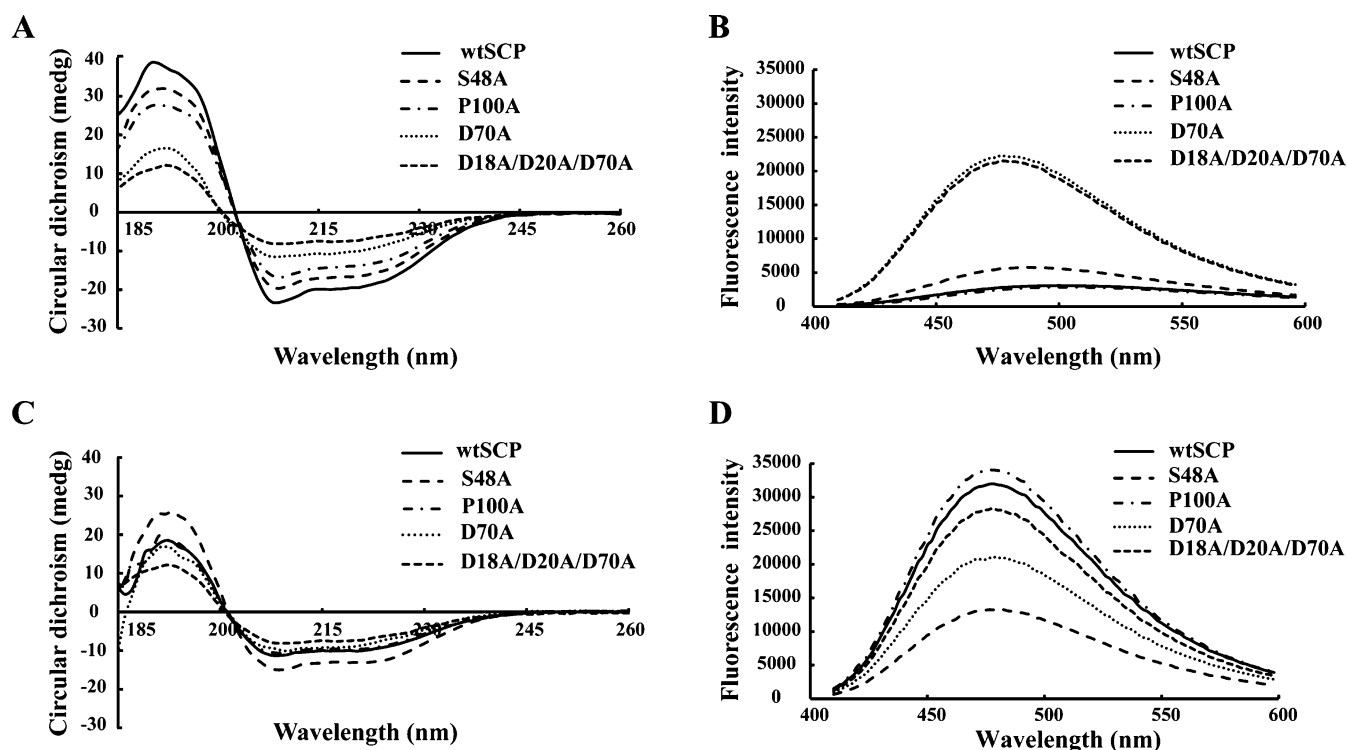


Figure 6. Secondary structure and hydrophobicity analysis of mSCPs. (A) CD spectra of wtSCP and mSCPs recorded under native conditions. (B) Hydrophobicity analysis of wtSCP and mSCPs recorded under native conditions. (C) CD spectra of wtSCP and mSCPs recorded after the addition of EDTA. (D) Hydrophobicity analysis of wtSCP and mSCPs recorded after the addition of EDTA.

starts from Asp and ends with Glu.³⁷ Therefore, sequence analysis confirmed that amino acids 18–29 and 70–81 of the SCP sequence were part of the CD and EF sites, respectively.

In this study, D18A/D20A was located in the CD site and D70A was located in the EF site. Comparing D18A/D20A with D70A, the immunoreactivity of D70A was significantly lower. The calcium-binding ability of D70A was reduced by its change in secondary structure and enhanced hydrophobicity. Similarly, Raith et al.²⁴ determined that variants could significantly reduce the allergenicity of Phl p 7 if they impaired its calcium-binding capacity. As a result, it can be inferred that the lack of calcium-binding ability was one of the causes of low immunoreactivity of D70A. However, after adding the calcium chelator EDTA, the ability of D18A/D20A to bind calcium was retained, which further explained the observation that its immunoreactivity did not change.

In addition, the integrity of the secondary structures of the allergens was correlated with their allergenicities. Research has shown that when the α -helical content of egg allergen ovalbumin³⁸ and peanut allergen Ara h 1³⁹ decreased, sensitization was lower. The α -helical content of D70A decreased, and it tended to form agglomerates when heated. Therefore, the reduced α -helical content and thermal instability were also responsible for the differences in the immunoreactivity of D70A.

In this study, D₁₈/D₂₀/D₇₀ were simultaneously mutated but no significant differences in immunoreactivity or other characteristics between D18A/D20A/D70A and D70A were found. This indicated that there is no synergy or antagonism between the D₁₈/D₂₀ and D₇₀ sites. These results confirmed that D₇₀ is indeed the key amino acid at the calcium binding site, which also has a substantial influence on the immunoreactivity of SCP.

Although the antigenicity of an allergen depends on its epitopes,⁴⁰ critical structural areas of certain types of allergens also play a significant role. Mei et al.⁴¹ mutated the key amino acids of conformational epitopes and the disulfide bond in arginine kinase and found that the IgE binding abilities of the structural mutants were lower than those of the epitope mutants. In this study, the effect of mutation on the calcium-binding sites was greater than that on the conformational epitopes of SCP. Perhaps, the polyclonal nature of the antibodies means that individual mutations are insufficient for decreasing immunoreactivity rather gross changes such as those induced by loss of calcium binding are required. This further verified the importance of the calcium-binding sites in the EF-hand family.

In conclusion, the key amino acids of the conformational epitopes and calcium-binding sites were mutated to reduce the immunoreactivity of SCP from *S. Paramamosain*. Two effective variants (D70A and D18A/D20A/D70A) were generated that exhibited low immunoreactivity by iELISA and BAT. Furthermore, after adding the calcium chelator EDTA, the calcium binding ability was found to be related to allergen immunoreactivity through the secondary structure and hydrophobicity analysis. As the allergic activity was significantly reduced, the protein displayed a lower risk of inducing anaphylaxis. It would be practical to use mSCPs together with a new adjuvant⁴² or combined with an immune adjuvant for allergen-specific immunotherapy.⁴³ Therefore, we believe that SCP mutants can be used for the prevention and control of shellfish allergy.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.0c05733>.

Construction and purification of the SCP variants, IgE/IgG binding activity analysis of mSCPs (T38A, E41A, N53A, C90A, and D18A/D20A), and secondary structure and hydrophobicity analysis of D18A/D20A (PDF)

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Notes

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■ ACKNOWLEDGMENTS

This work was supported by grants from the National Key R Program of China (2019YFD0901703), the Grant from the National Natural Scientific Foundation of China (31871720), the science and technology program of Fujian province (2018N5009, 2018R0071), and the Marine Scientific Research Special Foundation for Public Sector Program (DY135-B2-07, 201505026-03).

■ ABBREVIATIONS USED

ANS, 8-anilino-1-naphthalenesulfonic acid; BAT, basophil activation test; BSA, bovine serum albumin; CD, circular dichroism; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; Ig, Immunoglobulin; mSCP, sarcoplasmic calcium-binding protein variants; MFI, mean fluorescence intensity; SCP, sarcoplasmic calcium-binding protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; wtSCP, wild-type sarcoplasmic calcium-binding protein

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