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Two hypo-allergenic derivatives lacking the dominant linear epitope of Scy p 1 and Scy p 3

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ABSTRACT

Scylla paramamosain frequently elicits IgE-mediated type-I hypersensitivity reactions. Molecular candidates for crab allergen-specific immunotherapy have not been studied previously. In this study, reduced and alkylated (red/alk) derivatives with destroyed conformational epitopes and mutant derivatives (mtALLERGEN) with deleted heat/digestion-stable linear epitopes were produced of tropomyosin and myosin light chain. Structural changes and the allergenicity of derivatives was analyzed. Compared with wild-type allergens, red/alk derivatives had dramatically altered protein structures, whereas mtALLERGEN showed slightly structural effects. Enzyme linked immunosorbent assay revealed the heterogeneous epitope-recognition patterns with derivatives among 29 crab-sensitised patients, of whom 13% and 62% recognised conformational and linear epitopes, respectively, whereas 25% recognised both epitope types to the same extent. Furthermore, mtALLERGEN could not bind to IgE or induce basophil activation in some patients. These results imply that hypo-allergenic derivatives of crab myofibril allergens that specifically lacked linear epitopes may serve as viable candidates for immunotherapy.

1. Introduction

Shellfish allergies are characterised as IgE-mediated, type-I hypersensitivity reactions, which typically persist throughout the lives of affected individuals (Sicherer, Muñoz-Furlong & Sampson, 2004). Anaphylaxis occurs mainly because of the presence of allergens, and multiple shellfish allergens have been reported, for instance tropomyosin (TM) and the myosin light chain (MLC) of myofibril proteins (Faber et al., 2017; Li et al., 2019). According to recent statistics, the recognition rate of MLC is second only to the main allergen TM in patients with a shellfish allergy (Ayuso et al., 2010). Furthermore, TM and MLC allergies often show clinical cross-reactions with the corresponding allergens from other species (i.e., mites and *Blattella germanica*), mainly because of high sequence homology or similar IgE epitopes (Kamath et al., 2020; Ruethers et al., 2018). Hence, allergic reactions caused by TM and MLC should not be ignored.

The epitopes of an allergen are related to its antigenicity. Epitopes can be classified as linear or conformational: linear epitope is continuous in the primary sequences of allergens, while conformational epitope is comprised of amino acid residues/peptides that are higher ordered structures by protein folding (Willison, et al., 2013). It has been reported that IgE epitopes are mostly conformational (Ayuso et al., 2012); however, in recent, it is widely accepted that food processing and digestion often destroy the conformational ones. Furthermore, although the linear

Abbreviations: AIT, allergen-specific immunotherapy; ANS, 8-anilino-1-naphthalenesulfonic acid; BAT, basophil-activation test; BSA, bovine serum albumin; CD, circular dichroism; ELISA, enzyme-linked immunosorbent assay; H-wtScy p 1, heat-treated wild-type Scy p 1; H-wtScy p 3, heat-treated wild-type Scy p 3; IAA, iodoacetamide; red/alk-wtScy p 3, reduced and alkylated wild-type Scy p 3; red/alk-wtScy p 1, reduced and alkylated wild-type Scy p 1; red/alk-mtScy p 3, reduced and alkylated mutant Scy p 3; red/alk-mtScy p 1, reduced and alkylated mutant Scy p 1; Ig, immunoglobulin; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MLC, myosin light chain, named Scy p 3 by WHO/IUIS; mtALLERGEN, mutant allergen; mtScy p 3, mutant Scy p 3; mtScy p 1, mutant Scy p 1; OD, optical density; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SI, stimulation index; TM, tropomyosin, named Scy p 1 by WHO/IUIS; wtALLERGEN, wild-type allergen; wtScy p 3, wild-type Scy p 3; wtScy p 1, wild-type Scy p 1.

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epitopes are more likely to reach the immune system in a reactive state, conformational structures/epitopes may also occur, even upon harsh processing and digestion (Liu & Sathe, 2018). Hence, determining whether conformational or linear epitopes dominate is a timely and difficult topic in the field of food allergens. To date, only a few studies have compared the relative importance of conformational and linear epitopes in peanut allergens (Bernard et al., 2015; Tscheppe et al., 2020). Furthermore, epitope characterization is important for comprehending the mechanisms of food-allergy reactions. Two heat-stable myofibril allergens Scy p 1 (tropomyosin, TM) and Scy p 3 (myosin light chain, MLC) were found to have both conformational and linear epitopes (Liu et al., 2018; Li et al., 2019; Liu et al., 2021). However, only a limited number of studies have analyzed the heterogeneous epitope-recognition patterns in patient serum samples; identifying the correlative epitopes in crab allergies can provide a reference for allergy immunotherapy and for developing modified myofibril allergens that reduce or eliminate allergenicity.

Although avoiding exposure to allergenic foods is currently the most effective way to prevent food allergies, allergen-specific immunotherapy (AIT) is slowly evolving into a major strategy for modifying the disease (Akdis & Akdis, 2014). AIT modifies the cellular and humoral responses of allergic patients to achieve long-term improvement of allergy symptoms. AIT involves several approaches for counteracting food allergies, including oral, sublingual, epicutaneous, and subcutaneous administration (Jones, Burks & Dupont, 2014; Pajno et al., 2018). At present, AIT is not available for crab allergies. However, a survey in China displayed that the prevalence of crab allergies is higher than for other shellfish (Hao et al., 2018; Wai et al., 2020). Hence, it is urgent to investigate AIT for crab allergies.

AIT is potentially a curative therapy, and it may increase the amount of food that the patient can tolerate, preventing allergic symptoms and reducing the risk of potentially life-threatening allergic reactions. The therapy consists of the administration of gradually increasing doses of food allergens via the oral, sublingual or subcutaneous routes (Schoos et al., 2020). In most cases, different routes and/or doses of AIT delivery results in similar immunologic changes (Schoos et al., 2020). The ultimate goal of food allergy-AIT is to achieve post-discontinuation effectiveness so that a patient can eat a normal serving of the trigger food without symptoms. This is also known as “tolerance” or “sustained unresponsiveness”. These terms all imply that the food allergen can be ingested without the appearance of allergic symptoms despite a period of absence of exposure. Furthermore, AIT uses the immune system of the patient to establish a counterimmune response, antagonizing the allergic immune response by vaccination with the disease-causing allergens or derivatives thereof (Jones, Burks & Dupont, 2014). However, since allergens may produce allergic side-effects during clinical applications, hypo-allergens with low IgE binding capacity have been considered as candidate molecules for AIT against food allergies (Freidl et al., 2017). Most hypo-allergenic derivatives were obtained using site-directed mutagenesis (Chen et al., 2021), but this is time-consuming process with highly unpredictable results (Pomés, 2010). The development of hypo-allergenic derivatives based on the deletion or destruction of IgE epitopes but retained *T*-cell epitopes is emerging as a common approach for obtaining ideal proteins and analyzing the effects of epitopes (Kleij et al., 2019). Tscheppe et al. (2020) generated hypo-allergenic derivatives that could be used in immunotherapy based on chemically modified epitopes, and they determined the leading role of conformational epitopes of Ara h 2. Zhang et al. (2020) also transformed the relative linear epitopes of oyster TM into hypo-allergenic derivatives. Therefore, hypo-allergenic derivatives of crab myofibril allergens can be explored through modification of IgE epitopes and retention of *T*-cell epitope, which might produce a candidate molecule for AIT.

This study was designed to analyze the effects of conformational and linear epitopes on allergenicity and to produce the corresponding hypo-allergenic derivatives of crab myofibril allergens. For these purposes, Scy p 1 and Scy p 3 molecules were modified, and multiple derivatives were constructed by deleting linear epitopes and destroying conformational epitopes. Secondary structures and surface hydrophobicities were analyzed by circular dichroism (CD) using fluorescent 8-anilino-1-naphthalenesulfonic acid (ANS) probes. Moreover, IgE binding capacity were analyzed by studying inhibition in enzyme linked immunosorbent assays (ELISA) with sera from 29 crab-sensitised patients. Furthermore, the allergenicity of multiple derivatives were evaluated by conducting basophil-activation tests (BAT).

2. Materials and methods

2.1. Materials

Horseradish peroxidase-labeled goat anti-human IgE and goat anti-rabbit IgG antibodies were purchased from Southern Biotech (Birmingham, Alabama, USA). ANS was purchased from Sigma-Aldrich (St Louis, Missouri, USA). All other reagents were of analytical grade.

2.2. Collection of whole blood and serum samples from patients

All serological processes were executed with reference to the guidelines for the care and use of human specimens (Women and Children’s Hospital Affiliated with Xiamen University), and experiments were approved by the Human Ethics Committee (approval number KY-2019-014). Blood donors provided signed, informed consent. The specificity of detection for crab (f23) IgE was evaluated *in vitro* using ImmunoCAP (Phadia AB, Rapsgatan, Uppsala, Sweden); samples with an IgE value of $\geq$ 0.35 kU/L were stored. Meanwhile, ELISA was further employed to recognize Scy p 1-positive sera and Scy p 3-positive sera with a cut-off value of OD450 nm $>$ 0.12, and at least 2-fold higher than that of the negative control serum. The sensitivity profiles and clinical symptoms of the patients enrolled in this study are shown in Table 1. Pooled serum was prepared by mixing equal amounts of undiluted serum samples (Table 1).

2.3. Design and identification of derivatives

The conformational epitopes of Scy p 1 and Scy p 3 were destroyed by reduced and alkylated (red/alk) following iodoacetamide (IAA) treatment, as described according the reference (Tscheppe et al., 2020). To generate mutant allergens (mtALLERGEN), we deleted amino acids located at stable heat/digestion linear epitopes, plus *N*-term and C-term with the surface negative electrostatic potential. The oligonucleotide primers used in PCR amplification are presented in Table S1. Red/alk wild-type Scy p 1 (red/alk-wtScy p 1), mutant Scy p 1 (mtScy p 1), and red/alk-mtScy p 1 (red/alk-mtScy p 1) were collectively referred to as Scy p 1 derivatives. Furthermore, red/alk wild-type Scy p 3 (red/alk-wtScy p 3), mutant Scy p 3 (mtScy p 3), and red/alk-mtScy p 3 (red/alk-mtScy p 3) were designated as Scy p 3 derivatives. Experimental details are presented in the Methods section of the supplementary material.

Allergen derivatives were analyzed by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis, using rabbit anti-crab Scy p 1 (1:1 $\times$ 10^5 dilution) and rabbit anti-crab Scy p 3 (1:8 $\times$ 10^4 dilution) polyclonal antibodies. Furthermore, red/alk-mtScy p 1 and red/alk-mtScy p 3 were submitted to Shenzhen Company for alkylation analysis by liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis.

2.4. CD spectra and labeled with fluorescent ANS probes analysis of derivatives

The changed secondary structure of derivatives was checked by CD spectrum (Applied Photophysics, Ltd., Surrey, UK), the procedures were described previously (Li et al., 2019), which revealed some minor changes. Each derivative was prepared at a concentration of 0.30 mg/

Table 1
Clinical and laboratory characterizations of 29 crab-sensitised patients and 4 non-allergic individuals.

Serum sample number	Age (years)	Sex	sIgE crab (f23) by ImmunoCAP (kUA/L)	Symptoms	Specific allergen
1	35	M	0.07	ND	–
2	25	F	0.13	ND	–
3	27	M	1.73	D, Ed	Scy p 1
4	31	F	0.80	Ec, P, U	Scy p 1
5	18	F	0.75	De, Ec	Scy p 1
6	31	F	1.34	Ed	Scy p 1
7	37	M	0.97	Em, N	Scy p 1
8	24	M	19.85	Is	Scy p 1
9	6	M	0.64	D, N	Scy p 1
10	23	F	7.52	U, De, P	Scy p 1
11	23	F	0.51	N, Em	Scy p 1
12	10	M	0.83	U, Ed	Scy p 1
13	47	F	1.40	Ar	Scy p 1
14	16	F	4.61	P, U	Scy p 1
15	55	F	3.72	Ec, Is	Scy p 1
16	9	F	0.10	ND	–
17	30	M	0.08	ND	–
18	31	F	1.03	Ed	Scy p 3
19	37	M	4.91	N, De	Scy p 3
20	24	M	0.90	P, De, U	Scy p 3
21	17	F	0.61	U, Is	Scy p 3
22	23	F	0.65	D, U	Scy p 3
23	21	F	1.11	N, Em	Scy p 3
24	10	M	7.80	P, U, Is	Scy p 3
25	42	F	1.40	R	Scy p 3
26	26	F	0.56	P, U	Scy p 3
27	35	F	0.72	U, Ec, Is	Scy p 3
28	29	F	1.61	D, Ec	Scy p 3
29	17	M	0.47	De, E, N	Scy p 3
30	25	M	0.97	P, N	Scy p 3
31	56	F	0.45	N, Em	Scy p 3
32	33	F	9.87	R, N	Scy p 3
33	30	M	5.17	P, U	Scy p 3

D, diarrhea; De, dermatitis; Ec, eczema; Ed, edema; Em, emesis; F, female; Is, itchy skin; M, male; N, nausea; ND, not determined/unknown; P, pruritus; R, rhinitis; sIgE, specific IgE; U, urticaria.

mL in 20 mM phosphate-buffered saline (PBS). The thermal-denaturation temperature was detected.

The surface hydrophobicity of derivatives was detected using a fluorescent ANS probes, methods were based on previous report (Tong et al., 2012), with some modifications. The concentration of each protein was adjusted to 0.30 mg/mL with 200 μL of 20 mM PBS. Experimental details are presented in the Methods section of the supplementary material.

2.5. Thermal-stability analysis of derivatives

Thermal-stability analysis of the derivatives generated in this study was incubated at the temperature range from 30 to 100 °C for 30 min. Proteins were diluted to 0.30 mg/mL and then analyzed by 15% SDS-PAGE and western blotting. The control groups incubated at 4 °C.

2.6. ELISA analysis of derivatives with serum from crab-sensitised patients

The IgE binding capacity of derivatives were analyzed using ELISA with serum samples from 29 crab-sensitised patients as the primary antibody, with a 1:3 dilution. More details were provided by Liu et al. (2018). In addition, inhibited IgE binding capacity of derivatives was tested by ELISA analysis, based on the method described by Tscheppe et al. (2020), with some modifications. Wild-type allergens (wtALLERGEN, 200 ng) were immobilised to ELISA plates and incubated with pooled, undiluted serum. Experimental details are presented in the Methods section of the supplementary material.

Furthermore, the specific IgE binding capacity of heat-treated wtALLERGEN (H-wtScy p 1 and H-wtScy p 3) were determined by dot blot analysis after heating at 100℃ for 2 h, using serum samples from 29 crab-sensitised patients as the primary antibody.

2.7. BAT analysis of derivatives

The effects of derivatives on the patients' effector cells were explored by BAT. Different concentrations of derivatives (20 μg/mL, 50 μg/mL, and 100 μg/mL) were used for the experiments. Therewith, the most appropriate concentration was prepared to induce basophil activation. The experimental method was according to the reference (Kleij et al., 2019), with some modification. Experimental details are presented in the Methods section of the supplementary material.

2.8. Statistical analysis

Each experiment was repeated more than three times, and all results are presented as the mean ± standard deviation (SD). One-way repeated-measures analysis of variance was performed as described by Li et al. (2019).

3. Results

3.1. Preparation and identification of derivatives

Linear epitopes (AA 99–109 and AA110-123 of Scy p 1; AA 51–61, AA 96–107 and AA 135–147 of Scy p 3) plus *N*-term and C-term with the surface negative electrostatic potential were deleted from the primary sequences of mtALLERGEN (Fig. 1A and 1C). To explore the effects of conformational epitopes without deleting linear epitopes, the allergens were treated with IAA to generated red/alk forms. As shown in Fig. 1B, red/alk-wtScy p 1 retained linear epitopes (red shading), mtScy p 1 had conformational epitopes (green and blue shading), and red/alk-mtScy p 1 lacked both types of epitopes. Fig. 1D shows that wtScy p 3 folded into a compact structure consisting of 8 α-helixes, with a disulfide bond between Cys36 and Cys130. Compared with wtScy p 3, mtScy p 3 retained 7 α-helixes with disulfide bonds. However, the structures of red/alk-wtScy p 3 and red/alk-mtScy p 3 became loose and the disulfide bonds were reduced.

The formation of allergen derivatives was confirmed by SDS-PAGE and western blot analysis. Compared with the wtALLERGEN, the purified derivatives had approximately the same molecular weights (Fig. 2A and 2C). Fig. 2B shows that wtScy p 1 had strong reaction specifically, whereas the IgG-binding abilities of the Scy p 1 derivatives were significantly reduced with polyclonal antibody, meanwhile, red/alk-mtScy p 1 showed the weakest band. However, all Scy p 3 derivatives reacted strongly with polyclonal antibody (Fig. 2D). Fig. 2E and 2F display the alkylation results for red/alk-mtScy p 1 and red/alk-mtScy p 3. The *N*-terminal, C-terminal, and linear epitopes of the wtALLERGEN were missing, which indicated the successful construction of the mtALLERGEN. In addition, several amino acid residues (D, E and M) were modified *via* alkylation in red/alk-mtScy p 1 (Fig. 2E). In Fig. 2F, Cys36, Cys130, and Cys131 (red boxes) had carbamidomethylation modifications with DTT and IAA, indicating that Cys of the thiol groups were reduced, thereby couldn't form disulfide bond, which preliminary reflected the reducted and alkylated of red/alk-mtScy p 3.

3.2. Secondary structures and hydrophobicity analysis of derivatives

Structural changes in the allergen derivatives were analyzed by CD (Fig. 3A and 3G). The wtALLERGEN and mtALLERGEN presented a typical α-helix structure. However, the peak values of the mtALLERGEN were lower than those of the wtALLERGEN, which suggested the slightly change of protein structure. In addition, the curves of four other derivatives tended to be flat. These findings showed they were

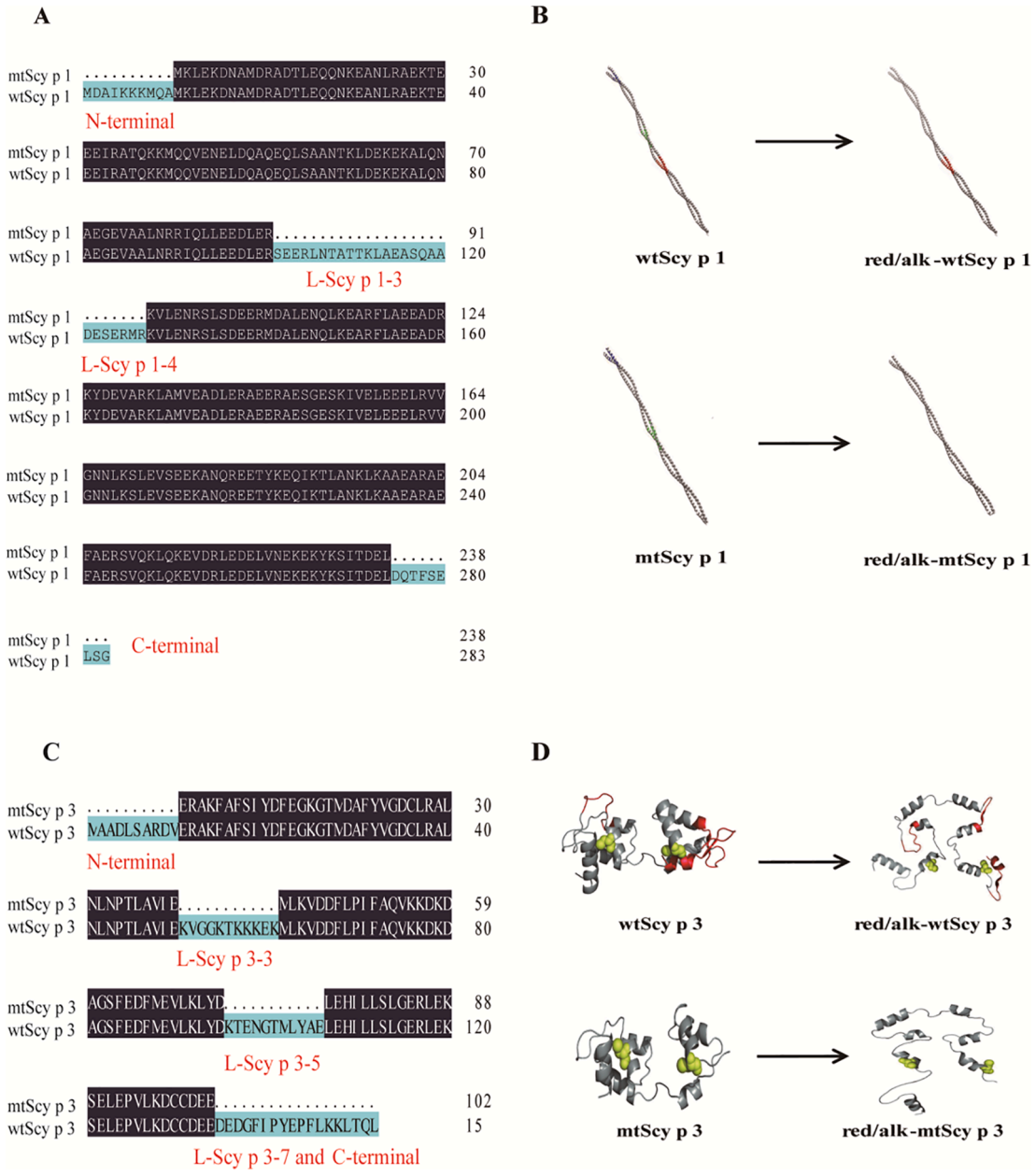

Fig. 1. Design strategy for constructing derivatives with destroyed conformational epitopes and/or deleted linear epitopes. (A) Amino acid-sequence alignment showing the deletion of the *N*-terminal, C-terminal, and critical linear epitopes in mtScy p 1. (B) Structural representations of Scy p 1 derivatives. The red shading represents critical linear epitopes in wtScy p 1 and red/alk-wtScy p 1. The green and blue shading represent conformational epitopes in wtScy p 1 and mtScy p 1. (C) Amino acid-sequence alignment showing the removal of *N*-terminal, C-terminal, and critical linear epitopes in mtScy p 3. (D) Structural representations of Scy p 3 derivatives. The red positions represent critical linear epitopes in wtScy p 3 and red/alk-wtScy p 3. The yellow balls represented the disulfide bond between residues Cys36 and Cys130 residues in wtScy p 3 and mtScy p 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

unstructured proteins due the disulfide bond breaks after 50 mM DTT and 200 mM IAA treatment, which confirmed that they were fully red/alk.

The thermal-denaturation temperature values of the derivatives were analyzed using Global 3.0 software. Fig. 3C–3F indicate that the thermal-denaturation temperature values of wtScy p 1 (79.2 ± 0.8℃) and mtScy p 1 (77.3 ± 0.7℃) were higher than those of red/alk-wtScy p 1 (45.4 ± 1.0℃) and red/alk-mtScy p 1 (42.1 ± 0.4℃), further indicating that the secondary structures of red/alk-wtScy p 1 and red/alk-mtScy p 1 were observably altered. The thermal-denaturation temperature values of the Scy p 3 derivatives (Fig. 3I–3L) were similar to those of the Scy p 1 derivatives.

Regarding the surface hydrophobicity (Fig. 3B), no significant difference was found in terms of the fluorescence intensities of wtScy p 1 and mtScy p 1 at 500 nm. However, the fluorescence intensities of red/alk-wtScy p 1 and red/alk-mtScy p 1 were enhanced, and the fluorescence peak shifted to the left. Compared with wtScy p 1, the fluorescence intensity of red/alk-wtScy p 1 was 2.0 times higher at 495 nm and that of red/alk-mtScy p 1 was 6.8 times higher at 485 nm. The surface hydrophobicity of the Scy p 3 derivatives (Fig. 3H) were similar to those of the Scy p 1 derivatives, which showed that the structure was obviously altered. In addition, the allergen derivatives were relatively stable, even at 100 °C (Figs. S1 and S2).

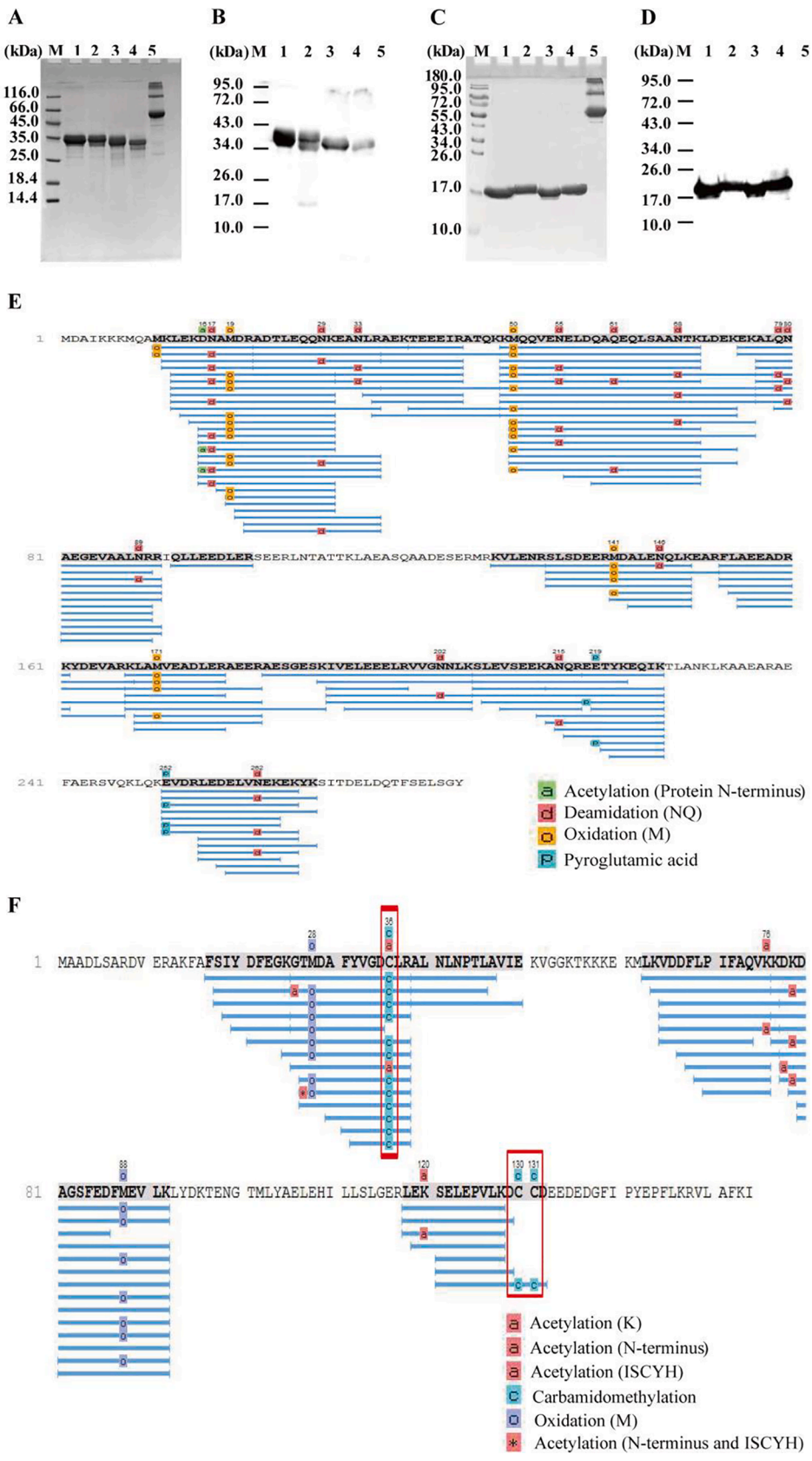

Fig. 2. Analysis and identification of derivatives using SDS-PAGE, western blot analysis, and LC–MS/MS. (A) SDS-PAGE analysis of Scy p 1 derivatives. Lane M, protein marker; lane 1, wtScy p 1; lane 2, red/alk-wtScy p 1; lane 3, mtScy p 1; lane 4, red/alk-mtScy p 1; lane 5, BSA. (B) Western blot validation of Scy p 1 derivatives. Lane 1, wtScy p 1; lane 2, red/alk-wtScy p 1; lane 3, mtScy p 1; lane 4, red/alk-mtScy p 1; lane 5, BSA. (C) SDS-PAGE analysis of Scy p 3 derivatives. Lane 1, wtScy p 3; lane 2, red/alk-wtScy p 3; lane 3, mtScy p 3; lane 4, red/alk-mtScy p 3; lane 5, BSA. (D) Western blot validation of Scy p 3 derivatives. Lane 1, wtScy p 3; lane 2, red/alk-wtScy p 3; lane 3, mtScy p 3; lane 4, red/alk-mtScy p 3; lane 5, BSA. (E) Alkylation-modification analysis of red/alk-mtScy p 1 by LC-MS/MS. (F) Alkylation-modification analysis of red/alk-mtScy p 3 by LC-MS/MS. The red boxes represent carbamidomethylation modifications of Cys36, Cys130, and Cys131. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

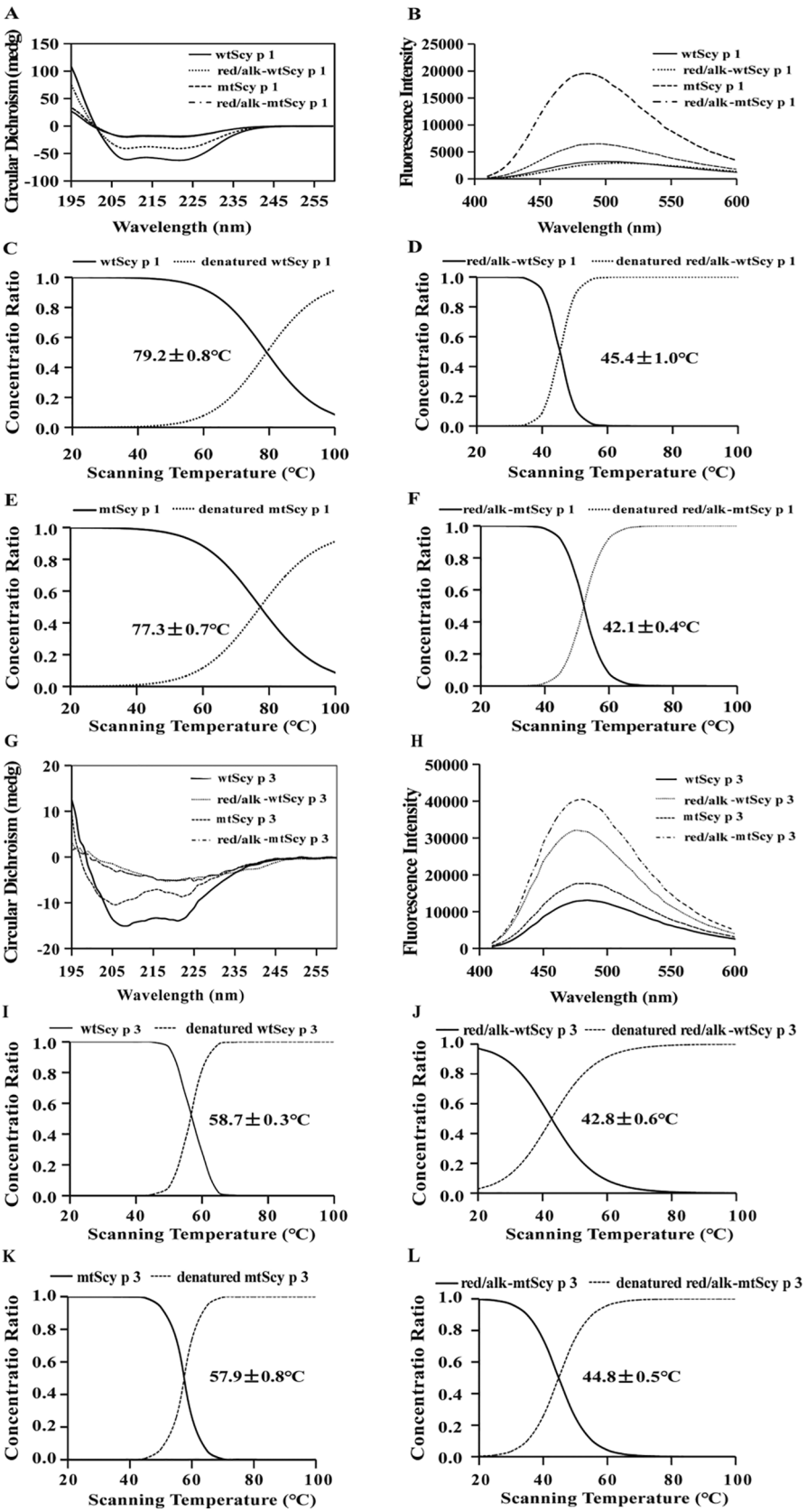

Fig. 3. Structural analysis of derivatives determined by CD spectroscopy and ANS probe. (A) Secondary structural analysis of Scy p 1 derivatives by CD spectroscopy. (B) Hydrophobicity analysis of Scy p 1 derivatives by ANS fluorescent probe. (C) Monitoring thermal-denaturation temperature value of wtScy p 1 by CD spectroscopy. (D) Monitoring the thermal-denaturation temperature value of red/alk-wtScy p 1 by CD spectroscopy. (E) Monitoring the thermal-denaturation temperature value of mtScy p 1 by CD spectroscopy. (F) Monitoring the thermal-denaturation temperature value of red/alk-mtScy p 1 by CD spectroscopy. (G) Secondary structural analysis of Scy p 3 derivatives by CD spectroscopy. (H) Hydrophobicity analysis of Scy p 3 derivatives using a fluorescent ANS probe. (I) Monitoring the thermal-denaturation temperature value of wtScy p 3 by CD spectroscopy. (J) Monitoring the thermal-denaturation temperature value of red/alk-wtScy p 3 by CD spectroscopy. (K) Monitoring the thermal-denaturation temperature value of mtScy p 3 by CD spectroscopy. (L) Monitoring the thermal-denaturation temperature value of red/alk-mtScy p 3 by CD spectroscopy.

3.3. IgE binding capacity analysis of derivatives

IgE binding to allergen derivatives was detected with serum from 29 crab-sensitised patients (Table 1). Compared with wtScy p 1, the IgE binding capacity of red/alk-wtScy p 1 decreased. mtScy p 1 showed the lowest IgE binding capacity among all serum samples (patients' serum sample numbers 3–15), and no intergroup difference was observed with red/alk-mtScy p 1 (Fig. 4A). Moreover, IgE binding to mtScy p 1 in serum from three patients (patients' serum sample numbers 8, 9, and 11) was consistent with that of non-allergic individuals. Regarding the Scy p 3 derivatives (Fig. 4E), red/alk-mtScy p 3 had the lowest IgE binding capacity, and no intergroup difference was found with mtScy p 3. Furthermore, IgE binding to red/alk-mtScy p 3 in the serum of four patients (patients' serum sample numbers 18, 20, 29, and 30) was destroyed.

The reduced IgE binding capacity of the allergen derivatives were further confirmed by inhibition-ELISA. An increase in the protein concentration gradually enhanced the inhibition rate. The inhibition rates of wtScy p 1 and wtScy p 3 were the highest, whereas those of mtScy p 1 and red/alk-mtScy p 3 were the lowest (Fig. 4B and 4F), when studying multiple protein concentrations. Furthermore, the inhibitory effect of red/alk-mtScy p 1 did not show an intergroup difference with mtScy p 1.

Fig. 4C and Fig. S3A showed heterogeneous epitope-recognition patterns for Scy p 1 derivatives in patient serum samples. Of them, 15% (patients' serum sample numbers 6 and 7) and 62% (patients' serum numbers 3, 4, 8, 9, 10, and 13–15) preferentially recognised the conformational and linear epitopes, respectively, whereas 23% (patients' serum sample numbers 5, 11, and 12) recognised both epitope types to the same degree due the optical density (OD) 450 values of red/alk-wtScy p 1 and mtScy p 1 were consistent. Regarding the Scy p 3 derivatives (Fig. 4G and Fig. S3B), 12.5% (patients' serum sample numbers 19 and 21) and 62.5% (patients' serum sample numbers 18, 20, 24, 25, and 28–33) preferentially recognised conformational and linear epitopes, respectively, whereas 18.75% (patients' serum sample numbers 22, 26, and 27) recognised both epitope types to the same degree due the OD 450 values of red/alk-wtScy p 3 and mtScy p 3 were consistent.. Furthermore, compared with wtScy p 3, OD value of one sample (patients' serum sample numbers 23, 6.25%) did not change. None of the allergen derivatives were recognised by IgE from serum obtained from non-allergic individuals (patients' serum sample numbers 1, 2, 16, and 17).

Dot blot analysis was performed to further verify the trend that the predominant epitope type of the wtALLERGEN. As shown in Fig. 4D and 4H, wtScy p 1 and wtScy p 3 had strong IgE binding capacity with all serum samples from sensitised patients. H-wtScy p 1 and H-wtScy p 3 retained strong IgE binding capacity with all samples, except for those from four patients (patients' serum sample numbers 6, 7, 19, and 21). Hence, wtScy p 1 and wtScy p 3 also could bind specifically to IgE after structural failure.

3.4. Allergenicity analysis of derivatives

BAT was used to analyze the activation of basophils from selected crab-sensitised patients, with a concentration of 50 μg/mL for each derivative. The BAT results were analyzed by flow cytometry analysis (Figs. S4 and S4). As shown in Fig. 5A–5D, compared with the negative control, wtScy p 1 and wtScy p 3 significantly increased the levels of CD63 ($p < 0.05$) and CD203c ($p < 0.05$) on the surfaces of basophils. Nevertheless, the stimulus index (SI) of the derivatives in allergens decreased. The SI values of red/alk-wtScy p 1 and red/alk-wtScy p 3 were higher than those of the other four derivatives. red/alk-mtScy p 1 and red/alk-mtScy p 3 had the lowest SI values, but no intergroup difference was observed compared to the mtALLERGEN, and the results showed the ability of these derivatives to induce basophil-activation was significantly reduced.

4. Discussion

In this study, we determined structural changes, analyzed the heterogeneous epitope-recognition patterns, and produced the corresponding derivatives of crab myofibril allergens with lower IgE-binding and basophil-activation capacities.

It was reported that in protein sequence, not only Cys, but also other amino acid residues can undergo alkylation modification using IAA (Boja & Fales, 2001). In our paper, although Cys does not exist in Scy p 1 sequence, Asp, Glu and Met are indeed modified by LC-MS/MS. It was worth noting that IAA may also affect linear epitopes, and we will explore in the future. Ultimately, the alkylation reduction reaction of wtScy p 1 was occurred and with the structure affected. Different physicochemical characteristics can impact on the allergenicity of proteins, including the structural integrity, the organisational level, heat stability, chemical activities, among others (Costa et al., 2021). Chen et al. (2021) discovered that reduced α-helical content and increased surface hydrophobicity were responsible for the hypo-allergenicity of sarcoplasmic calcium-binding protein from crab. In this study, compared with wtScy p 3, the α-helical contents of all derivatives decreased, and their allergenicity were lower, which were similar to the reported in the literature (Blanc et al., 2011; Chen et al., 2021). Meanwhile, inhibition ELISA results showed no loss of IgE binding capacity, despite significant structural damage to red/alk-wtScy p 1, mostly due to the presence of linear epitopes (Carnés et al., 2007; Kamath et al., 2013). Interestingly, the allergenicity of red/alk allergens was higher compared to mtALLERGE, suggested that the high allergenicity of red/alk allergens could not be consequence of some molecules being modified but others not. Furthermore, the predominant epitope type of allergens were determined by comparing the IgE binding capacity of fold proteins and denatured proteins (Zhang et al., 2015). Protein denaturation can include chemical treatment (Kleij et al., 2019), heat treatment (Fang et al., 2018) among others. In this study, comparing wtALLERGEN with H-wtALLERGEN is expected to further analyze the trend that wtALLERGEN recognised linear or conformational epitopes, which indicating linear epitope was the mainly epitope type of crab myofibril allergens to bind IgE. Hence, for heat-stable allergens, the presence of stable heat/digested epitopes has a greater influence on allergenicity than structural destruction.

The epitope is the part of an antigen that interacts with its corresponding paratope by non-covalent forces (Liu & Sathe, 2018). The interaction may serve as the molecular basis for IgE cross-linking with allergens on the surfaces of mast cells or basophils, and subsequent degranulation (Murphy & Weaver, 2017). In addition, patients with egg or milk allergies who recognised conformational epitopes are usually no longer allergic in adulthood (Järvinen et al., 2002). Nevertheless, patients do not outgrow peanut allergies because the linear epitopes of peanut allergens are still recognise (Tscheppe et al., 2020). In this study, the recognition of linear IgE epitopes was predominant in crab myofibril allergens, with a minor involvement of conformational epitopes. The results showed that crab allergy, like peanut allergy, are lifelong conditions because of the presence of stable linear epitopes. For allergens with a tight conformation, it is generally believed that they mainly have conformational epitopes (Tscheppe et al., 2020). Interestingly, Scy p 3 folded into a compact structure with one disulfide bond, but its linear epitopes were primarily recognised. This result suggests that determining the dominance of conformational epitopes during IgE binding based on the level of structural complexity may be unreliable. Furthermore, Zhang et al. (2015) found that conformational epitopes in shrimp MLC were primarily recognised, rather than linear epitopes. The main reason is that the spatial structures of Scy p 3 and shrimp MLC are significantly different, as shrimp MLC lacks disulfide bonds. Crab muscles contain salt-soluble myofibril and water-soluble myosinogen (Yang et al., 2019). Previous data showed that conformational epitopes in crab myosinogen allergens are mainly recognised (Yang et al., 2019). The current findings revealed that linear epitopes are mainly recognised in

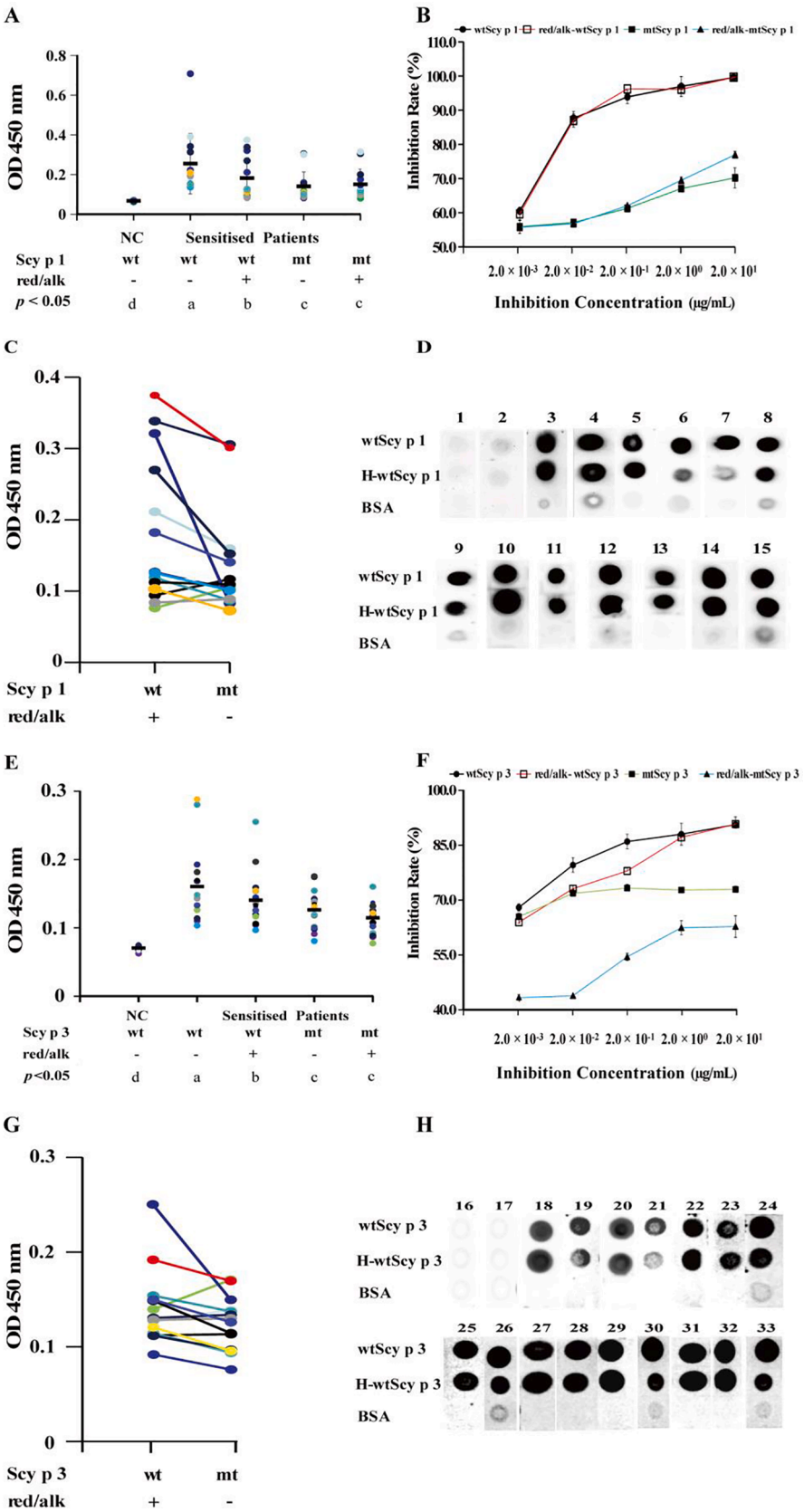

Fig. 4. Analysis of the IgE binding capacity of derivatives using serum samples from 29 crab-sensitised patients. All data are expressed as the mean ± SD (n = 3). NC represents the negative control (OD 450 nm < 0.1), and wtALLERGEN (OD 450 nm > 0.1) was used as the positive control. The horizontal lines represent the average values. (A) Distributions of ELISA OD values with Scy p 1-sensitised patients (patients' serum sample numbers 3–15). All data are expressed as the mean ± SD (n = 3). (B) Inhibition ELISA analysis using undiluted, serum from Scy p 1-sensitised patients (patients' serum sample numbers 3–15). (C) Epitope-recognition patterns were determined by comparing IgE binding to red/alk-wtScy p 1 (lacking conformational epitopes), and mtScy p 1 (lacking linear epitopes). Different colored data points represent different serum samples of patients. (D) Dot blot analysis of the IgE binding capacity of wtScy p 1 and H-wtScy p 1 using serum from non-allergic individuals (patients 1 and 2) and Scy p 1-sensitised patients (patients' serum sample numbers 3–15). wtScy p 1 served as a positive control (i.e., without processing), H-wtScy p 1 was studied in the experimental group after heating wtScy p 1 to 100℃ for 2 h, and BSA served as the negative control. (E) Distributions of ELISA OD values for Scy p 3-sensitised patients (patients' serum sample numbers 18–33). (F) Inhibition ELISA analysis using undiluted, pooled serum from Scy p 3-sensitised patients (patients' serum sample numbers 18–33). (G) Epitope-recognition patterns were determined by comparing IgE binding to red/alk-wtScy p 3 (lacking conformational epitopes), and mtScy p 3 (lacking linear epitopes). Different colored data points represent different serum samples of patients. (H) Dot blot analysis of the IgE binding capacity of wtScy p 3 and H-wtScy p 3 using serum from non-allergic individuals (patients 15 and 16) and Scy p 3-sensitised patients (patients' serum sample numbers 17–33). wtScy p 3 was studied as a positive control (i.e., without processing, H-wtScy p 3 was studied in the experimental group after heating wtScy p 3 to 100℃ for 2 h, and BSA served as the negative control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

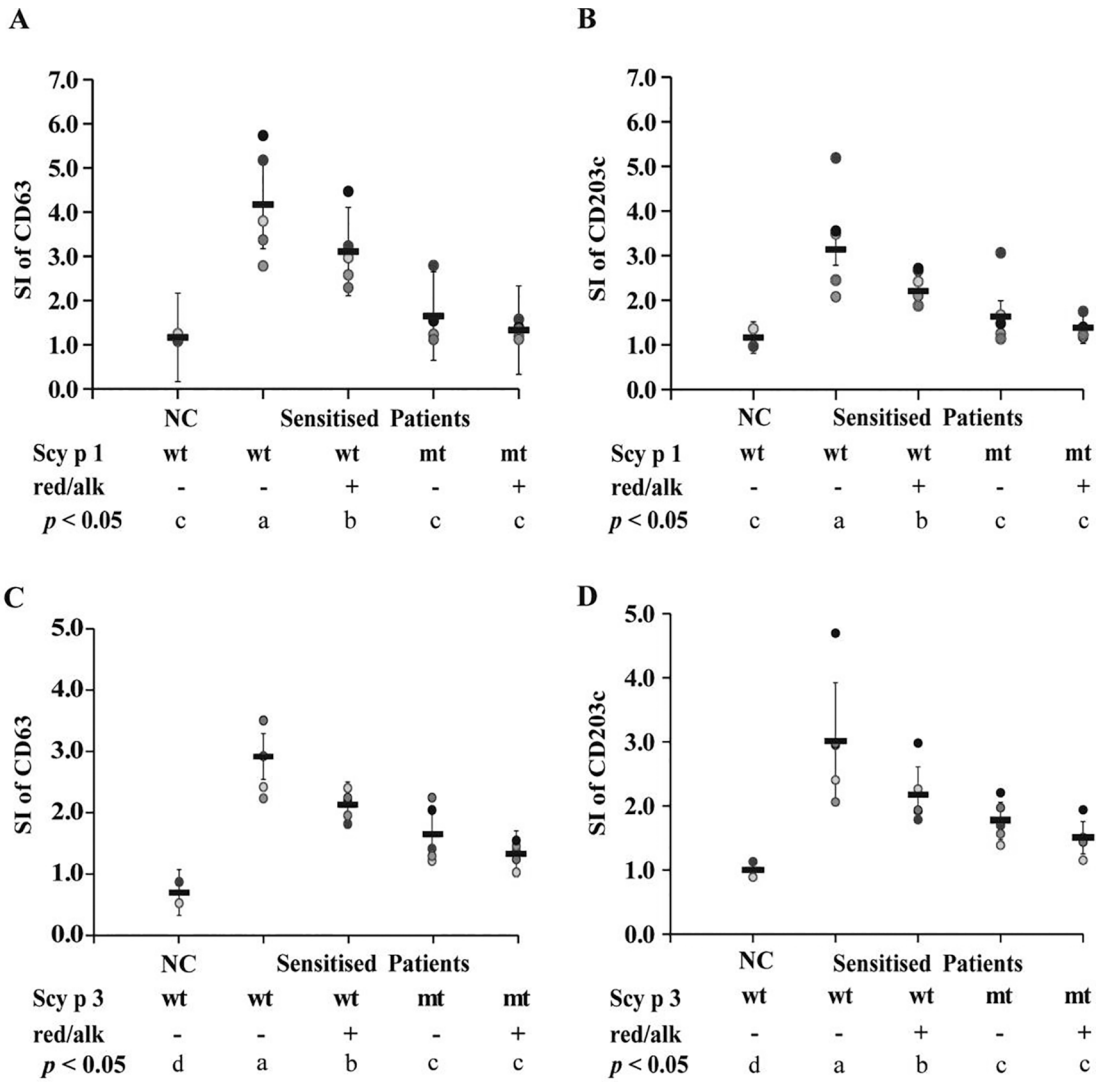

Fig. 5. Allergenicity analysis of derivatives by BAT with 10 crab-sensitised patients. (A) Effects of Scy p 1 derivatives on surface CD63 expression on basophils from 5 patients selected 13 Scy p 1-sensitised patients. NC represents non-allergic individuals, and the horizontal lines represent the average SI values. $p < 0.05$, compared with wtScy p 1. (B) Effects of Scy p 1 derivatives on surface CD203c expression on basophils from 5 patients selected from 13 Scy p 1-sensitised patients. NC represents non-allergic individuals, and the horizontal lines represent the average SI values. $p < 0.05$, compared with wtScy p 1. (C) Effects of Scy p 3 derivatives on surface CD63 expression on basophils from 5 patients selected from 16 Scy p 3-sensitised patients. NC represents non-allergic individuals, and the horizontal lines represent the average SI values. $p < 0.05$, compared with wtScy p 3. (D) Effects of Scy p 3 derivatives on surface CD203c expression on basophils from 5 patients selected from 16 Scy p 3-sensitised patients. NC represents non-allergic individuals, and the horizontal lines represent the average SI values. $p < 0.05$, compared with wtScy p 3.

crab myofibril allergens and provided information regarding the recognition of IgE epitopes in crab allergens. Furthermore, knowledge of the epitope characteristics and types is vital for comprehending nosogenesis and exploring detection, diagnostic, and therapeutic strategies for crab allergies.

It was interesting to recall that a number of antigenic sites to bind strongly to antibodies were earlier observed to exhibit significant negative potentials (López-Torrejón et al., 2007). In our study, the surface electrostatic potential of crab myofibril allergens were calculated. For Scy p 1, deleted epitope 3 (AA 99–109), epitope 4 (AA110-123) and C-term were mostly negatively charged. Similarly, deleted epitope 2 (AA 51–61), epitope 4 (AA 96–107), epitope 7 (AA 135–147), *N*-term and C-term of Scy p 3 also were mostly negatively charged and larger areas. These results indicated that the above epitopes, also *N*-term and C-term may be more easier to bind to antibodies. Subsequently, the binding of the above epitopes to antibody were further analyzed through experiments. Liu et al. (2021) found compared with other epitopes of Scy p 1, deleted epitope 1 and epitope 2 had the greatest inhibition efficiency at different concentrations. For Scy p 3, deleted epitope 1, epitope 2 and epitope 3 displayed the stronger IgE binding capacity than other epitopes by inhibition ELISA (Li et al., 2019). Hence, these epitopes with stronger IgE binding capacity were considered deleting to obtain hypoallergenic derivatives. However, new molecular vaccination and tolerance induction strategies of AIT require a detailed knowledge of IgE and *T*-cell epitopes (Curin et al., 2018). In our study, *T*-cell epitope of crab myofibril allergens were predicted. *T*-cell epitope 1 and *T*-cell epitope 2 of Scy p 1 were partially overlapped with IgE epitope (AA 39–48) and IgE epitope (AA 153–162), respectively. In Scy p 3, *T*-cell epitope 1, *T*-cell epitope 2 and *T*-cell epitope 3 were also partially overlapped with IgE epitope (AA 35–47), IgE epitope (AA 66–80) and IgE epitope (AA 109–120), respectively. In order to preserve the *T*-cell proliferation of the hypo-allergenic derivatives, peptides with both *T*-cell and IgE epitopes in Scy p 1 and Scy p 3 should be preserved. To sum up, by deleted linear epitopes plus *N*-term and C-term, hypo-allergenic derivatives of crab myofibril allergen but retained *T*-cell activation were generated.

In recent years, increasing studies have been conducted to

investigate ways to regulate food allergies, with the main focus being on AIT. Yet, despite advances in AIT for inhalant allergens, the development of AIT for food allergies has been at a stalemate not only due to frequently reported anaphylactic side effects during treatment, but also due to the lack of criteria for evaluating clinical effects (Schoos et al., 2020; Wai et al., 2019). Furthermore, food allergens are easily digestible in the gastrointestinal tract, and digestive products still contain>20 epitopes bound to IgE (Schoos et al., 2020; Wai et al., 2019). To address these issues, allergens with low IgE binding capacity and basophil activation were applied by modifying IgE epitopes. This is similar to some naturally occurring polymorphisms. In addition, developing product candidates with low side effects (such as through genetic engineering or chemical modifications) is the first prerequisite for clinically acceptable treatment (Kleij et al., 2019; Valenta, Campana, Focke-Tejkl & Niederberger, 2016). In this study, we prepared derivatives of crab myofibril allergens by deleting heat/digestion-stable linear epitopes through genetic engineering and destroying conformational epitopes *via* chemical modification. And either epitopes deletion or chemical modification, can reduce the IgE binding capacity of wtALLERGEN. Meanwhile, the derivatives with deleted linear epitopes showed lower allergenicity than those with destroyed conformational epitopes. However, it was worth noting that the derivatives produced by deleted linear epitopes did not abrogate binding completely, illustrating the influence of other linear epitopes is not negligible. Certainly, these results can help to provide an accurate template for the analysis of the IgE binding. In the future, we will delete all IgE epitopes to prepare mutants. Furthermore, it has been reported that hypo-allergenic derivative of Cra g 1 and Ara h 2 has been constructed by partial epitope deletion (Tscheppe et al., 2020; Zhang et al., 2020). And these hypo-allergenic derivatives may be safer immunotherapy regiments to reduce the potential risk of allergic side-effects during treatment through inhibition of mediators release in activated mast cells or reduced anaphylactogenic potency. In our study, hypo-allergenic derivatives can reduce basophil activation in the whole blood of sensitised patients, which makes them suitable candidate molecules for further development of a crab AIT vaccine with less side effects. Patent protection for the prepared methods and products of the derivatives has been applied for in China, under patent application number 202011083369.5. Study has shown that the induction of functional blocking antibodies such as IgG4 in human is tightly associated with successful immunotherapy, which it can act to induce and sustain immunological tolerance to foods (Oliver et al., 2017). Hence, future research in our laboratory will focus also on evaluating the therapeutic potentials of Scy p 1 and Scy p 3 derivatives for crab AIT *in vivo*, using a mouse model.

5. Conclusion

Heat/digestion-stable epitopes of crab myofibril allergens influenced allergenicity to a greater degree than structural destruction. Furthermore, we analyzed the heterogeneous epitope-recognition patterns and identified correlative epitopes in crab allergies that can provide a reference for allergy immunotherapy and developing modified myofibril allergens that reduce or eliminate allergenicity. The most important outcome is that derivatives of crab myofibril allergens were successfully constructed; they showed hypo-allergenicity, which makes them suitable candidate molecules for further development of a crab AIT vaccine with less side effects.

CRediT authorship contribution statement

Mengsi Li: Methodology, Investigation, Writing – original draft. **Fei Xia:** Conceptualization, Methodology. **Yiyu Chen:** Investigation, Data curation. **Meng Liu:** Conceptualization. **Qingmei Liu:** Investigation. **Yang Yang:** Investigation. **Guixia Chen:** Resources. **Li Wang:** Supervision. **Minjie Cao:** Supervision. **Guangming Liu:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article appendix A can be found online at https://doi.org/10.1016/j.foodchem.2021.131588.

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Online Supplementary Material

Supplementary Methods

Expression and purification of wild-type and mutant allergens

The expression strains, BL21-pET-22b-*Scy p 1* (Liu et al., 2018) and BL21-pET-22b-*Scy p 3* (Li et al., 2019), were induced by isoprophyl-β-d-thiogalactoside (IPTG) after 16 h at 16℃. Wild-type allergens (wtALLERGEN) were purified using Ni-NTA resin. For mutant Scy p 1 (mtScy p 1), amino acids of N-terminus (1-10), C-terminus (275-283) and the critical linear epitope regions (102-127) were removed. Similarly, mutant Scy p 3 (mtScy p 3) was deleted the amino acids of N-terminus (1-10), C-terminus (143-153) and the critical linear epitope regions (51-61, 96-106 and 135-147). The oligonucleotide primers used in PCR amplification are presented in Table S1. The BL21 expression strain containing mutant allergens (mtALLERGEN) gene were induced by IPTG after 16 h at 16℃, and then they were purified by Ni-NTA resin.

Reduction and alkylation of wild-type and mutant allergens

wtALLERGEN and mtALLERGEN were reduced and alkylated to destroy conformational epitopes. The experimental steps were described according the reference (Tscheppe et al., 2020). Briefly, the protein samples were incubated with 50 mM dithiothreitol (DTT) at 60 °C for 1 hour, followed by 200 mM iodoacetamide (IAA) at room temperature for 2 hours. To inactivate unbound IAA, 37 mM DTT were added, and the reaction was incubated for 20 min. Proteins were dialyzed against PBS (pH 7.6).

CD spectra and labeled with fluorescent ANS probes analysis of derivatives

The changed secondary structure of derivatives was checked by CD spectrum (190–260 nm) using a circular dichroism spectrophotometer (Applied Photophysics,

Ltd., Surrey, UK). The procedures were described previously (Li et al., 2019) with some modification. Each spectrum represents the scan at 25 °C with protein concentration adjusted to 0.30 mg/mL. The operating parameters were as follows: scan rate, interval and bandwidth were set to 100 nm/min, 0.25 s and 1.0 nm, respectively.

The effects of temperature on derivatives secondary structure were detected from the circular dichroism spectra by monitoring the thermal denaturation temperature. The operating parameters were as follows: scan rate, interval, bandwidth, temperature range, and heating rate were set to 100 nm/min, 0.25 s, 1.0 nm, 20–100 °C, and 0.5 °C/min, respectively.

The surface hydrophobicity of derivatives was detected using a fluorescent ANS probes (Tong et al., 2012), with some modification. The concentration of each protein was adjusted to 0.30 mg/mL with 200 μL of 20 mM PBS. The fluorescence value was measured at an excitation wavelength of 375 nm, and the emission wavelength range was 400 to 600 nm.

ELISA analysis of derivatives with serum samples from crab-sensitised patients

The IgE binding capacity of derivatives were analyzed using ELISA with serum samples from 29 crab-sensitised patients as the primary antibody, with a 1:3 dilution. The procedures were described previously (Liu et al., 2018), with some modification. The goat anti-human IgE-HRP antibody ($1{:}2\times10^{3}$ dilution) was incubated as a secondary antibody. The OD value was measured at an wavelength of 450 nm.

Inhibited IgE binding capacity of derivatives was also tested by ELISA analysis, based on the method described by Tscheppe et al. (Tscheppe et al., 2020), with some modifications. wtALLERGEN (200 ng) were immobilised to ELISA plates. The derivatives as the inhibitor were diluted to different concentrations ($2.0\times10^{-3,}$ 2.0×10^{-2},

2.0×10^{-1}, 2.0×10^{0} and 2.0×10^{1}), and then pre-incubated with the serum pool of crab-sensitised patients without dilute for 16 h at 4 °C. The goat anti-human IgE-HRP antibody ($1{:}2\times10^{3}$ dilution) was incubated as a secondary antibody. The OD value was measured at an wavelength of 450 nm and the inhibition rates were calculated as follows:

{1 − [(experimental group − negative control)/(positive control − negative control)]} × 100%

BAT analysis of derivatives

The serum verified by dot blot was used to further detect the effect of allergen on the effector cells of the patients with BAT. The experimental method was according to the reference (Gadermaier et al., 2018), with some modification. The effect of derivatives-induced basophil activation was measured by detecting the level of CD203c and CD63 upregulation using flow cytometry. Allergen-induced CD203c and CD63 upregulation was calculated using the mean fluorescence intensities (MFIs) obtained with stimulated (MFIstim) and unstimulated (MFIcon) cells and was expressed as the stimulation index (SI), defifined as MFIstim/MFIcon.

References

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Supplementary Figure captions

Figure S1. Thermal-stability analysis of Scy p 1 derivatives. (A) SDS-PAGE analysis of the thermal stability of wtScy p 1. (B) Western blot for the validation of the thermal stability of wtScy p 1. (C) SDS-PAGE analysis of the thermal stability of red/alk-wtScy p 1. (D) Western blot for the validation of the thermal stability of red/alk-wtScy p 1. (E) SDS-PAGE analysis of the thermal stability of mtScy p 1. (F) Western blot for the validation of the thermal stability of mtScy p 1. (G) SDS-PAGE analysis of the thermal stability of red/alk-mtScy p 1. (H) Western blot for the validation of the thermal stability of red/alk-mtScy p 1. In each image, lane M is the protein marker.

Figure S2. Thermal-stability analysis of Scy p 3 derivatives. (A) SDS-PAGE analysis of the thermal stability of wtScy p 3. (B) Western blot for the validation of the thermal stability of wtScy p 3. (C) SDS-PAGE analysis of the thermal stability of red/alk-wtScy p 3. (D) Western blot for the validation of the thermal stability of red/alk-wtScy p 3. (E) SDS-PAGE analysis of the thermal stability of mtScy p 3. (F) Western blot for the validation of the thermal stability of mtScy p 3. (G) SDS-PAGE analysis of the thermal stability of red/alk-mtScy p 3. (H) Western blot for the validation of the thermal stability of red/alk-mtScy p 3. In each image, lane M is the protein marker.

Figure S3. Epitope-recognition patterns analysis using ELISA with serum samples from 29 crab-sensitised patients. All data are expressed as the mean ± SD (n = 3). (A) Individual epitope recognition patterns demonstrated by comparing IgE binding to wtScy p 1, red/alk-wtScy p 1 (lacking conformational epitopes), and mtScy p 1 (lacking linear epitopes). (B) Individual epitope recognition patterns demonstrated by comparing IgE binding to wtScy p 3, red/alk-wtScy p 3 (lacking conformational epitopes), and mtScy p 3 (lacking linear epitopes).

Figure S4. Allergenicity analysis of Scy p 1 derivatives by BAT using flow cytometry. The white, black, and gray coloring represent cell populations stimulated with PBS, wtScy p 1, and Scy p 1 derivatives, respectively. NC represents non-allergic individuals.

Figure S5. Allergenicity analysis of Scy p 3 derivatives by BAT using flow cytometry. The white, black, and gray coloring represent cell populations stimulated with PBS, wtScy p 3, and Scy p 3 derivatives, respectively. NC represents non-allergic individuals.

Figure S1

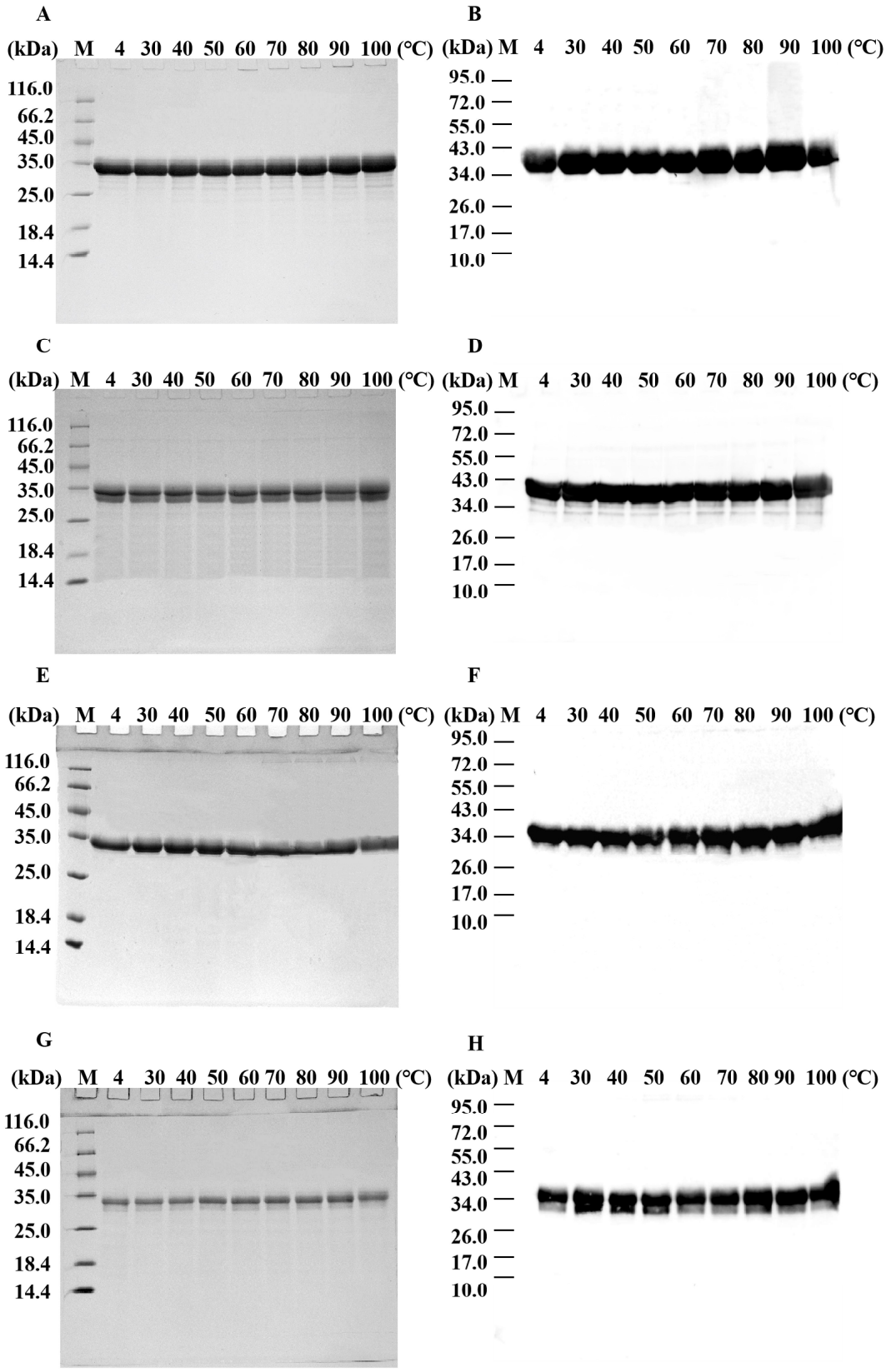
A
(kDa) M 4 30 40 50 60 70 80 90 100 (°C)
116.0
66.2
45.0
35.0
25.0
18.4
14.4
B
(kDa) M 4 30 40 50 60 70 80 90 100 (°C)
95.0
72.0
55.0
43.0
34.0
26.0
17.0
10.0
C
(kDa) M 4 30 40 50 60 70 80 90 100 (°C)
116.0
66.2
45.0
35.0
25.0
18.4
14.4
D
(kDa) M 4 30 40 50 60 70 80 90 100 (°C)
95.0
72.0
55.0
43.0
34.0
26.0
17.0
10.0
E
(kDa) M 4 30 40 50 60 70 80 90 100 (°C)
116.0
66.2
45.0
35.0
25.0
18.4
14.4
F
(kDa) M 4 30 40 50 60 70 80 90 100 (°C)
95.0
72.0
55.0
43.0
34.0
26.0
17.0
10.0
G
(kDa) M 4 30 40 50 60 70 80 90 100 (°C)
116.0
66.2
45.0
35.0
25.0
18.4
14.4
H
(kDa) M 4 30 40 50 60 70 80 90 100 (°C)
95.0
72.0
55.0
43.0
34.0
26.0
17.0
10.0

Figure S2

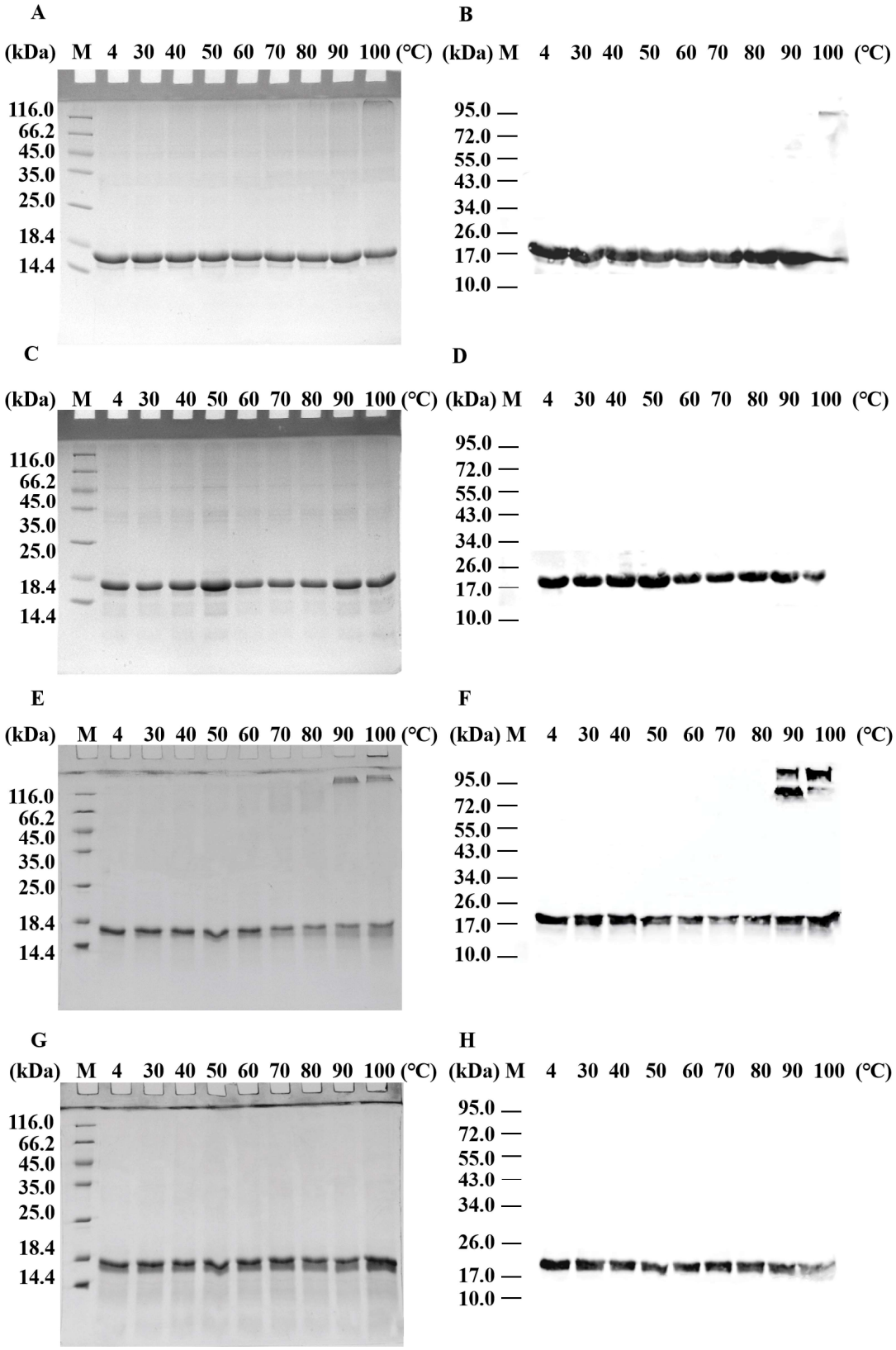
A
(kDa) M 4 30 40 50 60 70 80 90 100 (°C)
116.0
66.2
45.0
35.0
25.0
18.4
14.4
B
(kDa) M 4 30 40 50 60 70 80 90 100 (°C)
95.0
72.0
55.0
43.0
34.0
26.0
17.0
10.0
C
(kDa) M 4 30 40 50 60 70 80 90 100 (°C)
116.0
66.2
45.0
35.0
25.0
18.4
14.4
D
(kDa) M 4 30 40 50 60 70 80 90 100 (°C)
95.0
72.0
55.0
43.0
34.0
26.0
17.0
10.0
E
(kDa) M 4 30 40 50 60 70 80 90 100 (°C)
116.0
66.2
45.0
35.0
25.0
18.4
14.4
F
(kDa) M 4 30 40 50 60 70 80 90 100 (°C)
95.0
72.0
55.0
43.0
34.0
26.0
17.0
10.0
G
(kDa) M 4 30 40 50 60 70 80 90 100 (°C)
116.0
66.2
45.0
35.0
25.0
18.4
14.4
H
(kDa) M 4 30 40 50 60 70 80 90 100 (°C)
95.0
72.0
55.0
43.0
34.0
26.0
17.0
10.0

Figure S3

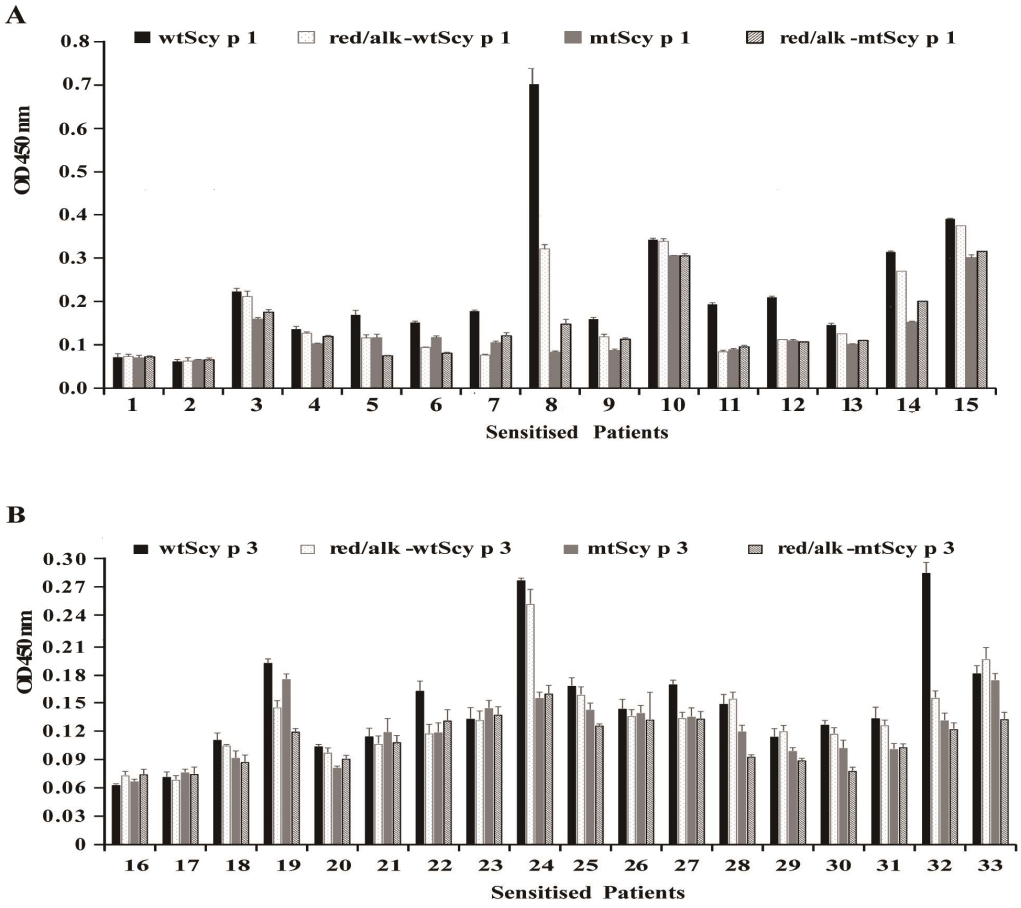
A
wtScy p 1
red/alk-wtScy p 1
mtScy p 1
red/alk -mtScy p 1
OD450 nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Sensitised Patients
B
wtScy p 3
red/alk -wtScy p 3
mtScy p 3
red/alk -mtScy p 3
OD450 nm
0.30
0.27
0.24
0.21
0.18
0.15
0.12
0.09
0.06
0.03
0
16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
Sensitised Patients

Figure S4

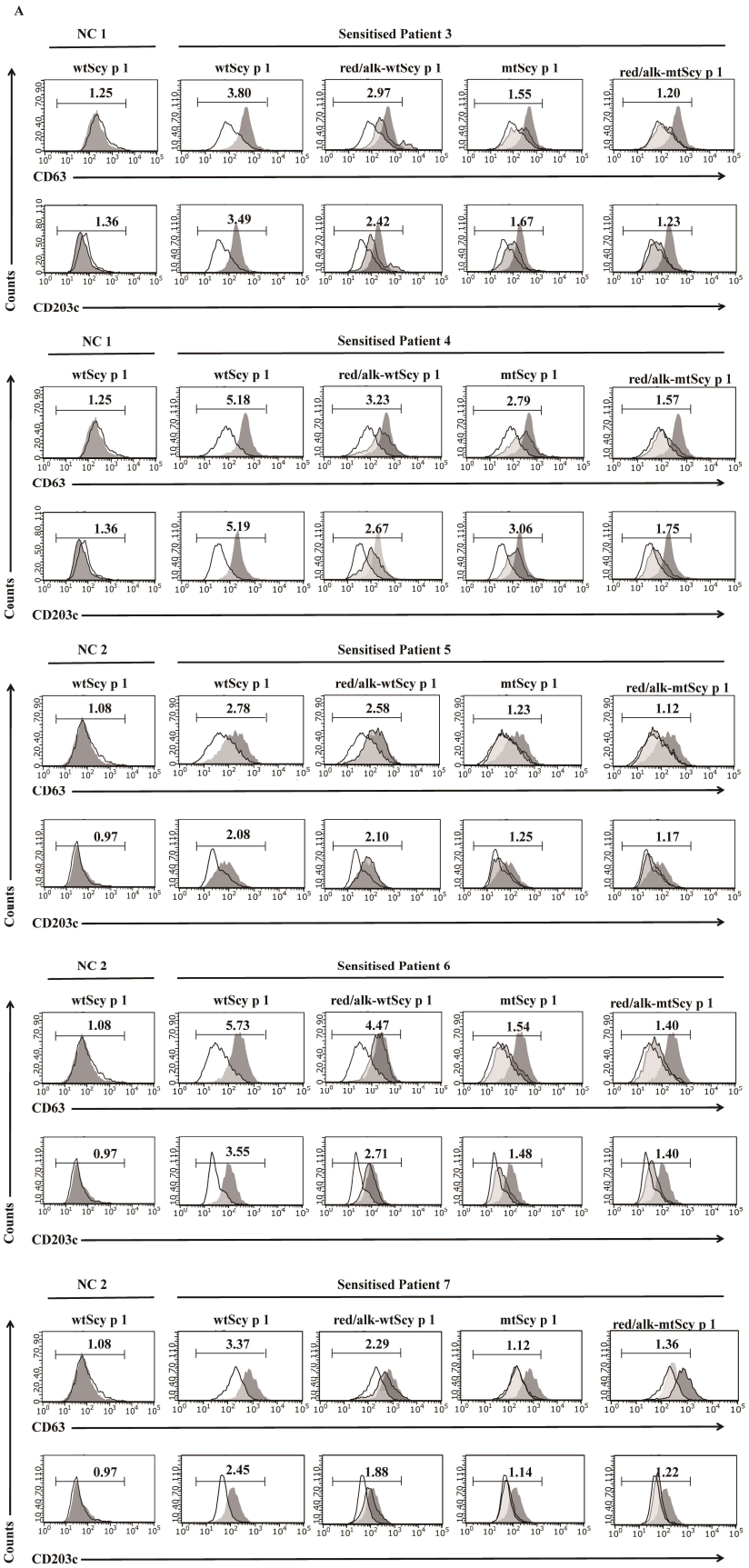
A
NC 1
Sensitised Patient 3
wtScy p 1
wtScy p 1
red/alk-wtScy p 1
mtScy p 1
red/alk-mtScy p 1
1.25
3.80
2.97
1.55
1.20
CD63
1.36
3.49
2.42
1.67
1.23
Counts
CD203c
NC 1
Sensitised Patient 4
wtScy p 1
wtScy p 1
red/alk-wtScy p 1
mtScy p 1
red/alk-mtScy p 1
1.25
5.18
3.23
2.79
1.57
CD63
1.36
5.19
2.67
3.06
1.75
Counts
CD203c
NC 2
Sensitised Patient 5
wtScy p 1
wtScy p 1
red/alk-wtScy p 1
mtScy p 1
red/alk-mtScy p 1
1.08
2.78
2.58
1.23
1.12
CD63
0.97
2.08
2.10
1.25
1.17
Counts
CD203c
NC 2
Sensitised Patient 6
wtScy p 1
wtScy p 1
red/alk-wtScy p 1
mtScy p 1
red/alk-mtScy p 1
1.08
5.73
4.47
1.54
1.40
CD63
0.97
3.55
2.71
1.48
1.40
Counts
CD203c
NC 2
Sensitised Patient 7
wtScy p 1
wtScy p 1
red/alk-wtScy p 1
mtScy p 1
red/alk-mtScy p 1
1.08
3.37
2.29
1.12
1.36
CD63
0.97
2.45
1.88
1.14
1.22
Counts
CD203c

Figure S5

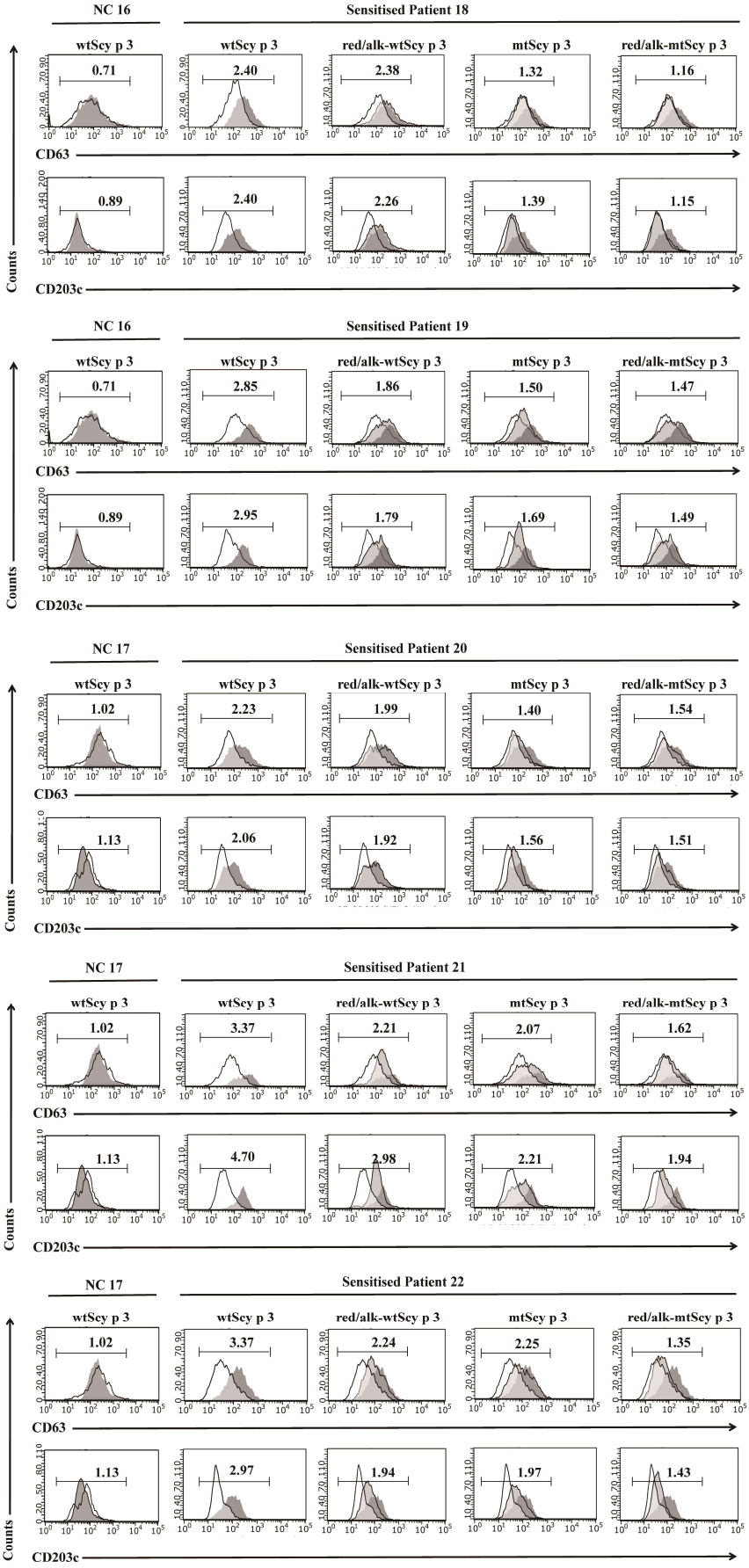
NC 16
Sensitised Patient 18
wtScy p 3
wtScy p 3
red/alk-wtScy p 3
mtScy p 3
red/alk-mtScy p 3
0.71
2.40
2.38
1.32
1.16
CD63
0.89
2.40
2.26
1.39
1.15
Counts
CD203c
NC 16
Sensitised Patient 19
wtScy p 3
wtScy p 3
red/alk-wtScy p 3
mtScy p 3
red/alk-mtScy p 3
0.71
2.85
1.86
1.50
1.47
CD63
0.89
2.95
1.79
1.69
1.49
Counts
CD203c
NC 17
Sensitised Patient 20
wtScy p 3
wtScy p 3
red/alk-wtScy p 3
mtScy p 3
red/alk-mtScy p 3
1.02
2.23
1.99
1.40
1.54
CD63
1.13
2.06
1.92
1.56
1.51
Counts
CD203c
NC 17
Sensitised Patient 21
wtScy p 3
wtScy p 3
red/alk-wtScy p 3
mtScy p 3
red/alk-mtScy p 3
1.02
3.37
2.21
2.07
1.62
CD63
1.13
4.70
2.98
2.21
1.94
Counts
CD203c
NC 17
Sensitised Patient 22
wtScy p 3
wtScy p 3
red/alk-wtScy p 3
mtScy p 3
red/alk-mtScy p 3
1.02
3.37
2.24
2.25
1.35
CD63
1.13
2.97
1.94
1.97
1.43
Counts
CD203c

Table S1. The mutated oligonucleotide primers of Scy p 1 and Scy p 3

Primers	Oligonucleotide sequence (5'-3')
Scy p 1-F1	***CATATG***ATGAAGCTGGAGAAGGACA
Scy p 1-R1	TACGCTCGTCCTCCTCGAGGAGCTG
Scy p 1--F2	GAGGACGAGCGTATGCGTAAGGTGC
Scy p 1--R2	***GAATTC***CAGCTCGTCGGTAATTGAC
Scy p 3-F1	***CATATG***GAGAGGGCGAAATTCGCCT
Scy p 3-R1	AGCATCTCTATGACGGCCAGGGTGG
Scy p 3-F2	TCATAGAGATGCTCAAGGTCGACGA
Scy p 3-R2	CTCAAGCTCGTACAGCTTCAGCACC
Scy p 3-F3	TGTACGAGCTTGAGCACATTCTCCT
Scy p 3-R3	***GCGGCCGC***CTCCTCGTCGCAGCAGT

CATATG, GCGGCCGC, and ***GAATTC*** restriction sites for *Nde I, Not I* and *EcoR I* enzymes, respectively.