



Insight into the mechanism of allergenicity decreasing in recombinant sarcoplasmic calcium-binding protein from shrimp (*Litopenaeus vannamei*) with thermal processing via spectroscopy and molecular dynamics simulation techniques

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ABSTRACT

In the present study, sarcoplasmic calcium-binding protein (SCP) was first expressed in *E. coli* BL21 (DE3), and then identified based on immunoblotting and SCP amino acid sequencing of shrimp (*Litopenaeus vannamei*) using mass spectrometry (MS). The recombinant SCP (rSCP) was treated with different temperature conditions to investigate its immunological properties, *in vitro* digestibility and structural changes with enzyme-linked immunosorbent assay (ELISA), immunoblotting, spectrophotometry and molecular dynamics simulation techniques. The immunoglobulin (Ig) E-binding activity of the rSCP could remain stable until 80 °C, whereas the higher thermal processing temperatures resulted in a significant decrease in IgG/IgE-binding capacity coupled with alterations in the secondary and tertiary structures. Notably, the maximum reduction of IgG/IgE reactivity and *in vitro* digestibility were observed in the autoclaved rSCP. The decrease in the potential allergenicity of rSCP not only correlated well with the decreasing of α -helix, epitopes masking and exposure of more protease cleavage sites, but also with the destruction of Ca²⁺ binding sites due to the unfolding of the rSCP with heating treatments, which was supported by the thermal-induced changes of the secondary and tertiary structures. These findings indicate that autoclaved treatment may be an effective and promising approach for producing hypoallergenic seafood.

1. Introduction

Shellfish play an important role in human nutrition and health due to the ample high-quality proteins and nutrients (Fu et al., 2019; Ruethers et al., 2018). Shellfish consumption has become increasingly popular in numerous countries with the ever-increasing international trade in seafood products. However, as the intake of crustacean shellfish increases, more people are experiencing allergic reactions to shellfish (Fu et al., 2019; Ruethers et al., 2018). Shellfish allergy can cause a long-lasting and potentially life-threatening disorder, affecting up to 2.5% of the general population worldwide (Ayuso et al., 2009; Fu et al., 2019).

Despite the high prevalence of shellfish allergy, there is no effective treatment available, and dietary avoidance is the only therapy

recommended. The majority of shellfish species that cause allergic responses are from the Crustacea class, which includes shrimp, prawn, lobster, and crab, with shrimp being the most prevalent of all shellfish (Ayuso et al., 2009; Ayuso et al., 2010). Considering the hazard of shrimp allergies, numerous shrimp allergens were identified to date including tropomyosin (TM), arginine kinase (AK), myosin light chain (MLC), sarcoplasmic calcium-binding protein (SCP), troponin C and triosephosphate isomerase (Ayuso et al., 2010; Ruethers et al., 2018). Among the aforesaid allergens, TM and SCP have been identified as the important highly heat-stable allergens in crustacean shellfish, eliciting 72–98% and 29–50% positive IgE responses in shrimp-allergic patients, respectively (Ayuso et al., 2009; Ayuso et al., 2010; Ruethers et al., 2018).

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Additionally, unlike TM was well characterized and extensively explored in various shrimps, there is still limited information regarding SCP (Ayuso et al., 2010; Han et al., 2020; Zhao et al., 2022c). Available studies indicated that the SCP, an invertebrate EF-hand calcium-binding protein, was first identified as a shellfish allergen of 20-kDa in black tiger prawn (Pen m 4) and pacific white-leg shrimp (Lit v 4) (Ayuso et al., 2009; Shiomi et al., 2008). Subsequently, its sequence (Accession number: ACM89179.1), and three IgE binding epitopes from *Litopenaeus vannamei* shrimp SCP also were identified with 53 subjects including 34 children, and 19 adults (Ayuso et al., 2009; Ayuso et al., 2010). SCP is also considered to be a highly stable allergen, which displayed common co-sensitization with TM reached up to 31% (Ruethers et al., 2018; Wai et al., 2020). The SCP isolated red swamp crayfish (*Procambarus clarkii*) and *Scylla paramamosain* had high thermal stability with slight IgG or IgE-binding reactivity reduction up to 100 °C for 30 min (Chen et al., 2013; Hu et al., 2017).

When more than one allergen is involved in hypersensitivity to a single food, the effect of thermal processing on its allergenicity is dependent on the stability of allergen as detected by the IgE. According to our recent studies, only two major proteins around 20 kDa (SCP) and 37 kDa (TM) exist in soluble extracts from different cooked shrimps (*Litopenaeus vannamei*), including boiling, baking, autoclaving, steaming, frying, and microwaving (Zhao et al., 2022a,b). Significantly, a condensable reduction in IgG/IgE binding activity was observed in the SCPs derived from various treated shrimp extracts, which may have been contributed by structural alterations. However, the structural changes and reduced IgG/IgE binding activity caused by thermal processing on the molecular level are scarce. To design an effective allergy control procedure and reduce consumer risk, the underlying causes of thermal processing on the target protein must be highlighted. Therefore, the aim of this study was to evaluate the effects of heating on structure alterations of SCP coupled spectroscopic and molecular dynamics (MD) simulation techniques.

2. Materials and methods

2.1. Materials

Ovalbumin (OVA), 8-Anilino-1-naphthalenesulfonate (ANS), porcine pepsin and trypsin were acquired from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA), isopropyl- β -D-thiogalactoside (IPTG), loading buffer, colormixed protein marker (11–180 kDa), Tween 20, 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Rabbit anti-shrimp rSCP IgG was obtained from Hubei Proteingene Technology Co., Ltd. (Wuhan, China) with titer of 2.56×10^6 . Horseradish peroxidase (HRP) conjugated goat antihuman IgE and goat anti-rabbit IgG was purchased from Zhongshan Jinqiao Biotechnology Co., Ltd (Beijing, China). All chemicals used in the study are of analytical grade.

2.2. Shrimp-allergic patient sera

Ten shrimp-allergic patient sera ($n = 10$) and two normal human sera ($n = 2$) were collected from the Affiliated Hospital of Qingdao University (Qingdao, China). The shrimp-allergic patient sera were documented by clinical allergic history, and specific IgE levels tested by the ImmunoCAP system (Phadia AB, Uppsala, Sweden) (S Table 1). A written informed consent was required from each patient, and the study was approved by the ethics committee of the Affiliated Hospital of Qingdao University (File No. QD-PF-20210325026). Two normal sera ($n = 2$) without an allergic history were used as the negative control. All positive sera ($n = 10$) were mixed into a positive pooled shrimp-allergic patient serum.

2.3. Cloning, expression, purification and identification of rSCP

The gene sequences for shrimp (*Litopenaeus vannamei*) SCP

(Accession number: ACM89179.1) were obtained from National Center for Biotechnology Information (NCBI) and synthesized for *E. coli* Top 10 expression by Tsingke Biotechnology Co., Ltd., (Beijing, China). The detailed protocols for cloning, expression, purification and identification of rSCP are described in S 2. Material and methods as [supplementary material](#).

2.4. Thermal processing of rSCP and SDS-PAGE analysis

rSCP sample was dissolved into 10 mM PBS (pH 7.4, 0.5 M NaCl) to a protein concentration of 1.0 mg/mL. Then, rSCP sample was heated at 60 °C, 80 °C, 100 °C, and autoclaved condition (0.104 MPa at 121 °C) for 15 min, respectively. The sample treated at 25 °C was considered a control. The concentration was determined using bicinchoninic acid (BCA) reagent assay kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) with BSA as standard.

Gel electrophoresis of the samples was performed as described by Zhao et al. (2019) with 5% resolving gel and 15% stacking gel. Each sample was mixed and boiled with $4 \times$ loading buffer in a 3:1 (v/v) ratio for 5 min. Afterwards, samples (2 μ g) were loaded into the lanes of the gels, and electrophoresed at 80 V for 30 min, and then at 120 V for 1 h.

2.5. Assessment of IgG/IgE-binding ability

The effects of thermal processing on the IgG/IgE-binding ability were evaluated using western-blotting, dot-blotting analysis and indirect and inhibition ELISA analysis as previously described by Zhao et al. (2022a, b). The protocols for western-blotting, dot-blotting, indirect and inhibition ELISA analysis were described in S 2. Material and methods.

2.6. In vitro digestion assay

In vitro simulated gastrointestinal digestion of rSCP was performed as previously described by Zhao et al. (2022a), and the detailed protocol was described in the S 2 Material and methods.

2.7. CD spectra analysis

The CD spectrum data between 190 and 260 nm were collected from raw and treated rSCP (0.2 mg/mL) using a Jasco-810 spectropolarimeter (JASCO, Japan Spectroscopic Co. Inc., Tokyo, Japan) at 25 °C. Each sample was scanned three times at a 1200 nm/min scanning speed with a 1 mm path length quartz cuvette. The secondary structure contents were calculated using CDNN Program (Applied Photophysics Ltd., Surrey, UK).

2.8. UV absorption spectra measurement

The UV spectrum data of raw and treated rSCP (0.2 mg/mL) was recorded from 200 to 320 nm at 0.5 nm wavelength interval using a UV-vis spectrophotometer (UV-4802, Unico Instrument Co., Ltd. USA) at 25 °C with PBS as the blank.

2.9. Intrinsic and extrinsic fluorescence spectra measurements

Intrinsic and extrinsic fluorescence spectrum of raw and treated rSCP (0.2 mg/mL) were determined using F-4600 spectrofluorometer (Horiba Ltd., Kyoto, Japan) as reported by He et al. (2020). Before ANS fluorescence measurements, each sample (2.0 mL) was reacted with 25 μ L of 8.0 mM ANS solution for 5 min. The spectrum data were obtained by intrinsic fluorescence emission between 300 and 400 nm at a 280 nm excitation, while ANS fluorescence emission between 400 and 600 nm at a 380 nm excitation. The excitation and emission slit width were set at 5 nm.

2.10. Structure modelling and calcium binding site prediction of SCP

The shrimp (*Litopenaeus vannamei*) rSCP sequence was first retrieved from the NCBI protein database using the ID: ACM89179.1, then its structure are generated using comparative modeling method with the template PDB: 2SAS.1 using Robetta service (<https://robetta.bakerlab.org/>) (Kim et al., 2004). 3D structure of the modeled SCP was visualized using Discovery Studio Visualizer Client (2019 version, Accelrys Inc., San Diego, CA, USA). The stereochemical quality assessment of the predicted model was accessed using ERRAT, PROCHECK, and Verify 3D

online tools (<https://saves.mbi.ucla.edu>).

Patch Dock server, an automatic and online server for molecular docking (Schneidman-Duhovny et al., 2005), was performed to identified the putative Ca^{2+} binding site of SCP according to the method as described by Srinivasan et al. (2020). The ligand Ca^{2+} (Substance ID: 271) was retrieved from PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). Clustering RMSD was set at 4.0 Å. The result was obtained based on the patch dock score and atomic contact energy (ACE) value.

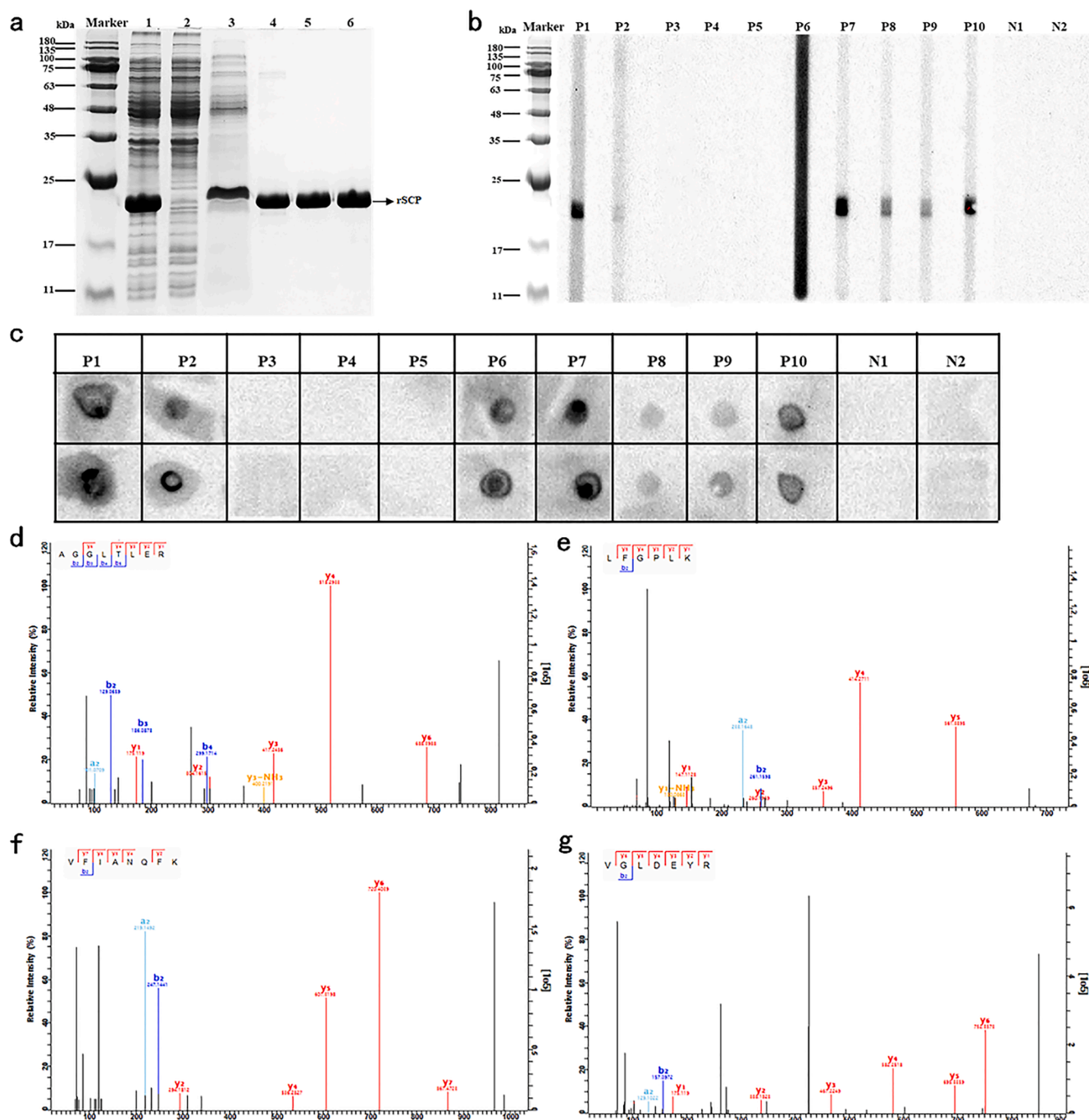


Fig. 1. Expression, purification, and identification of shrimp (*Litopenaeus vannamei*) recombinant sarcoplasmic calcium-binding protein (rSCP). (a) SDS-PAGE analysis of rSCP. Lane M: Protein marker. Lane 1: The supernatant of cell lysate. Lane 2: Effluent solution in affinity purification. Lane 3: Impurity washing solution. Lane 4: Eluted by 20 mM imidazole. Lane 5: Eluted by 50 mM imidazole. Lane 6: Eluted by 500 mM imidazole. (b) Western blotting assays (c) and dot blotting assays with 10 shrimp-allergic patient serum and 2 normal human serum. Each sample was performed in two replicates in dot blotting assays. The MS/MS spectra of the identified (d) peptide 157–164: AGGLTLER; (e) peptide 185–190: LFGPLK; (f) peptide 104–111: VFIANQFK; (g) peptide 121–127: VGLDEYR.

2.11. MD simulations analysis

All MD simulations were performed using GROMACS 2019.5 software and parameters generated by the CHARMM36 force field (Huang et al., 2017). The effect of temperature on secondary structure of the rSCP was analyzed using STRIDE algorithm implemented in visual molecular dynamics (VMD) (Humphrey, Dalke, & Schulten, 1996). Root mean square deviations (RMSD) and root mean square fluctuations (RMSF) were analyzed using GROMACS analyzing tools. The detailed protocol for MD simulations was also described in S 2. Material and methods.

2.12. Statistical analysis

All assays were carried out in triplicates and the data was presented as mean $\pm$ standard deviations. Analyses of variance (ANOVA) and Duncan's means comparison test was applied with a significance level of 0.05 by using SPSS software (version 25, SPSS Inc. Chicago, IL, USA).

3. Results

3.1. Cloning, expression and identification of rSCP

The gene sequence coding for *Litopenaeus vannamei* shrimp SCP

(Accession numbers: ACM89179.1) was synthesized and cloned into pET30a expression vector, and then the recombinant vector was transferred into *E. coli* Top 10. After IPTG induction and affinity purification, a single band (around 20 kDa) eluted by 50 mM and 500 mM imidazole was obtained with the purity higher than 95% confirmed by Image Lab software (Fig. 1a). The above eluted fractions were mixed and used to carry out the following experiments.

Western and dot-blotting assay were employed to confirm the IgE-binding reactivity of the purified rSCP with 10 positive shrimp-allergic patient sera and 2 normal human sera. As shown in Fig. 1b and c, a strong IgE binding to 7/10 shrimp-allergic patient sera was observed except for sera P3, P4 and P5, and no IgE-binding activity was detected with 2 negative control sera.

Purified rSCP was further identified by mass spectrometry. As shown in Fig. 1d-g and S Table 2, peptide mapping resulted in four peptide fragments, including 157–164: AGGLTLER, 185–190: LFGPLK, 104–111: VFIANQFK, and 121–127: VGLDEYR, which were special peptides of previously identified SCP isoforms (Accession number: ACM89179).

3.2. IgG/IgE binding capacity analysis

SDS-PAGE results indicated that rSCP remained structurally intact even when heated to 100 °C, whereas mild degradation and an increase in molecular weight (MW) occurred in the autoclaved rSCP (Fig. 2a). To

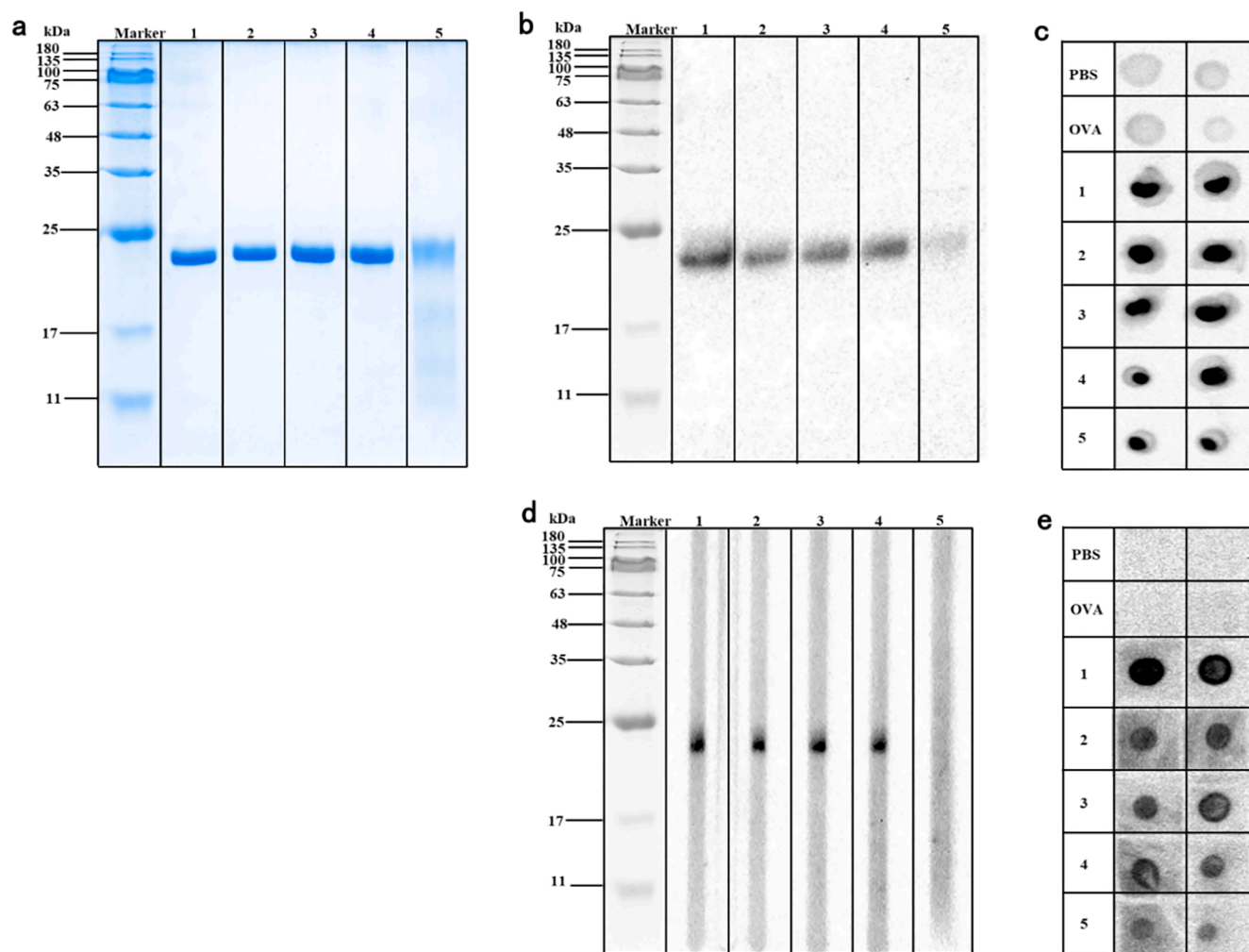


Fig. 2. The immunoblotting analysis of IgG/IgE binding ability of shrimp (*Litopenaeus vannamei*) rSCP heated at different temperatures. (a) SDS-PAGE analysis. Lane 1, 25 °C; Lane 2, 60 °C; Lane 3, 80 °C; Lane 4, 100 °C; Lane 5, Autoclaved; (b) Western blotting assays with rabbit anti-SCP sera. (d) Western blotting assays (e) and dot blotting assays with the pooled shrimp-allergic patient serum. Each sample was performed in two replicates in dot blotting assays. PBS, blank control; OVA, Negative control; Number 1, 25 °C; Number 2, 60 °C; Number 3, 80 °C; Number 4, 100 °C; and Number 5, Autoclaved.

further evaluate the alterations in rSCP IgG/IgE binding ability when treated at different temperatures, western-blotting, dot-blotting, indirect and inhibition ELISA were performed using SCP rabbit antisera and shrimp-allergic patient sera (Figs. 2 and 3). The IgG immunoblotting intensity of rSCP decreased slightly in the range of 60 to 100 °C compared with control, whereas the IgE immunoblotting intensity displayed insignificant difference. Nevertheless, an obvious decreased IgG and IgE immunoblotting intensity was observed in the autoclaved samples (Fig. 2b-e).

As shown in Fig. 3, only slight reduced IgG binding values and inhibition rate were found in the samples treated with 60 to 100 °C for 15 min (Fig. 3a and c). The raw and various heated rSCP presented varying levels IgE binding in 7/10 shrimp-allergic patient sera with negligible IgE binding to OVA, and no positive binding was found in P3, P4, P5 and two negative sera. Specifically, rSCP only demonstrated slightly reduction (5.69% to 13.10%) in IgE activity up to 80 °C in P2, P7, P8, P10, and pooled sera with insignificant changes of IgE binding values in P1, P6, and P9, whereas the IgE-binding activity began to decrease when the temperature exceeded 80 °C (Fig. 3b and d). Significantly, both IgG and IgE binding activity displayed significantly decreasing ($> 50\%$) ($P < 0.05$) in autoclaved samples.

3.3. In vitro digestibility analysis

The *in vitro* digestibility of rSCP in SGF and SIF was analyzed by SDS-PAGE (Fig. 4a and b). In the SGF digestion process, the raw and treated (80 °C) shrimp rSCP displayed similar digested trend, and two major fragments of 14 and 12 kDa were produced after 1 min digestion.

The native band (20 kDa) from the raw and treated (80 °C) rSCP were undetectable after 40 min digestion. In comparison, more diffused and wider range of digested fragments were observed in autoclaved rSCP. Significantly, native band and its digested bands from autoclaved rSCP disappeared within only 20 min digestion (Fig. 4a). For the SGF digestion process, there was a significant change in band intensity of raw and treated thermal treated rSCP with the expansion of digestion time. After 40 min, 20 min, and 1 min of digestion, the native band from untreated, treated at 80 °C, and autoclaved rSCP was no longer visible (Fig. 4b).

Inhibition ELISA assay was further performed to evaluate the IgG/IgE binding ability of digestion products from raw and treated rSCP at different digested time. As shown in Fig. 5, the IgG/IgE binding inhibition decreased gradually with the expansion of digestion time in both SGF and SIF digestion process. In comparison, the treated rSCP digested fragments, especially for autoclaved rSCP, displayed lower IgG/IgE binding inhibition. It is worth noting that even at autoclaved rSCP, the digested products after 1 h SGF/SIF digestion retained specific IgG/IgE binding capability.

3.4. Structural change analysis

3.4.1. CD spectra

As shown in Fig. 6a, two apparent negative peaks at around 208 nm and 222 nm were observed in the CD curves, which are typical characteristic of the α -helix of protein secondary structure (He et al., 2021). In contrast to the raw rSCP CD spectra, the negative intensities at 208 nm and 222 nm displayed insignificant alterations with thermal treatments up to 80 °C. There is an obvious decrease in two negative peaks

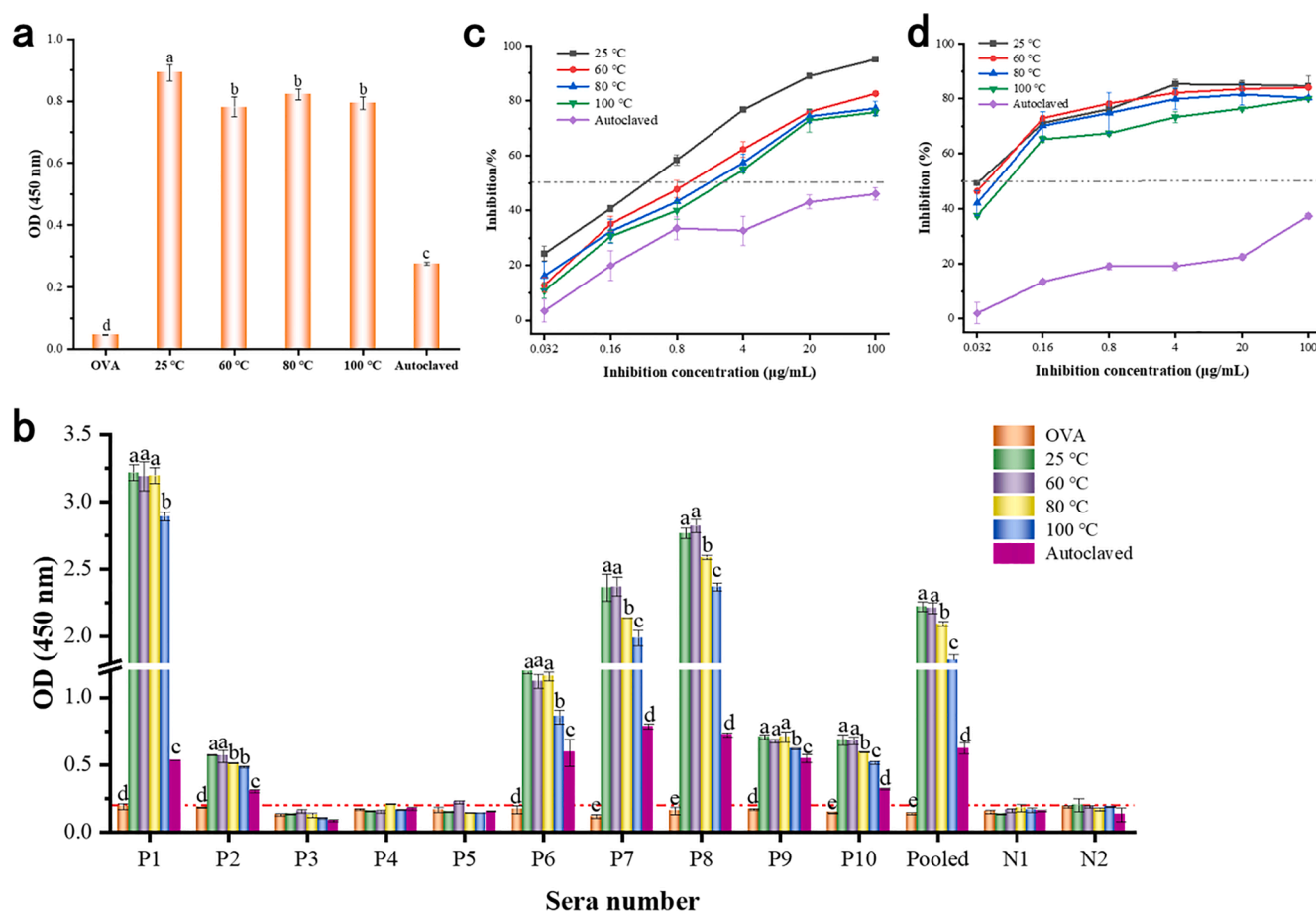


Fig. 3. The indirect and inhibition ELISA analysis of IgG/IgE binding ability of shrimp (*Litopenaeus vannamei*) rSCP heated at different temperatures. Indirect ELISA analysis with rabbit anti-SCP sera (a) and 10 shrimp-allergic patient serum and 2 normal human serum (b). Inhibition ELISA analysis with rabbit anti-SCP sera (c) and pooled shrimp-allergic patient serum (d). Different lowercase letters in column mean significant differences ($P < 0.05$) among different heat-treated samples.

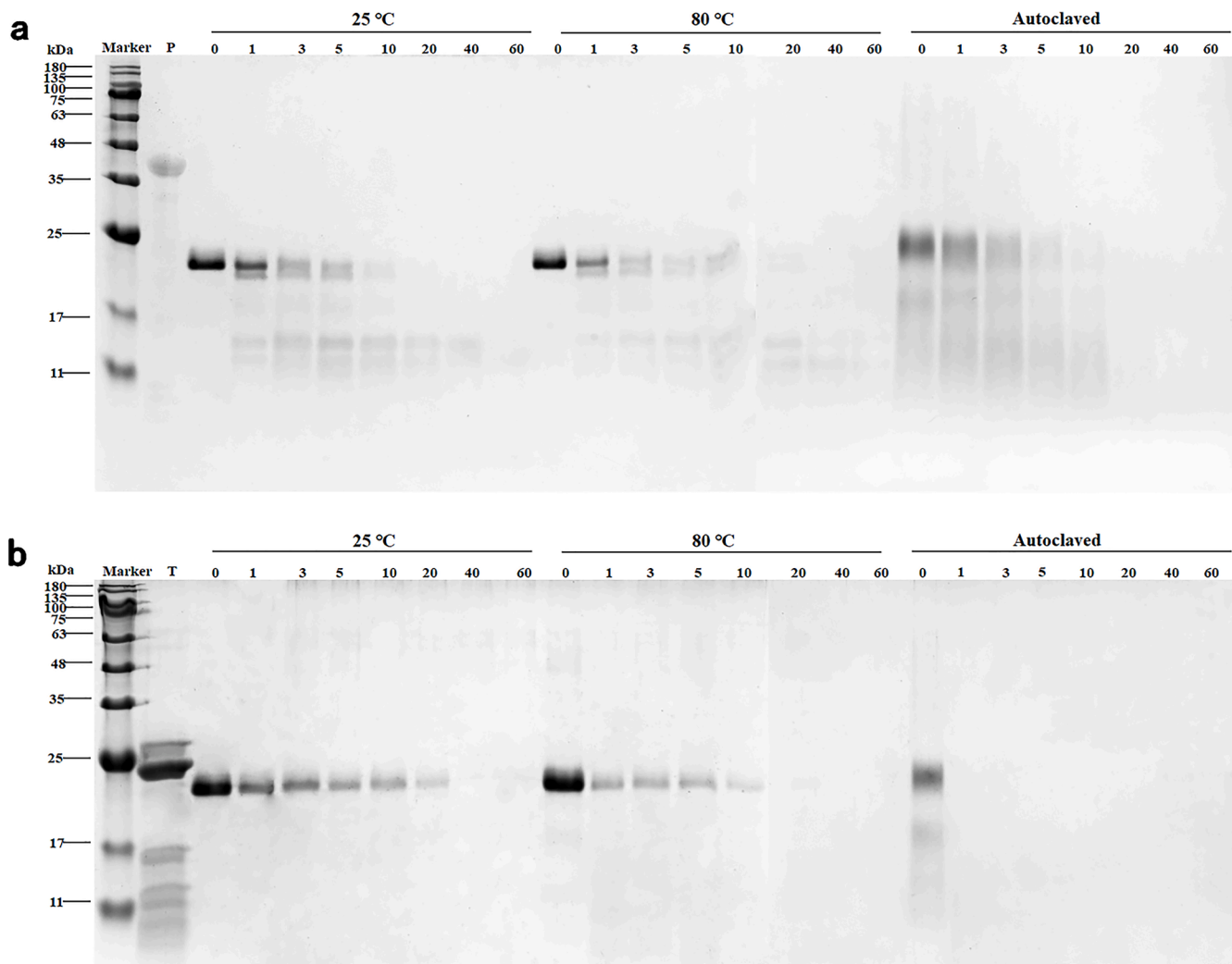


Fig. 4. The digestibility of different heat-treated shrimp (*Litopenaeus vannamei*) rSCP in simulated gastric fluids (SGF) digestion (a) and intestinal fluids (SIF) digestion (b), respectively, assessed by SDS–PAGE. Lane P, pepsin; Lane T, trypsin; Lane 0, 1, 3, 5, 10, 20, 40, and 60, SGF or SIF digestion for 0, 1, 3, 5, 10, 20, 40, and 60 min, respectively.

intensity with evident blue shift when the temperature exceeds 80 °C.

The content of various secondary structural elements in raw and different treated-rSCP was calculated using the CDNN programs. The α -helix contents of rSCP decreased from 52.5% (25 °C) to 27.1% (autoclaved) which were induced by thermal treatments, and the percentage of β -sheet, β -turn, and random coil contents decreased from 10.6% to 20.2%, 14.3% to 18.5% and 22.6% to 34.2% with the thermal processing (Fig. 6b).

3.4.2. UV absorption spectra

The maximum UV absorbance peak of raw rSCP was observed to be around 280 nm (Fig. 6c). An obvious increased absorbance intensity in the maximum absorbance peak without any shift was found with temperature increasing. The autoclaved shrimp rSCP showed the maximum UV absorption intensity.

3.4.3. Intrinsic fluorescence spectra

The raw shrimp rSCP showed the maximum fluorescence intensity (3562 a.u.) at 332 nm (Fig. 6d). With gradually increased temperature, treated rSCP presented an apparent decrease in the maximum fluorescence intensity along with significant red shifts (332–337 nm) in the maximum fluorescence emission ($\lambda_{\max}$). Autoclaved rSCP displayed the lowest fluorescence intensity at $\lambda_{\max}$ 337 nm.

3.4.4. Extrinsic fluorescence spectra

The untreated rSCP with minimum ANS fluorescence intensity at $\lambda_{\max}$ 488.6 nm. The treated rSCP showed a significant increase in the ANS fluorescence intensity without obvious shift in $\lambda_{\max}$ compared to the unheated rSCP (Fig. 6e). The maximum fluorescence intensity was observed at autoclaved rSCP.

3.5. Modeled structure of SCP

SCP displayed six helix-loop-helix motifs, typical structural features of EF-hand proteins, which is the potential calcium ions (Ca^{2+}) binding sites (Fig. 7a and b). The quality of the predicted modeled SCP was checked by Ramachandran plots, ERRAT and VERIFY3D tools. As shown in Fig. 7c, 99.4% residues in the most favored and allowed regions with only 0.6% residues in the disallowed regions were found in the Ramachandran plot. Verify3D indicated that 86.01% of the residues have averaged 3D-1D score ≥ 0.2 (Fig. 7d). Further, a high overall quality factor of 99.4595 was obtained by ERRAT analysis (Fig. 7e). Based on the foregoing structure assessment programs, the modeled rSCP was found to be a structure of high acceptability.

3.6. Putative Ca^{2+} binding site

Modeled SCP interactions with Ca^{2+} ions were analyzed based on the

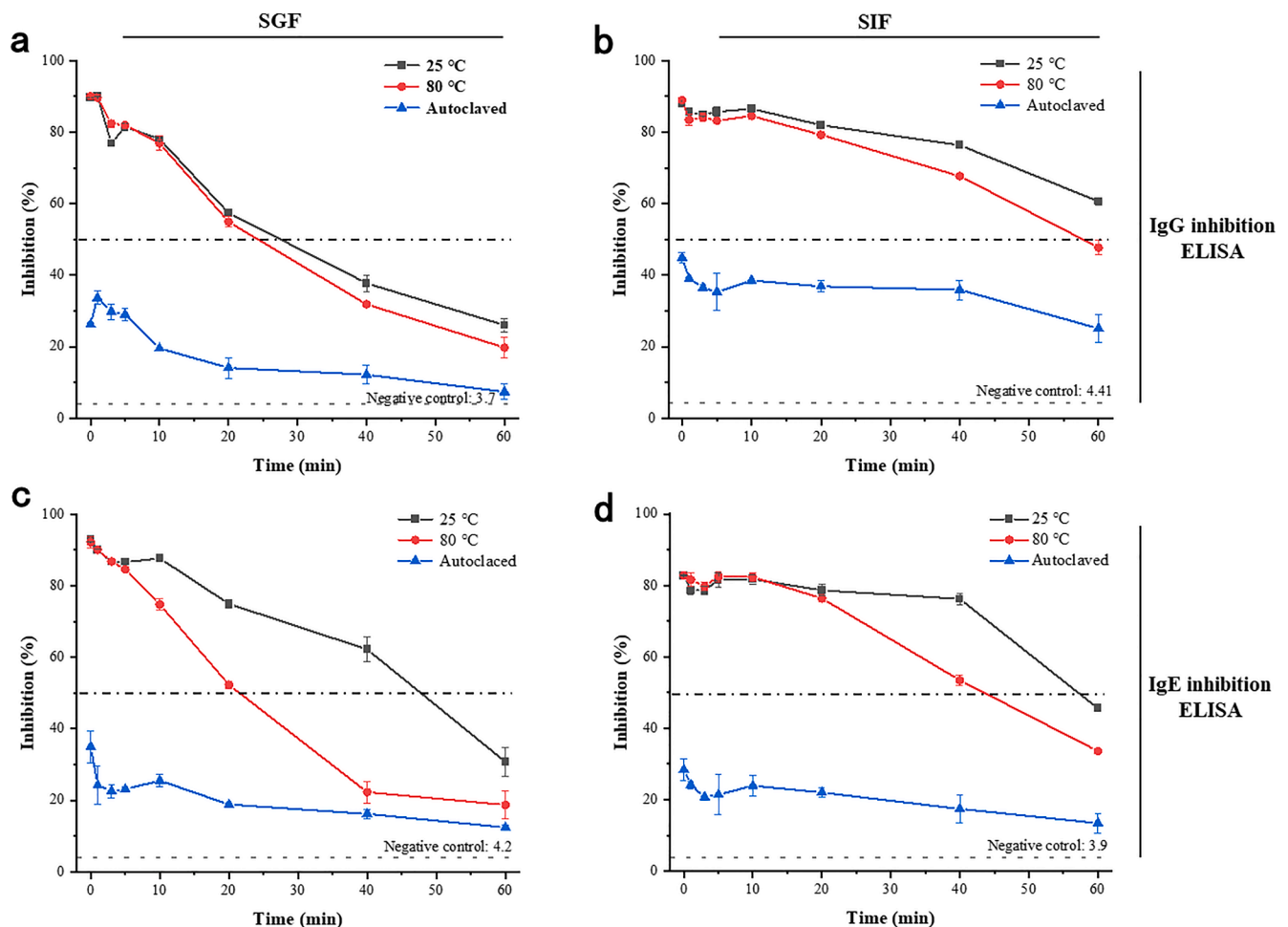


Fig. 5. Inhibition-ELISA analysis of the different digested fragments of shrimp (*Litopenaeus vannamei*) rSCP treated at different temperatures. IgG inhibition-ELISA analysis of SGF (a) and SIF (b) digested fragments; IgE inhibition-ELISA analysis of SGF (c) and SIF (d) digested fragments.

patch dock score and ACE value for the possible interaction (Table 1). The obtained top ten highest docking scoring alignments and the specific charged-charged interactions between Ca^{2+} and its surrounding amino acid residues were shown in Fig. 8. According to the molecular docking results, all Ca^{2+} ions docked with modeled SCP via charge-charge interaction, except for Complex No. 2, 5, 9 and 10. Therefore, Complex No. 1, 3, 4, 6, 7 and 8 were considered to be the putative Ca^{2+} binding sites of SCP.

3.7. MD simulations analysis

3.7.1. RMSD and RMSF

The RMSD of the protein backbone reached equilibrium within 5 ns at 25 °C with an average value of 0.2536 Å (Fig. 9a). With the simulated heating temperature increasing, although the equilibrium time displayed insignificant changes, considerable increase in the RMSD were found in various heating temperature conditions. For 60 °C, 80 °C, 100 °C and autoclaved SCP, the RMSD increases to an equilibrium value around 0.3286, 0.3735, 0.3758 and 0.4349 Å.

RMSF values of raw and various heated rSCP were plotted to evaluate the residue fluctuation regions of protein (Fig. 9c). An obvious alteration of fluctuation upon the temperature was observed, and the obvious fluctuation was observed with the increasing temperature, where much higher RMSF values were found in the autoclaved SCP with more trypsin cleavage site exposed (Fig. 9e). If the cut RMSF value was set at 0.20 Å, the positive predictive values to trypsinolysis for the susceptibility of target cut sites in rSCP are 8.69%, 21.74%, 21.74%,

34.78% and 39.13%, respectively, in various temperature conditions (25 °C, 60 °C, 80 °C, 100 °C and autoclaved). Moreover, all epitope regions, especially for E2: 49–72, and putative Ca^{2+} binding sites displayed higher RMSF values in higher temperature conditions (100 °C and autoclaved conditions) (Fig. 9c and S Fig. 1). Additionally, the increased steric hindrance with the reduction of α -helix content in IgE epitope regions was observed in structures of the MD simulated SCP in 80 °C and autoclaved conditions at the end of 20 ns MD simulations as compared to the MD simulated SCP in 25 °C (Fig. 9d).

3.7.2. Secondary structure

The analysis of time-dependent secondary structure fluctuations was performed using VMD software. The output was present in form of a graph with amino acid residues on the Y axis and the time on X axis as shown in the Fig. 10. Each color band corresponding to the residues represent the secondary structure orientation of the residue with respect to the duration of simulation. The rSCP molecule with a total of 193 amino acid residues contains 60.62% as α -helix, 4.15% as β -sheet, 12.95% as β -turn, and 22.28% as random coil. From Fig. 10, it displayed that heating caused obvious alterations in secondary structure contents between residues 90–99 and residues 117–122 with an increase in temperature from 25 to 60 °C. As the temperature increased further, more deviations in coil structures were observed in residues 18–22, 70–78, 90–100, and 150–160. Additionally, there were considerable disruptions between 116 and 160 residues which were mostly stable in the simulation conditions of 100 °C and autoclaving.

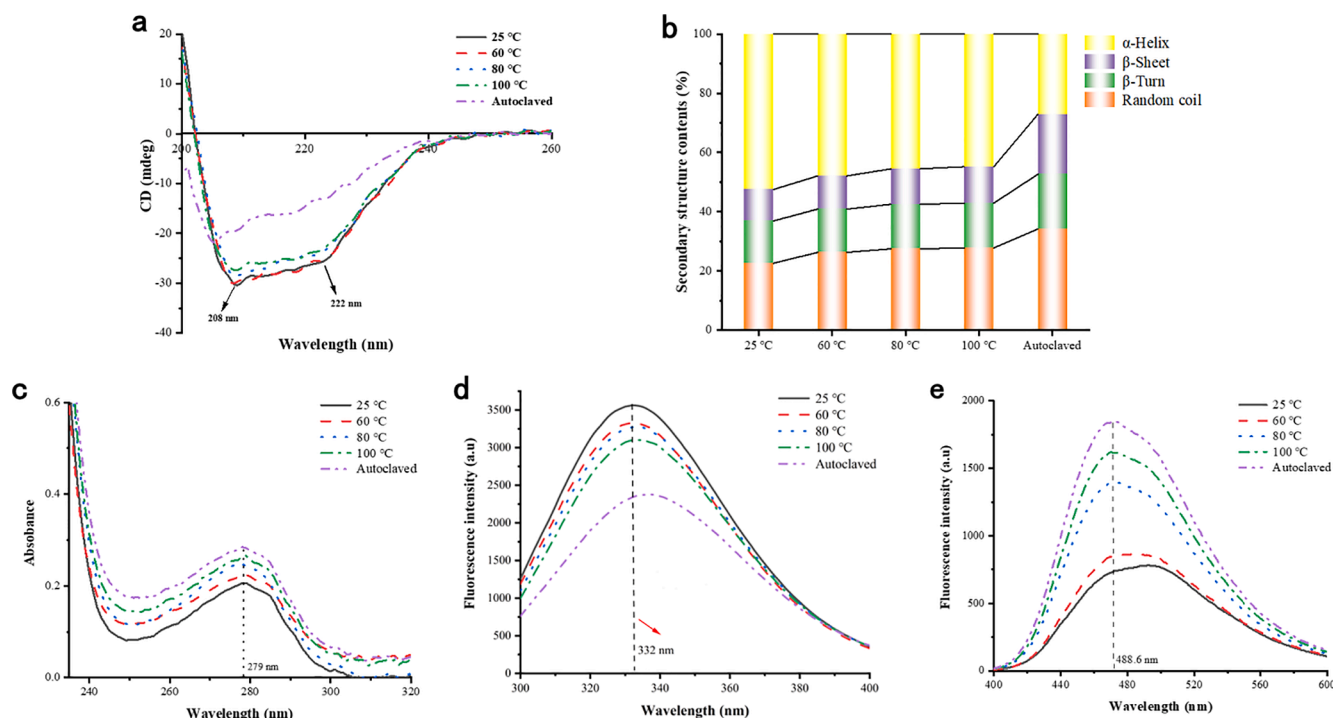


Fig. 6. The secondary and tertiary structures analysis of different heat-treated shrimp (*Litopenaeus vannamei*) rSCP using spectroscopic techniques. CD spectra (a) and secondary structural contents of rSCP at different temperatures (b); Tertiary structures changes of rSCP at different temperatures using UV spectra (c), intrinsic fluorescence spectra (d), and extrinsic fluorescence spectra analysis (e).

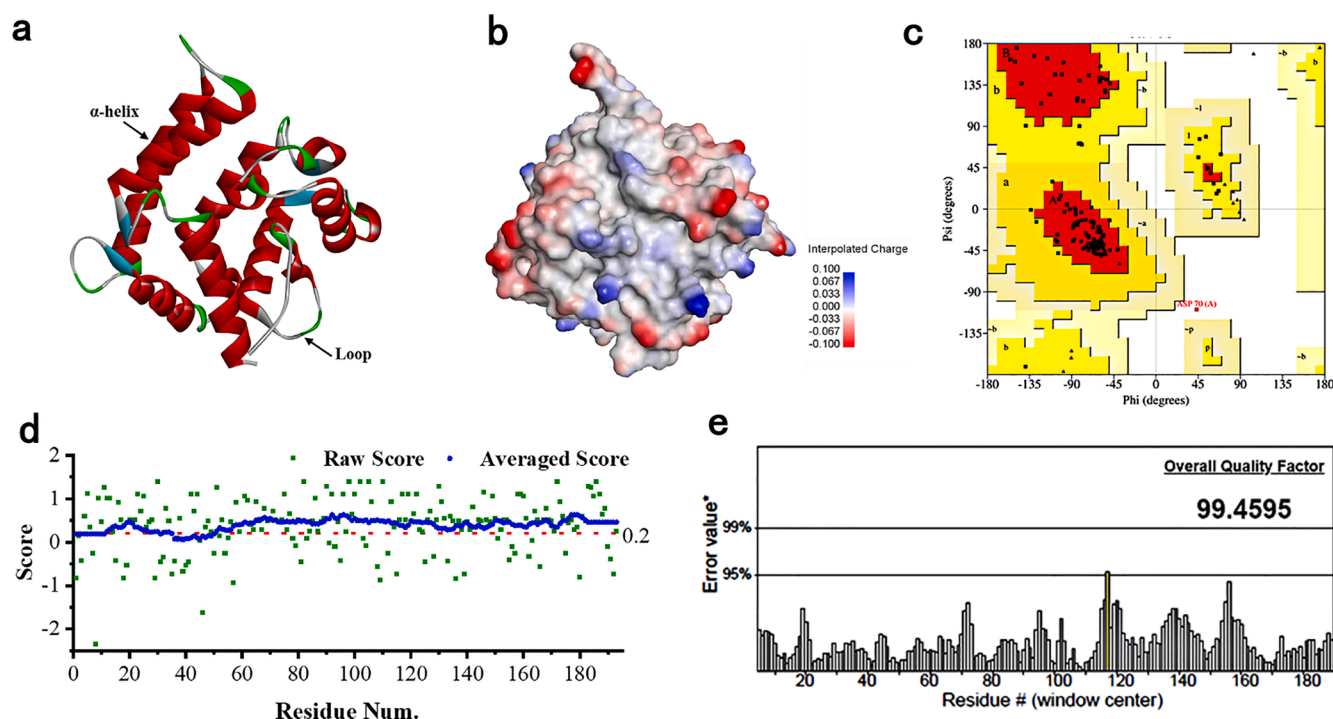


Fig. 7. The modeled 3-D structure and stereochemical quality evaluation of shrimp (*Litopenaeus vannamei*) SCP. (a) 3-D ribbon structure (b) interpolated charge structure of SCP predicted by Robetta server (<http://rosetta.bakerlab.org/>) with the comparative modeling method. Stereochemical quality evaluation of predicted SCP using various online tools, including Ramachandran plot (c), Verify 3D (d), and ERRAT (e) (<https://saves.mbi.ucla.edu>).

4. Discussion

Crustaceans, highly allergenic foods, can elicit profound allergic reactions among sensitized individuals (Ruethers et al., 2018). Despite the fact that shrimps are generally subjected to some level of thermal

treatments in our daily lives, processed shrimps might nevertheless produce an allergic reaction. According to the recent study, shrimp TM and SCP retained their extractability and IgE/IgG reactivity even after different cooking methods, which is likely the cause of allergic sensitization to both raw and cooked shrimps (Zhao et al., 2022b,c). However,

Table 1

Putative Ca²⁺ binding site predicted based on molecular interaction of modeled SCP with Ca²⁺ ions.

Complex No.	Dock scores	Area size	ACE value (kcal/Mol)	Charge interaction residues
1	932	104.7	-9.12	Tyr95, Gly96, Lys103, Asp167, Ala170, Gln171
2	930	110.4	-15.94	–
3	914	102.8	-0.99	Glu41, Arg43, Ala48, Ala107, Lys111, Asp114
4	904	98.2	-16.85	Val13, Arg14, Asp18, Asn21, Asn22, Gly23
5	834	96.3	-16.40	–
6	784	85.7	-9.82	Leu149, Thr151, Asp 154, Arg164, Asp167, Leu168
7	724	80.7	-8.99	Phe24, Leu25, Asp26, Asn72, Thr78, Glu81
8	724	80.3	-5.05	Arg133, Glu141, Pro188
9	718	79.5	-6.46	–
10	718	78.3	-9.38	–

–, No charge-charge interaction formed.

ACE, atomic contact energy.

despite the high prevalence of crustacean allergy among both children and adults, there is a lack of thorough study of some allergen characteristics during thermal treatments, especially for SCP.

In the present study, the SCP (*Litopenaeus vannamei*) was first expressed, purified and identified with 10 shrimp-allergic patient sera and 2 normal human sera. The MW of the purified rSCP (around 20 kDa) is in good agreement with previously identified SCP from *Litopenaeus vannamei* (Ayuso et al., 2009), *Penaeus monodon* (Shiomi et al., 2008), and *Scylla paramamosain* (Hu et al., 2017) (Fig. 1a). Additionally, the 20 kDa purified protein was recognized by serum IgE from 7 of 10 (70%) shrimp-allergic patient according to western- and dot-blotting results (Fig. 1b-c), and mass spectroscopic analysis demonstrated four peptides matched SCP from *Litopenaeus vannamei* shrimp (Fig. 1d-g). Some previous studies also indicated that the SCP allergen showed positive IgE binding in more than 50% of shellfish-allergic patients (Ayuso et al., 2009; Shiomi et al., 2008), and it may play a more significant role in causing severe allergic reaction than TM in some shellfish-allergic patient (Ayuso, 2011; Cheng et al., 2021). The above results confirmed that the 20 kDa purified protein was rSCP from *Litopenaeus vannamei* shrimps and had the same IgE reactivity as natural SCP (nSCP) of *Litopenaeus vannamei* shrimp, which could be attributed to the fact that rSCP in *E. coli* may have a similar folded protein structure as nSCP (Hu et al., 2017; Liu et al., 2021). Similarly, rSCP from pacific oyster (*Crassostrea gigas*) (Cheng et al., 2021), mud crab (*Scylla paramamosain*) (Hu et al., 2017), and black tiger shrimp (*Penaeus monodon*) (Mita et al., 2013) using *E. coli* expression system also has the same IgE reactivity as nSCP. Recombinant allergens are now regarded as a useful tool for investigating shellfish allergens, and producing diagnostic and immunotherapeutic agents for shellfish allergy subjects (Ayuso et al., 2009; Ayuso et al., 2010).

Allergens could get access to the human immune system depending on their stability to heat and digestive enzyme, mainly pepsin and trypsin (Pekar, Ret, & Untersmayr, 2018). Most food allergens can preserve their native three-dimensional pattern after chemical, physical or protease treatment over time (Cheng et al., 2021; Pekar et al., 2018; Ruethers et al., 2018). According to SDS-PAGE examination, the rSCP from shrimps (*Litopenaeus vannamei*) was stable until 100 °C and began to appear a dispersed band in the autoclaved rSCP (Fig. 2a). The results demonstrated the rSCP structure was much heat-stable. Similarly, our previous findings indicated that numerous proteins from shrimps (*Litopenaeus vannamei*) would be undissolved because of thermal treatments applied except of TM and SCP, including boiling, oven baking (180 °C), autoclaving (0.104 MPa at 121 °C for 15 min), steaming, deep frying, and microwaving, signifying they possessed high thermostability (Zhao

et al., 2022a,b). Similar thermostability were also confirmed in other various boiled crustacean shellfish extracts (Kamath et al., 2013; Nugraha, et al., 2021), which might make its role in shellfish-induced allergic reactions even more significant because shellfish usually are consumed cooked.

Subsequently, the IgG and IgE reactivity of rSCP with thermal treatments were analyzed by dot- and western-blotting assays, indirect ELISA and inhibition ELISA with rabbit anti-SCP sera and shrimp-allergic patient sera, respectively. Results demonstrated that IgG binding capacity of rSCP began to decrease at 60 °C, while no significant difference was observed in IgG reactivity in range of 60 °C to 100 °C (Figs. 2 and 3). Nevertheless, IgE-binding capacity displayed a marked decline (5.69% to 13.10% depending on different sera) ($P < 0.05$) up to 80 °C, suggesting that rSCP could retain their native tertiary structure till 80 °C. Previous findings also indicated that crustacean shellfish SCP most probably induce allergic sensitization to both raw and cooked shellfish, because it can maintain its IgG/IgE-binding capacity after various cooking treatments (Zhao et al., 2022a,b). Significantly, autoclaved treatment induced a more than 50% of reduction in IgG and IgE-binding capacity. A strong heat environment may elicit considerable conformational changes, terminating epitope surface exposure and causing a reduction in surface accessibility and flexibility for IgG and IgE recognition (Davis et al., 2001; Mills & Mackie, 2008; Zhao et al., 2019).

Shrimp or shrimp-derived foods are usually consumed after cooking, followed by gastrointestinal digestion with proteases. One of the main characteristics of allergen eliciting an allergic response via the gastrointestinal tract seems to be resistance to gastrointestinal digestion, that allows food proteins to retain intact epitopes that have the potential to bind IgE and trigger the sensitization (Pekar et al., 2018). The rSCP showed significantly different digestive patterns between the SGF and SIF. In comparison, much more digested fractions were observed in the range of 10 kDa to 20 kDa during SGF digestion process, while no minor digested fractions were found below 20 kDa in SIF digestion according to SDS-PAGE (Fig. 4 a and b). Similar digested pattern also could be observed in the investigation of SCP from pacific oyster (*Crassostrea gigas*) (Cheng et al., 2021). This mainly be attributed their different protease cleavage sites. Specifically, pepsin tends to cleave peptide bonds following phenylalanine (Phe) or tyrosine (Tyr), and trypsin mainly cleaves peptide bonds following lysine (Lys) or arginine (Arg) residues with higher specificity (Cheng et al., 2021). Furthermore, when compared to raw shrimp rSCP, the rSCP treated with thermal processing degraded quicker as temperature increased in both the SGF and SIF digestion processes, particularly in the SIF digestion process. Significantly, autoclaved shrimp rSCP showed the fastest degradation, and the main digested band was incompletely detected within 20 min of SGF digestion and 1 min SIF digestion, respectively, implying increased digestibility of rSCP because of the heat treatments. This phenomenon might be attributed to that heat-induced structural alterations with the exposure of more protease cleavage sites to make the protein more susceptible to proteolytic attack (Pekar et al., 2018; Zhao et al., 2019, 2022a).

To further examine the IgG/IgE-binding capabilities of rSCP-derived digestion fragments, inhibition ELISA was performed with rabbit anti-SCP sera and pooled shrimp-allergic patient sera. In comparison with undigested samples, the IgG/IgE reactivity of rSCP notably decreased with a prolonged digestion time (Fig. 5), which indicated that many conformational and linear epitopes of SCP could be degraded by protease due to the multiple accessible sites of SCP to pepsin/trypsin (Zhang et al., 2017). Allergens always remain relatively intact in gastrointestinal digestion and induce sensitive. However, there were still a relatively high IgG/IgE binding capacity in 1 hour's SGF and SIF digested fragments compared to negative control, which might be due to the presence of intact proteins or epitopes in the final digestion solutions (Chen et al., 2013; Pekar et al., 2018).

The secondary structural alterations of shrimp rSCP subjected to heat treatments was monitored by CD spectra, two obvious negative bands

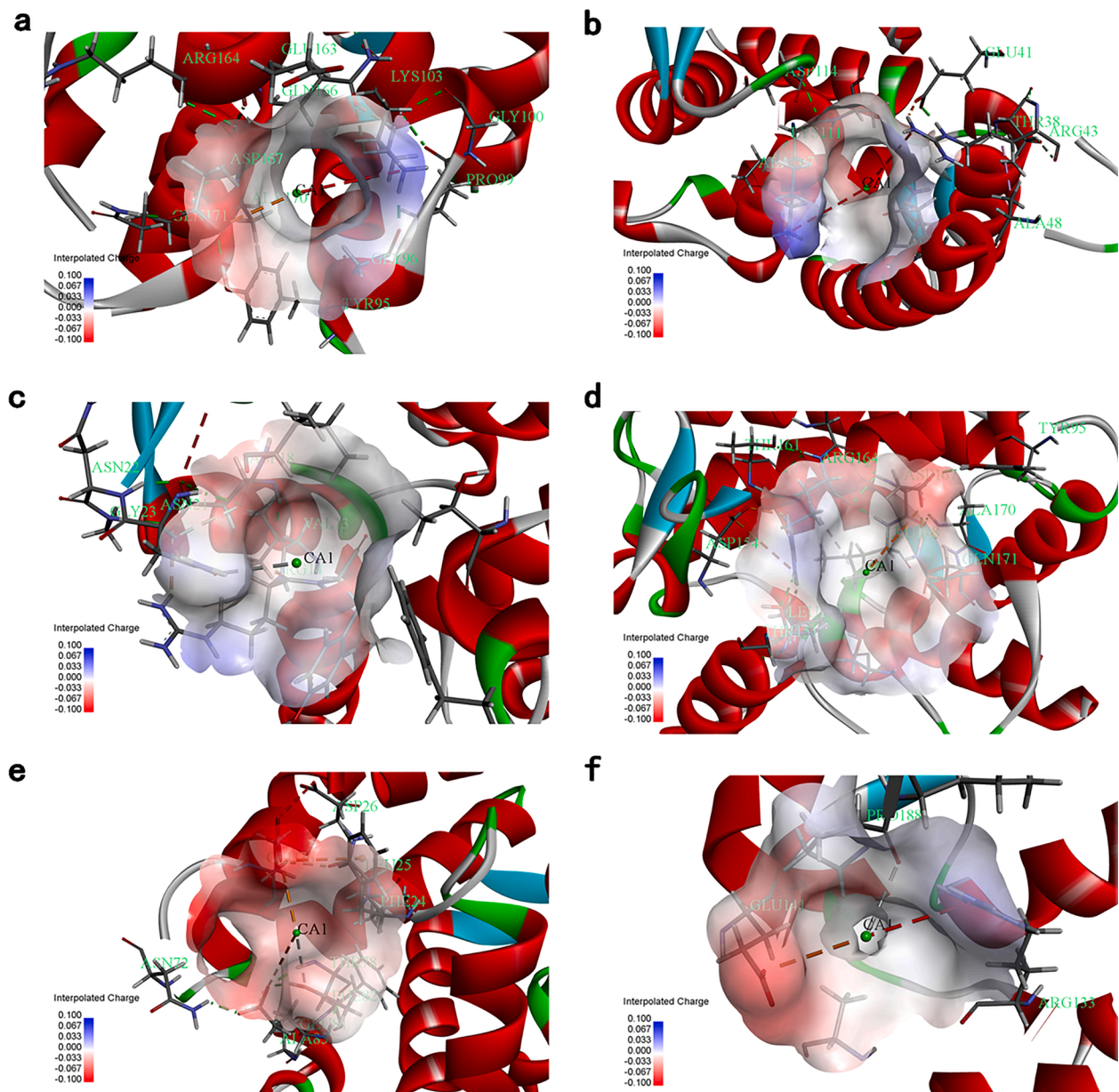


Fig. 8. Putative Ca^{2+} -binding site of 3-D modeled structure of shrimp (*Litopenaeus vannamei*) SCP. The receptor interaction with ligand (Ca^{2+}) is represented by Bright orange color by charge-charge interaction. Ca^{2+} labeled as CA1 and its surrounding amino acid residues were highlighted in green color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

around 208 and 222 nm, which were typical characteristic of the α -helix of protein secondary structure, were found in raw and different heated shrimp rSCP (He et al., 2020) (Fig. 6a). The α -helix contents of SCP significantly decreased with the temperature increasing till to 80 °C accompanied with increased percentage in β -sheet, β -turn, and random coil contents, and the percentage of α -helix was reduced to 27.1% in the autoclaved rSCP (Fig. 6b). These alterations in secondary structure were also confirmed by the following MD analysis results. Similar changes in secondary structure also were found in the investigation of thermal treated SCP from pacific oyster (*Crassostrea gigas*) and mud crab (*Scylla paramamosain*) (Cheng et al., 2021; Hu et al., 2017). It is noteworthy that the rSCP structural rearrangement might cause significant changes in tertiary structures of rSCP. Thus, the structural changes of heat-treated shrimp rSCP were also accessed via UV absorption spectra, intrinsic and extrinsic fluorescence spectra analysis. Shrimp rSCP exhibited significantly increased absorbance intensity in the maximum UV absorbance peak, decreased intrinsic fluorescence intensity, obvious red

shifts at λ_{max} , and clearly increased ANS fluorescence intensity with increasing temperature (Fig. 6c-e). These results suggested that more hydrophobic amino acid located in the protein interior exposed on the surface of protein resulting in the unfolding of the proteins because of the thermal processing, especially in the autoclaved shrimp rSCP (Lin et al., 2015; Zhao et al., 2019). Based on the basic role of epitopes forming, the hydrophobic regions seemed unlikely to be the epitopes of allergens (Zheng et al., 2011). Thus, these hydrophobic residues exposure might cause the epitopes masking, and induce shrimp rSCP unrecognized by IgG/IgE (Davis, Smales, & James, 2001; Zhao et al., 2022a). Combining the previous experimental findings, these structural modifications could result in the rSCP structural rearrangement and unfolding, which would bury or destroy some epitopes, and expose more protease cleavage sites, resulting in decreased allergenicity and digestion resistance (Cheng et al., 2021; Pekar et al., 2018).

A definite 3-D structure can provide more insights into understanding the structure and physicochemical properties of allergen. Since the

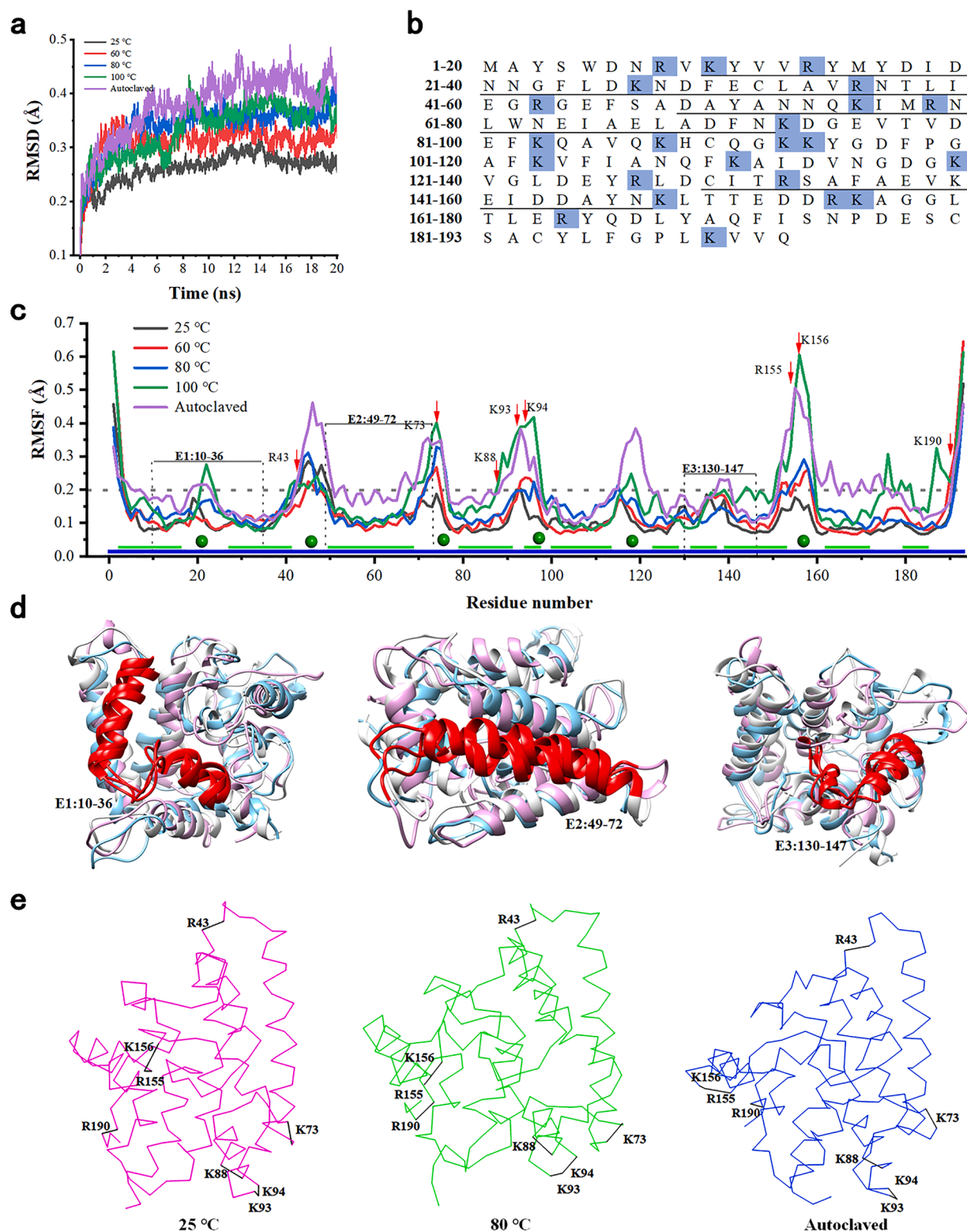


Fig. 9. RMSD, RMSF, trypsinolysis, and epitopes analyses of shrimp (*Litopenaeus vannamei*) SCP in different temperature environments during 20 ns MD simulation. (a) RMSD deviations observed in modelled SCP 3-D structure at different temperatures; (b) Complete amino acid sequence of shrimp (*Litopenaeus vannamei*) SCP (NCBI Accession Number: ACM89179). The IgE epitopes identified by Ayuso et al. (2010) are underlined. Trypsin cleavage sites were highlighted with light blue background; (c) RMSF curves showing the variations in the backbone residues of rSCP at different temperatures. Arrows represent the trypsinolysis of the RMSF. Green lines represent the α -helix regions. Green solid balls (●) represent the putative Ca^{2+} binding sites; (d) 3-D structures changes of rSCP at different temperatures depicting the epitopes. E1: 10–36, E2: 49–72, E3: 130–147. The structures of white, purple and pink are the simulated SCP in 25 °C, 80 °C and autoclaved conditions at the end of 20 ns MD simulations, the epitope regions were highlighted with red color; (e) Trypsin cleavage sites variations of the SCP in 25 °C, 80 °C and autoclaved conditions at the end of 20 ns MD simulations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

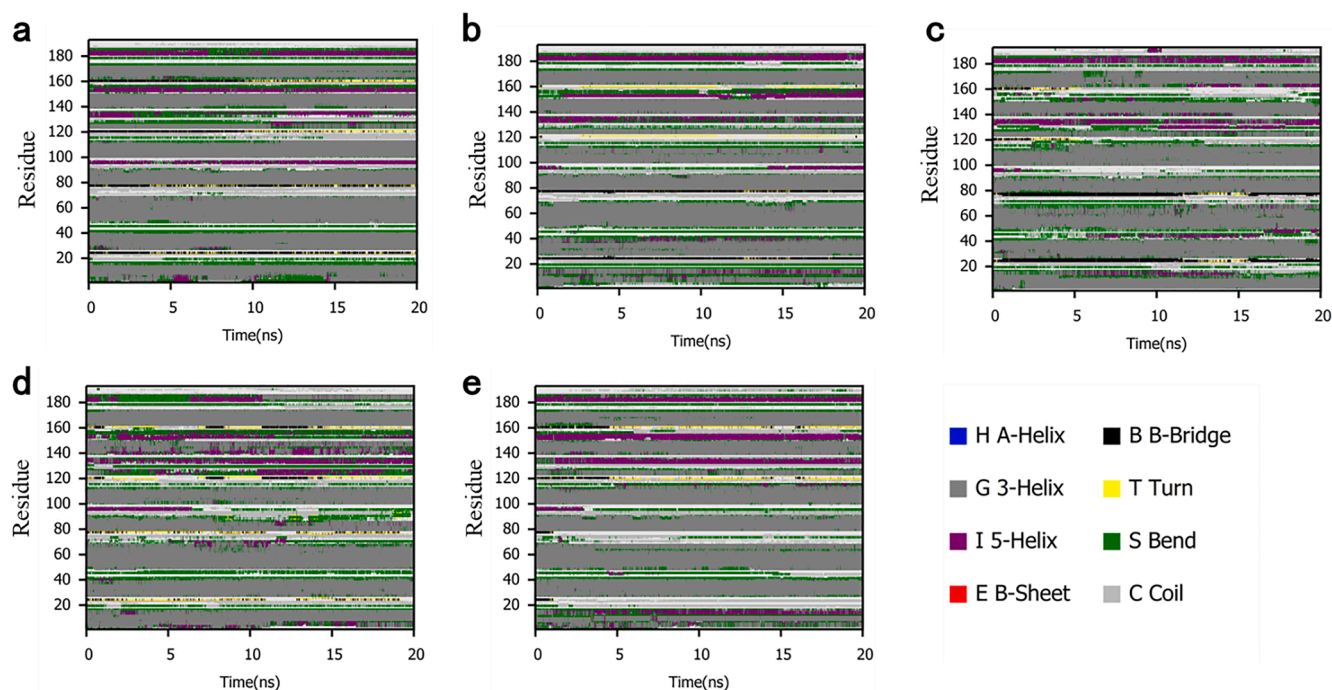


Fig. 10. STRIDE analysis showing the secondary structure changes of shrimp (*Litopenaeus vannamei*) rSCP in different temperature environments during 20 ns MD simulations. (a) 298.15 K (25 °C), (b) 333.15 K (60 °C), (c) 353.15 K (80 °C), (d) 373.15 K (100 °C), and (e) 394.15 K at 1.04 bar (autoclaved).

crystal structure of shrimp SCP were not successfully solved yet, a bio-informatics algorithm Robetta service (<https://robetta.bakerlab.org/>) was applied for obtaining its 3-D structure (Kim et al., 2004). The stereochemical evaluation by ERRAT, PROCHECK, and Verify 3D demonstrated the predicted SCP 3-D structure have high quality and acceptability (Fig. 7). SCP belongs to the family of Ca^{2+} -binding proteins that are identified by the presence of EF-hand motifs (helix-loop-helix motifs). The Ca^{2+} binding site in SCP plays a significant role in biological functions (Cheng et al., 2021; Han et al., 2021). To identify the relevant EF hand family allergens, it is necessary to understand the influence of thermal processing on the Ca^{2+} binding site, its IgG/IgE reactivity, and digestibility. Six putative Ca^{2+} binding site of SCP were identified using the molecular docking method with Patch Dock server in the present study (Fig. 8) (Schneidman-Duhovny et al., 2005). All the predicted Ca^{2+} binding sites located in the loop regions of SCP with the ionic force interactions between Ca^{2+} and its surrounding amino acid residues. Oxygen is the coordinating atom of choice and consequently EF-hands, and in particular EF-loops, are rich in the negatively charged amino acids, including Asp and Glu (Gifford et al., 2007). A previous report indicated that Asp is the most frequently used amino acid residue in Ca^{2+} -binding sites, and could be employed as the anchor ligand (Huang et al., 2007). Five of six predicated Ca^{2+} -binding sites contain Asp, and three of six predicated sites include Glu, which indicated the high accuracy in the prediction of Ca^{2+} -binding sites.

The MD simulations were performed in different temperature environments for 20 ns for further characterizing the structural changes of rSCP, especially in epitopes, Ca^{2+} -binding sites and protease cleavage sites, responsible for the decreased IgG/IgE binding ability with improved *in vitro* digestibility (Fig. 9). RMSD was first calculated for monitoring distance deviations during 20 ns MD simulations. Significantly increased backbone RMSD was observed under high temperature conditions (Fig. 9a). The final RMSD value increased 29.57% at 60 °C, 47.28% at 80 °C, 48.19% at 100 °C, and 71.49% at autoclaving conditions of RMSD at 25 °C when reached the simulated equilibrium at around 5 ns, which confirmed the 3-D structural alterations in high temperature conditions. Although similar studies are scarce, the increase of RMSD values was also observed in the peanut allergen Ara h 6,

bovine β -lactoglobulin and soybean trypsin inhibitor MD simulations at high temperature conditions (Euston, 2013; Vanga et al., 2015, 2018), which confirmed the structure unfolding due to high temperature. According to STRIDE analysis, the SCP MD simulation results in different temperatures also revealed a similar decrease in α -helix structures along with a simultaneous increase in the β -sheet, β -turn and random coil contents with temperature increasing, especially in such residues regions: 18–22, 70–78, 90–100 and 150–160 (Fig. 10). Besides, the results from MD simulations are in accordance with the above spectroscopic analysis. The thermal processing could induce the breaking of hydrogen bonds between the amino terminal hydrogen (NH–) and carbonyl terminal oxygen (–C=O), which probably play an important role in the formation and stabilization of the helical structure of SCP (He et al., 2021; Scholtz & Baldwin, 1992). A similar decrease in α -helix with reduced antigenicity under heating conditions, was also observed in thermal-treated rSCP from *Scylla paramamosain* and pacific oyster (*Crassostrea gigas*) (Cheng et al., 2021; Hu et al., 2017). The helical structure might play a critical role in antigenicity and digestibility, because it can significantly influence protein's 3-D structure, resulting in a corresponding alteration in epitopes, protease cleavage sites and Ca^{2+} -binding sites. Previous research found that the quantity of α -helices in the peanut allergen Ara h 2 and chicken ovalbumin decreased with antigenicity after heat treatments, implying that epitopes may be eliminated or hidden as a result of the loss of α -helical structure (Hu et al., 2011; Stănciuc et al., 2016; Zhang et al., 2016).

Thus, the variation of trypsin-sensitive target sites, three IgE epitopes of SCP reported by Ayuso et al. (2009) and putative Ca^{2+} binding site, were monitored according to RMSF analysis (Fig. 9). Our findings showed that under heating conditions, most trypsin cleavage sites, epitope regions and putative Ca^{2+} binding site displayed higher RMSF values in higher temperature conditions, especially at 100 °C and autoclaving conditions. The positive predictive values to trypsinolysis for the susceptibility of target cut sites in rSCP increased to 60.03% (60 °C), 60.03% (80 °C), 120.01% (80 °C) and 140.02% (autoclaving condition) of native control (25 °C), respectively, suggesting more trypsin cleavage sites were exposed because of the structure unfolding, thereby rendering the rSCP would be more susceptible to proteolytic

attack (Apostolovic et al., 2016; He et al., 2021). Furthermore, the putative Ca^{2+} binding site from thermal-induced rSCP showed considerable modifications, which would increase the potential of epitope degradation or unmasking, and hence impair IgG/IgE recognition.

5. Conclusions

Taken together, we first confirmed that significantly decreased potential allergenicity and improved digestibility will take place in heat-treated rSCP, and then elucidated its mechanisms. The degree of reduced IgG/IgE activity and enhanced digestibility varied according to the heating temperature applied, with autoclaved treatments showing the greatest reduction in IgG/IgE binding capability and digestion stability. This phenomenon was well correlated with the decreasing of α -helix, exposure of more protease cleavage sites and the destruction of Ca^{2+} binding sites, due to the unfolding of the rSCP subjected to heating treatments. Autoclaving may be an efficient method to reduce the allergenicity of shrimp SCP, and future studies could pay more attention to the *in vivo* experiments for examining the allergenicity and digestibility changes of SCP.

CRediT authorship contribution statement

Jinlong Zhao: Investigation, Methodology, Writing – original draft. **Wenye Zhu:** Validation, Resources. **Jianhua Zeng:** Validation, Resources. **Yuhai Liu:** Validation, Resources. **Hong Li:** Writing – review & editing, Formal analysis, Validation, Resources. **Hao Wang:** Validation, Resources, Methodology, Writing – review & editing, Supervision. **Ziye Zhang:** Methodology, Resources. **Hong Lin:** Data curation, Supervision. **Zhenxing Li:** Conceptualization, Methodology, 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 can be found online at <https://doi.org/10.1016/j.foodres.2022.111427>.

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