

Combination Processing Method Reduced IgE-Binding Activity of *Litopenaeus vannamei* by Modifying Lysine, Arginine, and Cysteine on Multiple Allergen Epitopes

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ABSTRACT: Allergic reactions occur after the whole food is ingested, rather than the purified allergen. The present study explores the low-allergenic food processing for *Litopenaeus vannamei* by analysis of macrostructure, digestibility, and immunoreactivity. Furthermore, the presence of modified amino acids on the reported IgE epitopes was analyzed by mass spectrometry. Results showed that the combination processing of Maillard reaction (shrimp meat with galactose) with high temperature–pressure at 115 °C obviously changed the macrostructure and increased the digestibility for the shrimp meat. Meanwhile, the processing significantly reduced the IgG/IgE-binding activity of the shrimp meat. The hypo-IgE-binding activity in processed shrimp may be due to the modification of lysine, arginine, and cysteine residues in antigen epitopes. This is a comprehensive assessment of the specific amino acid residues modified by glycation of multiple allergens in processed *L. vannamei*, which provides a new research method to explore the hypo-IgE-binding activity in food.

KEYWORDS: combination processing, glycation, IgE-binding activity, IgE epitopes, *Litopenaeus vannamei*

INTRODUCTION

Food allergy is a common and serious health issue worldwide, affecting about 8% of children and about 3% of adults.¹ Among the big eight allergenic food, shellfish is more crucial for residents located in coastal areas.² Shrimp allergy can trigger severe allergic reactions and usually persist throughout life.³ Like many other kinds of seafood, *Litopenaeus vannamei* remains a popular choice in daily life, for its nutrient value. The features of purified allergens in *L. vannamei*, including tropomyosin (TM), arginine kinase (AK), myosin light chain (MLC), and sarcoplasmic calcium-binding protein (SCP), were widely reported.⁴

Though avoidance of shrimp and shrimp products is the basic and common measure in managing shrimp allergies,³ a long-term strict prawn-free diet may lead to a diet imbalance. Thus, the hypo-immunoreactivity of food processing attracted research studies. There are many reports about the hypo-immunoreactivity processing, including boiling and high temperature pressure (HTP),^{5,6} which could reduce the allergenicity of food allergens. Allergic reactions occur after the whole food is ingested, rather than the purified allergen. Then, it is important and necessary to comprehensively assess the immunoreactivity of shrimp from the food itself.

Thermal processing is common for shrimp, and interactions occur between allergenic proteins and the other food components (such as the amino acids with the carbonyl groups from sugars).⁷ In some scenarios, Maillard reaction (MR) could reduce the allergenicity of shellfish TM,⁸ while it increased the IgE-binding activity for eggs.⁹ During the previous study, the single processing of HTP could obviously

decrease the immunoreactivity of mud crab¹⁰ and shrimp¹¹ for changing the food morphology and increasing the digestibility. Then, the research about the immunoreactivity of the shrimp meat after combined thermal processing, including MR and HTP treatments, is interesting and essential.

An allergen epitope, which is located on the spatial structure of the protein, is a prerequisite for activating the individual's immune system and developing effective processing methods that can mitigate food allergenicity.^{16,17} The higher frequency of amino acids on epitopes than the protein sequence is related to the allergenicity of allergens.¹⁸ Besides, the disulfide bond¹⁹ and the glycosylation site⁸ have been proven to be related to allergenicity. However, the research studies that focus on modifying specific amino acids on epitopes based on the whole processed shrimp are insufficient.

In the present study, the processing of MR, HTP, and MR combination with HTP (MR-HTP) was used to explore shrimp crude protein profiles by gel electrophoresis and western blotting. The processing temperatures of 110, 115, and 121 °C were optimized further. The macrostructure and texture profile analysis (TPA) of the processed shrimp meat were observed by scanning electron microscopy (SEM) and the TA-TX2 texture analyzer, respectively. Continuous gastro-

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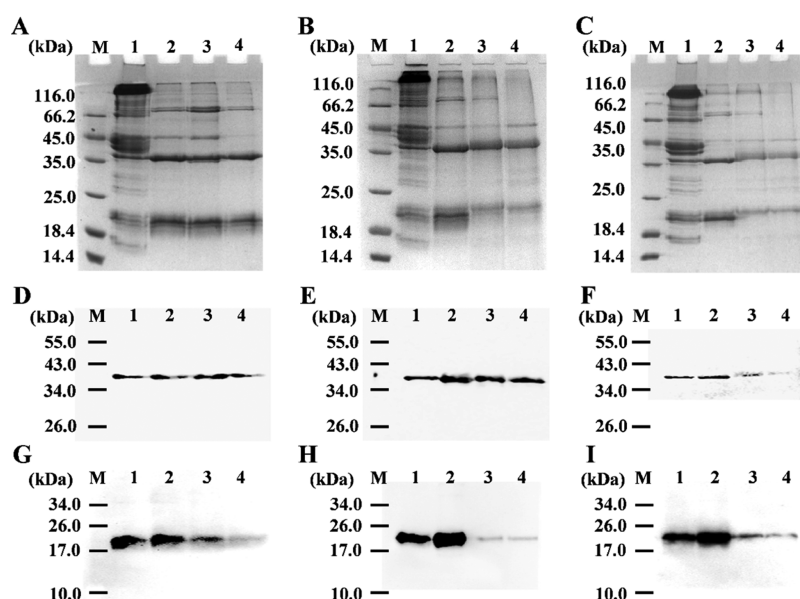


Figure 1. Protein profiles of shrimp crude extracts after different treatments. (A) SDS-PAGE of shrimp crude extracts after thermal processing at 110 °C. (B) SDS-PAGE of shrimp crude extracts after thermal processing at 115 °C. (C) SDS-PAGE of shrimp crude extracts after thermal processing at 121 °C. (D) Western blotting of shrimp TM by TM IgG pAb after thermal processing at 110 °C. (E) Western blotting of TM by IgG pAb after thermal processing at 115 °C. (F) Western blotting of TM by IgG pAb after thermal processing at 121 °C. (G) Western blotting of shrimp SCP by SCP IgG pAb after thermal processing at 110 °C. (H) Western blotting of SCP by IgG pAb after thermal processing at 115 °C. (I) Western blotting of SCP by IgG pAb after thermal processing at 121 °C. Lane M, protein marker. Lane 1, shrimp crude extracts in G1. Lane 2, shrimp crude extracts in G2. Lane 3, shrimp crude extracts in G3. Lane 4, shrimp crude extracts in G4.

intestinal digestion was employed to detect the digestibility of processed shrimp crude extracts. The IgE-binding activity of continuous gastrointestinal digestion products was measured by dot blotting. Then, the modified amino acid residues were analyzed by mass spectrometry to explore the mechanism of the optimized processing for low-allergenic seafood.

MATERIALS AND METHODS

Materials. Live shrimp (*L. vannamei*) were purchased from RT-Mart in Xiamen, China. Goat anti-rabbit IgG and goat anti-human IgE horseradish antibody, pepsin, and pancreatin were purchased from Sigma-Aldrich (Sigma-Aldrich, St Louis, USA). Rabbit anti-shellfish TM and SCP polyclonal antibodies (pAb) were prepared in our laboratory.¹¹ The other materials were of analytical grade.

Human Sera. Human sera from shrimp-sensitized patients were provided by the Women and Children's Hospital Affiliated to Xiamen University (the human ethical approval numbers are KY-2018-018 and KY-2019-014, Xiamen, Fujian, China). The informed consent was provided by the participator or the parents of participating children. The specificity of IgE levels to shrimp was assessed by ImmunoCAP (Phadia AB, Rapsatan, Uppsala, Sweden); sera with a positive value ≥ 0.35 kU_A/L were collected. The collected sera were recognized by enzyme-linked immunosorbent assay again using the specific allergen (purified TM and SCP), and the inclusion criterion was a positive allergen absorbance at 450 nm (value > 0.1). Meanwhile, two healthy volunteers, who had never shown symptoms of food allergy, were recruited for the control (specific IgE by ImmunoCAP < 0.35 kU_A/L and OD₄₅₀ < 0.1). The detailed information of individuals is shown in Table S1. The qualified sera were stored at -80 °C until use.

Food Processing of the Shrimp Meat and Preparation of Shrimp Crude Extracts. Live shrimp were transported to the laboratory and subjected to washing using plenty of water (~ 25 °C) to remove extraneous matter. The shrimp were peeled and divided into four groups for further processing.

Group 1 (G1). Raw shrimp without any processing were the control group.

Group 2 (G2). Shrimp (in G1) were soaked in an aqueous solution of D-galactose (galactose-to-water ratio, 1:3 w/w) for 2 h in 4 °C.⁸

Group 3 (G3). The shrimp (in G1) were subjected to pressure treatment (0.08 MPa) at different temperatures at 110, 115, and 121 °C for 6 min.

Group 4 (G4). The shrimp (in G2) were treated with thermal processing as described in G3.

The processed shrimp meat was mashed for 2 min and prepared with 10-fold volumes of ice-cold PBS buffer (pH 8.0) with 0.5 M NaCl as previously described.¹¹ The mixture was adjusted to a concentration of 2 mg/mL and stored at 4 °C for the following analysis as soon as possible.

In Vitro Gastrointestinal Continuous Digestion. The continuous digestion procedure was conducted as previously described.¹¹ In brief, the mixture of shrimp crude extracts was equally divided into aliquots of 20 mL (~ 4 mg/mL) in preparation for continuous digestion. Pepsin was added (enzyme-to-substrate ratio, 1:50 w/w) in the gastric digestion. Pancreatin was added (enzyme-to-substrate ratio, 1:200 w/w) in the duodenal digestion. The simulated gastric digestion and duodenal digestion lasted 60 and 240 min, respectively. The gastric digestion was stopped by raising the pH of the sample to 7.4 with NaOH. The duodenal digestion was stopped by boiling at 100 °C for 10 min. The digestion samples at different times were collected and stored at -20 °C for the following analysis.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis Analysis. The shrimp crude extracts were separated on 10-well 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. The digestion samples were separated on 10-well 15% SDS-PAGE gels. In brief, each extract (10 μ g/well) was mixed with reducing loading buffer containing SDS, glycerol, and bromophenol blue, heated at 95 °C for 10 min, and loaded onto a 12% or 15% polyacrylamide gel.

Immunobinding Activity Assay. Western blotting was used to evaluate the IgG-binding activity according to the reported methods.¹⁰ The primary antibody was rabbit anti-crab TM and SCP polyclonal antibodies (dilution 1:10⁵ and 1:4 $\times 10^4$, respectively). The secondary antibody used was HRP-labeled goat anti-rabbit IgG at a dilution of 1:20,000.

Dot blotting was employed to evaluate the IgE-binding activity. Briefly, 2 μ L of digestion products was added in duplicate on the nitrocellulose membrane (4.9 cm $\times$ 1.7 cm) and incubated for 2 h at

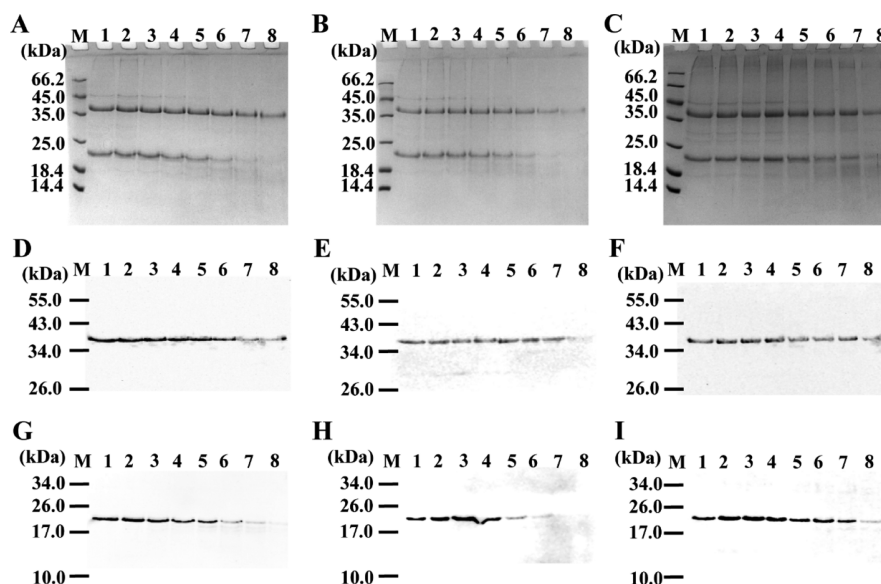


Figure 2. Protein profiles of the MR-HTP processed shrimp meat (G4) submitted to simulated gastric digestion *in vitro*. (A) SDS-PAGE of the shrimp meat in G4 at 110 °C. (B) SDS-PAGE of the shrimp meat in G4 at 115 °C. (C) SDS-PAGE of the shrimp meat in G4 at 121 °C. (D) Western blotting of TM in G4 at 110 °C. (E) Western blotting of TM in G4 at 115 °C. (F) Western blotting of TM in G4 at 121 °C. (G) Western blotting of SCP in G4 at 110 °C. (H) Western blotting of SCP in G4 at 115 °C. (I) Western blotting of SCP in G4 at 121 °C. Lane M, protein marker. Lane 1, the shrimp meat after processing and before gastric digestion. Lane 2, the shrimp meat was digested in the gastric phase for 0 min. Lane 3, the shrimp meat was digested for 1 min. Lane 4, the shrimp meat was digested for 2 min. Lane 5, the shrimp meat was digested for 5 min. Lane 6, the shrimp meat was digested for 15 min. Lane 7, the shrimp meat was digested for 30 min. Lane 8, the shrimp meat was digested for 60 min.

room temperature. The skimmed milk of 5% was added after the membrane was washed with the washing solution (TBS, 0.05% Tween 20). The milk was incubated for 2 h at room temperature. The membrane was added in a sealed sera bag, which was 8 mL of TM/SCP sensitized volunteers' sera pool (dilution 1:3). The primary antibody of sera was incubated overnight at room temperature. Then, the secondary antibody was horseradish peroxidase-conjugated goat anti-human IgE (dilution 1:20,000) and was added after washing the membrane and incubated for 2 h at room temperature. After washing the membrane again, the results were visualized by enhanced chemiluminescence.

Analysis of Modified Amino Acid Residues by Liquid Chromatography–Mass Spectrometry. Analysis of modified amino acid residues in processed protein extracts (G1–G4) was performed by Weinovate Bio. (Shenzhen, Guangdong, China) using EASY nLC 1200 and Q Exactive (Thermo Fisher, Waltham, Missouri, USA). The detection protocol was consistent with the reported methods.²⁰ Briefly, the trapping, desalting procedures were carried out with a volume of 20 μ L 100% solvent A (0.1% formic acid). Then, an elution gradient of 8–38% solvent B (80% acetonitrile, 0.1% formic acid) in 30 min was used. Data were acquired using an ion spray voltage of 2.4 kV and an interface heater temperature of 150 °C. The analysis software used was PEAKS Studio 8.5 (version 8.5, Bioinformatics Solutions Inc., Waterloo, Canada). Amino acid variable modifications include acetaldehyde (protein N-term), carbamidomethylation, carboxymethyl, furan, imidazolone, methylfuran, methyl, methoxy, methylglyoxal, N-glycopeptide, phenol, and pyrroline. The detailed information is provided in [Supporting Information](#).

Molecular Modeling, Epitopes, and Modified Amino Acid Graphics. The amino acid sequences of TM (IUIS entry: Lit v 1) and SCP (IUIS entry: Lit v 4) were obtained from the WHO/IUIS Allergen Nomenclature Database (<http://www.allergen.org/>). For more information, see [Supporting Information](#).

Based on the primary sequence, the three-dimensional (3D) structures of allergens were modeled using the Web Service Swiss Model (<http://swissmodel.expasy.org/>) by homology modeling. The

disulfide bonds were predicted on the website of Cyscon (<http://www.csbio.sjtu.edu.cn/bioinf/Cyscon/>).²¹

Epitopes of allergens were collected from the previous research.⁴ The identified epitopes and modified amino acids were displayed on the 3D surface of allergens using PyMOL software (San Carlos, California, USA).

RESULTS

Effects of Multiple Food Processing Methods on Crude Extracts and Allergens in the Whole Shrimp Meat. [Figure 1](#) shows protein profiles of different processed shrimp crude extracts by SDS-PAGE. There were several protein bands in raw shrimp (G1), which were distributed in molecular weight over 14 kDa. Compared with G1, the number of protein strips was reduced obviously in G2, especially for the higher molecular weight strips. Besides, the number of strips was less in G3 and G4. The main protein strips of G3 were observed of approximately >70, 45, and 38 kDa and a diffuse band of 20 kDa at 110 °C ([Figure 1A](#)). Protein bands of G3 were mainly observed of approximately 38 and 20 kDa at 115 °C ([Figure 1B](#)), which were similar to that shown in [Figure 1C](#). Protein bands of G4 were observed of approximately >70 and 38 kDa and a diffuse band of 20 kDa at 110 °C ([Figure 1A](#)), while they were observed of approximately 38 and 20 kDa at 115 and 121 °C ([Figure 1B,C](#)).

Western blotting was employed to further detect the IgG-binding activity of allergens in shrimp after thermal processing ([Figure 1D–I](#)). TM and SCP were widely recognized in all treated groups, while the bands in thermal processing groups were weaker at the three temperatures. Compared with SCP, the strips of TM were more stable in each group. In addition, the bands of TM and SCP in G4 were weaker than the other group.

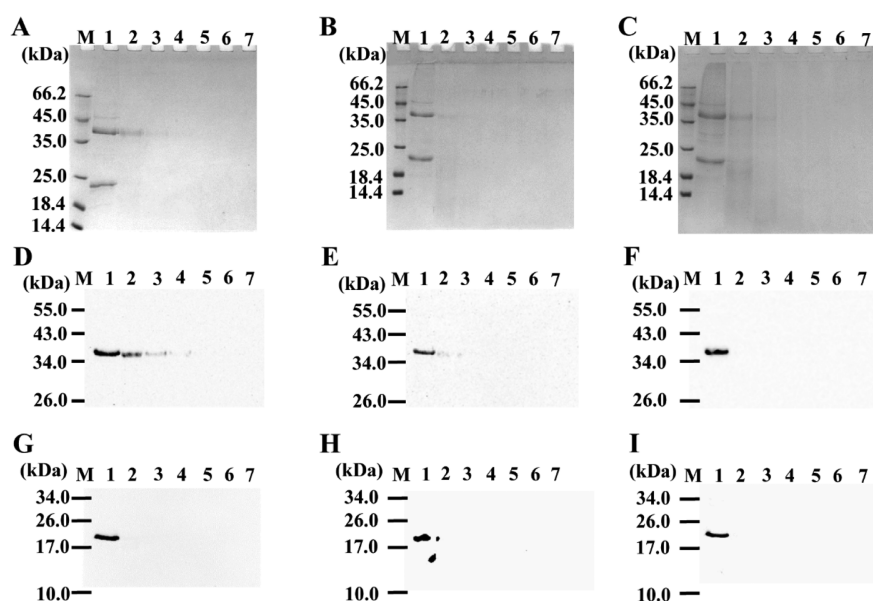


Figure 3. Protein profiles of the MR-HTP processed shrimp meat (G4) submitted to continuous duodenum digestion *in vitro*. (A) SDS-PAGE of the shrimp meat after MR-HTP processing at 110 °C. (B) SDS-PAGE of the shrimp meat after MR-HTP processing at 121 °C. (C) SDS-PAGE of the shrimp meat after MR-HTP processing at 115 °C. (D) Western blotting of TM after MR-HTP processing at 110 °C. (E) Western blotting of TM after MR-HTP processing at 115 °C. (F) Western blotting of TM after MR-HTP processing at 121 °C. (G) Western blotting of SCP after MR-HTP processing at 110 °C. (H) Western blotting of SCP after MR-HTP processing at 115 °C. (I) Western blotting of SCP after MR-HTP processing at 121 °C. Lane M, protein marker. Lane 1, the shrimp meat after gastric digestion and before duodenal digestion. Lane 2, the shrimp meat in duodenal digestion for 0 min after gastric digestion. Lane 3, the shrimp meat was digested for 10 min. Lane 4, the shrimp meat was digested for 60 min. Lane 5, the shrimp meat was digested for 120 min. Lane 6, the shrimp meat was digested for 180 min. Lane 7, the shrimp meat was digested for 240 min.

MR-HTP Processing Altered the Macrostructure and TPA Profile in the Shrimp Meat. Compared with the other three groups, G4 (MR-HTP) processing presented fewer protein bands in SDS-PAGE and weaker allergen bands in western blotting. Then, the processed temperature in G4 was further evaluated. Figure S1 shows the shrimp muscle tissue macrostructure by SEM at three amplifications, and the raw shrimp were presented as the control. The raw shrimp exhibited a dense and compact monolithic structure, while the thermally processed shrimp presented a loose structure with obvious faults and large gaps. Besides, the change of shrimp morphologies was the largest at 115 °C, especially in the 5000-fold amplification. The most amounts of gaps were presented in the shrimp morphologies at the 5000-fold amplification.

The TPA test was used to simulate chewing action to indicate sensory properties, and deformation parameters of the processed shrimp meat were measured. TPA parameters, including hardness, springiness, chewiness, gumminess, and cohesiveness, are shown in Table S2. The shrimp meat after processing at 110 °C showed the highest values in TPA. With the increase of processed temperature, the parameters (except cohesiveness) of the shrimp decreased gradually, which indicated that with higher temperature, the processed shrimp meat showed a softer texture. There was no obvious difference in cohesiveness in the three-temperature processed shrimp meat, which suggested that there was no difference when the processed shrimp meat withstands a second deformation relative to the first one.²²

MR-HTP Processing Increased the Digestibility and Reduced IgG-Binding Activity of Shrimp Meat Digestion Products. The complete digestion progress contains gastric-duodenal continuous digestion *in vitro*. Figure 2 shows

the digestion of the shrimp meat in the gastric passage. Two main strips were observed of approximately 38 and 20 kDa and presented similar digestion tendency among the combined processing. The main strip of nearly 38 kDa was stable after 60 min of digestion, while the strip of 20 kDa was dimmer and shallower on SDS-PAGE (Figure 2A–C). Most strips of shrimp crude extracts processed at 115 °C disappeared in 30 min, which means that the digestion was more thorough compared to the other two temperatures.

The bands in western blotting, recognized by TM pAb and SCP pAb, were weaker as the gastric digestion proceeded, which suggested that the sample was gradually digested by the digestive enzyme. The strip of TM was shallower after 30 min of gastric digestion, as shown in Figure 2D, while it was weaker after digestion for 60 min (Figure 2E,F). Compared with TM, the band of SCP was less stable. The strength of the SCP band was decreased obviously after the digestion of 15 min, as shown in Figure 2G. The trend of band degradation was more obvious, as shown in Figure 2H; the intensity of the SCP band was decreased obviously at 5 min. While the band of SCP was more stable as shown in Figure 2I, the strength was reduced significantly at 60 min.

During the duodenal continuous digestion that followed, the main bands of nearly 38 and 20 kDa degraded quickly (Figure 3A–C). After digestion for 10 min, the main bands degraded as shown in Figure 3A, vanished as shown in Figure 3B, and diffused as shown in Figure 3C. In addition, the TM-associated band was not detectable by western blotting after 120 min of duodenal digestion, as shown in Figure 3D. Furthermore, the strips of TM were not detectable at the beginning of the duodenal digestion, as shown in Figure 3E,F. Compared with TM, SCP showed weaker digestion stable activity (Figure 3G–

I), for the SCP-associated bands were not recognized at the beginning of the duodenal digestion.

MR-HTP Processing Reduced IgE-Binding Activity of Shrimp Meat Digestion Products. The IgE-binding activity is the prerequisite for allergens to cause food allergies. The allergen-specific sera pool was used to detect the continuous duodenal digestion products by Dot blotting, as shown in Figure 4. The strength of TM dots at 110 °C was gradually

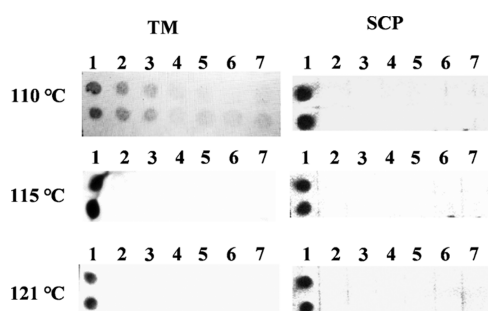


Figure 4. IgE-binding activity of continuous digestion products in G4 at different temperatures by specific sensitized sera. Lane 1, the shrimp meat after gastric digestion and before duodenal digestion. Lane 2, the shrimp meat in duodenal digestion for 0 min after gastric digestion. Lane 3, the shrimp meat in duodenal digestion for 10 min. Lane 4, the shrimp meat in duodenal digestion for 60 min. Lane 5, the shrimp meat in duodenal digestion for 120 min. Lane 6, the shrimp meat in duodenal digestion for 180 min. Lane 7, the shrimp meat in duodenal digestion for 240 min. The spots were the continuous digestion products added in duplicate on the nitrocellulose membrane that was recognized by sera.

weaker, which suggested that TM in the digestion products was gradually digested by the digestive enzyme. The other dots of TM and SCP at 115 and 121 °C were not detected by IgE during the digestion processing, indicating that compared with 110 °C, allergens at 115 and 121 °C were more thoroughly digested. Also, the dots recognized by individuals' sera showed no obvious difference between TM and SCP at 115 and 121 °C.

Mapping of Epitopes and Modified Amino Acid Residues on the 3D Structures of Allergens. In TM, 9 epitopes⁴ were mapped on the 3D structure (temple PDB: 1C1G), including amino acids (AA) 1–36, 37–63, 61–81, 82–105, 115–150, 142–162, 157–183, 190–210, and 246–284 (Figure 5A). In SCP, 3 epitopes⁴ were mapped to the modeled conformational structure (temple PDB: 2SAS), including AA 10–36, 49–72, and 130–147 (Figure 5B).

Then, the modified amino acid residues of digested products were detected by liquid chromatography–mass spectrometry (LC–MS/MS), as shown in Table 1, and mapped on the 3D structure of allergens. In TM, 19 lysine residues and 18 arginine residues were modified at 115 °C. In SCP, 7 lysine residues, 2 arginine residues, and 4 cysteine residues were modified at 115 °C. There were less modified amino acid residues located on the reported epitopes. In TM, 13 lysine residues and 16 arginine residues were modified on the 9 epitopes at 115 °C (Figure 5C). For SCP, 3 lysine residues, 2 arginine residues, and 1 cysteine residue were modified on the 3 epitopes at 115 °C (Figure 5D). Cys₉₀, Cys₁₃₀, Cys₁₈₀, and Cys₁₈₃ form two intramolecular disulfide bonds in SCP as predicted by the Cyson website.²¹ Interestingly, Cys₉₀, Cys₁₈₀, and Cys₁₈₃ were modified with acetaldehyde, which means that the disulfide bonds of SCP were destroyed at 115 °C.

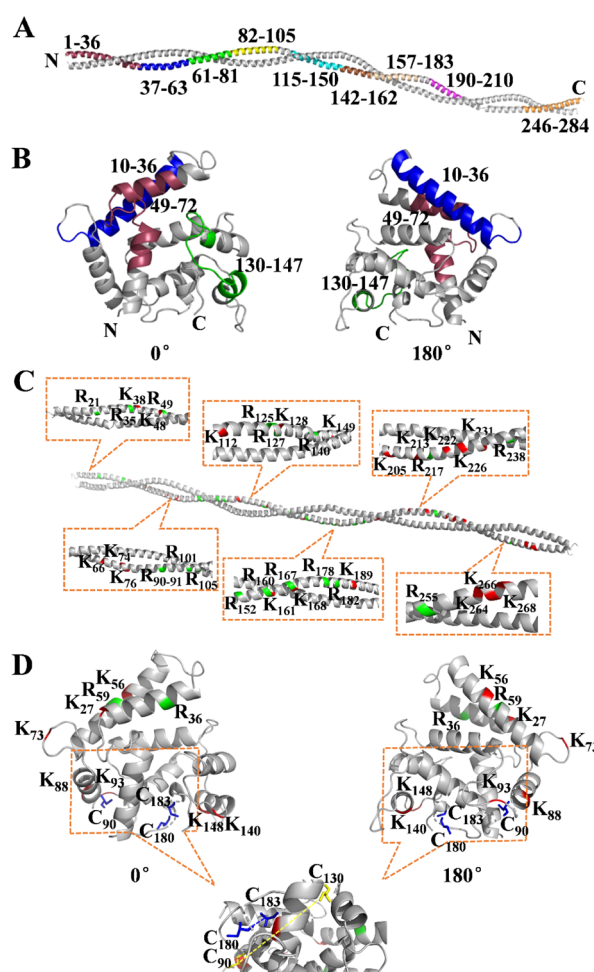


Figure 5. Structure modeling, epitopes, specific amino acid residues, and disulfide bond mapping on shrimp allergens. (A) Mapping of identified epitopes based on the 3D structure of TM. (B) Mapping of identified epitopes based on the 3D structure of SCP. The gray color shows the 3D structure of allergens. The other colors show the reported epitopes of allergens in the diagram. (C) Mapping of specific amino acid residues of TM by LC–MS/MS. (D) Mapping of specific amino acid residues and predicted disulfide bonds of SCP by LC–MS/MS. The gray color shows the 3D structure of allergens. The red color shows the modified lysine residues. The green color shows the modified arginine residues. The blue color shows the modified cysteine residues. The dotted lines in yellow and blue represent disulfide bonds.

In addition, the 3D structure and modified sites were researched for the other two allergens. In AK, 8 epitopes⁴ were mapped to the modeled special structure (temple PDB: 4BG4), including AA 1–18, 25–42, 64–96, 121–141, 142–159, 160–192, 232–255, and 319–342 (Figure S2A). Meanwhile, 3 cysteine residues (Cys₂₃, Cys₂₀₁, and Cys₂₇₁) were modified at 115 °C (Figure S2C). Cys₂₀₁ and Cys₂₇₁ form an intramolecular disulfide bond in AK as predicted by the Cyson website. There were no amino acids modified on the reported epitopes.

In MLC, 6 epitopes⁴ were mapped to the modeled conformational structure (temple PDB: 4IK4), including AA 13–30, 22–48, 49–66, 58–90, 79–99, and 118–141 (Figure S2B). Two lysine residues, 3 arginine residues, and 1 cysteine residue were modified at 115 °C (Table 1). Meanwhile, the

Table 1. Modified Amino Acid Residues in Shrimp Allergens Detected by LC–MS/MS^a

Allergens	Combination processing at 115 °C	Combination processing at 121 °C
TM	<u>R₂₁</u> , <u>R₃₅</u> , <u>K₃₈</u> , <u>R₄₈</u> , <u>R₄₉</u> , <u>K₆₆</u> , <u>K₇₄</u> , <u>K₇₆</u> ,	<u>R₂₁</u> , <u>K₃₀</u> , <u>K₄₈</u> , <u>R₄₉</u> , <u>K₆₆</u> , <u>K₇₄</u> , <u>K₇₆</u> , <u>R₉₀</u> ,
	<u>R₉₀</u> , <u>R₉₁</u> , <u>R₁₀₁</u> , <u>R₁₀₅</u> , <u>K₁₁₂</u> , <u>R₁₂₅</u> , <u>R₁₂₇</u> ,	<u>R₉₁</u> , <u>R₁₀₁</u> , <u>R₁₀₅</u> , <u>K₁₁₂</u> , <u>R₁₄₀</u> , <u>K₁₄₉</u> , <u>R₁₅₂</u> ,
	<u>K₁₂₈</u> , <u>R₁₄₀</u> , <u>K₁₄₉</u> , <u>R₁₅₂</u> , <u>R₁₆₀</u> , <u>K₁₆₁</u> , <u>R₁₆₇</u> ,	<u>R₁₆₀</u> , <u>K₁₆₁</u> , <u>R₁₇₈</u> , <u>R₁₈₂</u> , <u>K₂₀₅</u> , <u>K₂₁₃</u> , <u>R₂₁₇</u> ,
	<u>K₁₆₈</u> , <u>R₁₇₈</u> , <u>R₁₈₂</u> , <u>K₁₈₉</u> , <u>K₂₀₅</u> , <u>K₂₁₃</u> , <u>R₂₁₇</u> ,	<u>K₂₂₂</u> , <u>K₂₂₆</u> , <u>K₂₃₁</u> , <u>R₂₃₈</u> , <u>K₂₆₄</u> , <u>K₂₆₆</u> , <u>K₂₆₈</u>
	<u>K₂₂₂</u> , <u>K₂₂₆</u> , <u>K₂₃₁</u> , <u>R₂₃₈</u> , <u>R₂₅₅</u> , <u>K₂₆₄</u> ,	
	<u>K₂₆₆</u> , <u>K₂₆₈</u>	
SCP	<u>K₂₇</u> , <u>C₃₂</u> , <u>R₃₆</u> , <u>K₅₆</u> , <u>R₅₉</u> , <u>K₇₃</u> , <u>K₈₈</u> , <u>C₉₀</u> ,	<u>K₂₇</u> , <u>C₃₂</u> , <u>R₃₆</u> , <u>K₅₆</u> , <u>R₅₉</u> , <u>K₇₃</u> , <u>K₁₄₀</u> , <u>C₁₈₀</u> ,
	<u>K₉₃</u> , <u>K₁₄₀</u> , <u>K₁₄₈</u> , <u>C₁₈₀</u> , <u>C₁₈₃</u>	<u>C₁₈₃</u>
AK	<u>C₂₃</u> , <u>C₂₀₁</u> , <u>C₂₇₁</u>	<u>C₂₃</u> , <u>K₂₄</u> , <u>K₁₀₂</u> , <u>C₁₃₉</u> , <u>K₁₄₆</u> , <u>C₂₇₁</u> , <u>R₃₀₉</u> ,
		<u>R₃₁₂</u>
MLC	<u>R₃₀</u> , <u>K₃₆</u> , <u>R₄₄</u> , <u>K₄₆</u> , <u>C₁₂₁</u> , <u>R₁₂₅</u>	<u>R₃₀</u> , <u>K₃₆</u> , <u>K₄₆</u> , <u>C₁₂₁</u> , <u>R₁₂₅</u>

^aNote: the underline represents the amino acids, which are located in the allergen epitopes. The predicted disulfide bonds of cysteine residues were shadowed in gray color.

modified amino acids were located on the reported six epitopes (Figure S2D).

Compared with the amounts of modified amino acid residues at 115 °C (59 residues on proteins and 41 residues on epitopes), the modified amino acid residues were less at 121 °C (51 residues on proteins and 34 residues on epitopes) (Table 1).

DISCUSSION

IgE-mediated shrimp allergy represents an important and increasingly prevalent health issue in recent years. The shrimp production increased year by year in China,²³ which is one of the reasons for the increased prevalence of shrimp allergy. On the other hand, the wide cross-reactivity between shrimp and other crustaceans or mollusks may increase the risk of shrimp allergy.²⁴ Then, strict avoidance is common for shrimp allergy individuals to avoid food allergy, while the long period of strict abrosia is impractical in modern society. Food processing helps to reduce the allergenicity of allergens and has attracted researchers' attention in recent decades.¹² Then, the present paper focuses on the exploration and optimization of thermal processing conditions suitable for the preparation of low-allergenic shrimp products.^{13–15}

Thermal processing is common for shrimp before they are consumed, and it is believed to influence the allergenicity of food as it leads to the disruption of allergens.²⁵ Then, the thermal processing of single MR, HTP, and MR-HTP treatments were compared in the present study. The processed shrimp showed fewer kinds of proteins than the raw shrimp in the present research, which was consistent with previous studies that the cooked shrimp showed fewer protein bands than the raw shrimp.^{11,26} On the one hand, this might be a consequence of proteins that were not heat-stable and

dissolved in the broth after thermal processing.¹¹ On the other hand, the heating-mediated interaction of protein crosslinking, aggregation,¹⁷ and protein oxidation caused the change of the protein structure and reduced the species of proteins.²⁷ Furthermore, the additional amounts of D-galactose increased the development of the MR in the matrix and decreased the species of proteins in extraction samples.²⁸ Then, the combined processing in whatever kind of temperature showed less protein bands, of which were centered on 38 and 20 kDa. Although the heat-stable allergens of TM (molecular weight 38 kDa) and SCP (molecular weight 20 kDa) were retained, the bands were weaker after combined processing, which suggested that the IgG-binding activity of allergens was lower. Immunoreactivity highly relies on the protein structure,²⁹ and the changing of the IgG-binding activity means that the structure of allergens has been changed significantly. Vanga *et al.*¹² indicated that the higher the heat temperature, the greater the protein structure destruction, resulting in weaker bands and lower IgG-binding activity.

Heating temperature affects the whole food digestion parameters and the other properties for modifying the physicochemical state and immunoreactivity of the matrix.³⁰ MR-HTP processing destroyed the shapes and morphologies of the shrimp muscle according to SEM, which was consistent with the previous study. The obvious faults and large gaps may contribute to increasing the digestibility.^{10,11} With the increase of processed temperature, the macrostructure change is more obvious. Instrumental TPA has been used for the measurement of food textural properties many years ago.³⁰ Compared with 121 °C, the shrimp processed at 115 °C presented higher quality with regard to the suitable parameters of TPA. The results were similar to that of the previous study. Rong *et al.*³¹ compared the effect of cooking on peeled shrimp quality by

TPA and concluded that the processing at 121 °C and 5 min was not suitable for the shrimp products for the lower TPA parameters.

Resistance to gastrointestinal digestion is an important parameter to consider when assessing the residual immunoreactivity of allergens.³² The whole digestion progress should involve the continuous proteolytic action of pepsin and pancreatin.³³ The present research focuses on the continuous gastrointestinal digestion after thermal processing for shrimp. Compared with the other two processed temperatures, the processed shrimp at 115 °C showed higher digestibility for the strips degraded more thoroughly, which means that the shrimp digestibility did not show temperature dependence in the gastrointestinal digestion. The results of digestibility are not consistent with that of the previous report; the main reason speculated is that temperature plays a crucial role in the rearrangement of the products of the MR.²⁵ The higher temperature in heating changed the structure and properties of the protein, including the digestibility.^{10,11}

The key to the food remains sensitization is that the epitope still exist in the digestion products that can bind to IgE.²⁷ TM and SCP were chosen as representatives to detect the IgE-binding activity in digestion products, for they are heat- and digestion-stable allergens in the shellfish myofibrillar protein and myosinogen, respectively.¹¹ The combined processing at 115 °C presented lower IgE-binding activity of TM and SCP. There is still insufficient knowledge about the changing of the IgE-binding activity and protein structure in processed temperature higher than 115 °C.¹² One of the possible reasons for lower IgE-binding activity in combined processing was that allergens quickly degraded as peptides in the present digestion ratio.³⁴ The other reason might be that MR disrupted the epitopes accompanied by the changes of the secondary and tertiary structures that impaired the IgE-binding potential of allergens.²⁸

Ayuso *et al.*⁴ identified the IgE epitopes by means of peptide microarrays for IgE-binding with synthetic overlapping peptides spanning the sequences of four allergens in *L. vannamei*. The IgE epitopes are crucial for allergenicity, and the modification of specific amino acids on epitopes is related to the allergenicity of allergens.^{16,17} The MR takes place between the carbonyl group of reducing sugars and the N terminus, the ϵ -amino group of the protein.²⁵ Lysine and arginine, containing the free ϵ -amino group, were identified to participate in the glycation of food allergens.⁸ Meanwhile, cysteine, which formed the intermolecular disulfide bonds, helps to retain the spatial structure and immunological property.¹⁹ Shotgun proteomics were used to detect the modified amino acid residues in the present processed shrimp.⁸ There were more amounts of modified amino acid residues in shrimp allergens after combined processing at 115 °C, including lysine (28 residues on proteins and 18 residues on epitopes at 115 °C; 25 residues on proteins and 17 residues on epitopes at 121 °C), arginine (23 residues on proteins and 21 residues on epitopes at 115 °C; 19 residues on proteins and 15 residues on epitopes at 121 °C), and cysteine (8 residues on proteins and 2 residues on epitopes at 115 °C; 7 residues on proteins and 3 residues on epitopes at 121 °C), which means that the spatial structure was destroyed obviously at 115 °C and led lower immunoreactivity in the MR-HTP processed shrimp. Besides, the predicted disulfide bonds (Cys₂₀₁ and Cys₂₇₁ of AK; Cys₉₀, Cys₁₃₀, Cys₁₈₀, and Cys₁₈₃ of SCP)²¹ were modified by carbamidomethyl, which reduced the allergenicity

of allergens according to the previous reports.^{8,19} Meanwhile, C₃₂, R₃₆, R₄₃, C₁₀₀, K₁₁₁ and C₁₇₆ of SCP were modified sites, which in the MR products between SCP and xylose in *Scylla paramamosain* (data unpublished). Most of the reported modified amino acids by glycation overlapped with the present study, while the factors of reducing sugar, temperature, and processing time may be one of the reasons for the difference of modified amino acids.²⁵ In addition, the difference of amino acid sequences between *S. paramamosain* and *L. vannamei* may be the other reason (92.16% sequence homology in TM, 93.28% sequence homology in AK, and 79.27% sequence homology in SCP). Ayuso *et al.*³⁵ first identified MLC as the novel shrimp allergen in 2008; the present paper identified the modified specific amino acids of MLC for the first time.

In conclusion, the MR-HTP processed shrimp showed fewer kinds of proteins and allergens. After combined processing by D-galactose (galactose-to-water ratio, 1:3 w/w) at 0.08 MPa, 115 °C for 6 min, more amino acids (18 lysine residues, 21 arginine residues, and 2 cysteine residues) were glycosylated in four allergen epitopes, resulting in higher digestibility and lower IgE-binding activity of the processed products. Meanwhile, the processed shrimp meat showed good quality by TPA. The present study comprehensively evaluated the specific amino acids by glycation of various allergens in processed *L. vannamei*, clarified that the mechanism of processing reduced the allergenicity of foods to a certain extent, and provided new research methods for the exploration of hypoallergenic foods.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.1c00718>.

Additional experimental details; details of shrimp sensitized individuals and texture profile analysis; SEM of shrimp meat; and structure modeling, epitopes, and modified specific amino acid mapping of AK and MLC (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

AA, amino acids; AK, arginine kinase; HTP, high temperature–pressure; Ig, immunoglobulin; LC-MS/MS, liquid chromatography mass spectrometry–mass spectrometry; MLC, myosin light chain; MR, Maillard reaction; MR-HTP, MR combination with HTP; pAb, polyclonal antibodies; SCP, sarcoplasmic calcium-binding protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM, scanning electron microscope; TM, tropomyosin; TPA, texture profile analysis

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Supporting information

Methods

Macrostructure determination by SEM

The macrostructure of processed shrimp meat was determined by SEM according to the previous methods.¹ Briefly, the second sections of the abdomens of the shrimps were cut into smaller slices with 0.5 cm × 0.5 cm × 0.5 cm. The slices were fixed, dehydrated and dried for 2 h. Then the slices were coated with a gold layer and observed by Hitachi S-4800 (Zeiss, Dsseldorf, Germany) in different magnification times.

Instrumental texture profile analysis

TPA of shrimp meat was performed using a TA-TX2 texture analyzer, the procedure was similar with the reference.² Textural parameters, including hardness, springiness, chewiness, gumminess and cohesiveness were calculated with the help of TexturePro Lite software provided along with the instrument.

LC-MS/MS

The trapping, desalting procedure were carried out with a volumn of 20 µL 100% solvent A (0.1% formic acid). Then, an elution gradient of 5-38% solvent B (80% acetonitrile, 0.1% formic acid) in 60 min was used on an analytical column (Acclaim PepMap RSLC, 75µm×25 cm C18-2 µm 100 Å). Data-dependent acquisition mass spectrum techniques were used to acquire tandem MS data on a ThermoFisher Q Exactive mass spectrometer (ThermoFisher, USA) fitted with a Nano Flex ion source. The analysis software was PEAKS Studio 8.5 (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada). The local false discovery rate at PSM was 1.0% after searching

against *Scylla paramamosain* database with a maximum of two missed cleavages.

Precursor and fragment mass tolerance were set to 10 ppm and 0.05 Da, respectively.

Sequence alignment of epitopes and modified amino acids in AK and MLC

Allergen sequences of AK (Lit v 2, GenBank: DQ975203.1) and MLC (Lit v 3, GenBank: EU449515.1) were obtained from WHO/IUIS Allergen Nomenclature database (<http://www.allergen.org/>).

Based on the primary sequence, the three-dimensional (3D) structures of AK and MLC were modeled using the Web Service Swiss Model (<http://swissmodel.expasy.org>).

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Table S1. Information of shrimp sensitised individuals

ID	Age	Sex ^a	Symptom	Specific IgE (by ImmunoCAP, kU _A /L)	Specific IgE (OD 450 nm)	
					TM	SCP
1	7	F	anaphylactic rhinitis	36.3	0.236	-
2	5	F	eczema	52.4	0.345	-
3	5	M	urticaria	34.7	0.287	-
4	4	F	urticaria	29.5	0.179	-
5	6	M	diarrhea	32.7	0.206	-
6	3	F	diarrhea	28.9	0.152	-
7	2	M	pruritus and nausea	79.8	-	0.476
8	4	F	urticaria	33.4	-	0.209
9	5	F	diarrhea	27.8	-	0.175
10	4	F	asthma and dyspnea	49.7	-	0.228
11	3	F	asthma	35.6	-	0.132
12	7	M	diarrhea	43.0	-	0.295
13	24	M	- ^b	- ^c	-	-
14	16	F	- ^b	- ^c	-	-

Note: ^a M, male; F, female. - means value of absorbance at 450 nm < 0.100. -^b means the person was a non-allergic individual. -^c means value of specific IgE by ImmunoCAP < 0.35 kU_A/L.

Table S2. Texture profile analysis of shrimp meat after MR-HTP processing at different temperatures

Texture profile analysis	110 °C	115 °C	121°C
Hardness (gf)	133.2 ± 6.8	61.5 ± 6.0	57.8 ± 4.5
Springiness (mm)	0.9 ± 0.1	0.6 ± 0.1	0.4 ± 0.1
Chewiness (g)	73.7 ± 6.0	58.9 ± 4.8	17.6 ± 3.7
Gumminess (N)	86.1 ± 6.2	62.3 ± 5.8	44.5 ± 6.3
Cohesiveness	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.1

Figure legends

Figure S1. Scanning electron microscopy of shrimp meat in different horizon

Figure S2. Structure modeling, epitopes and modified specific amino acids mapping of AK and MLC

A) Mapping of identified epitopes base on the three-dimensional structure of AK.

B) Mapping of identified epitopes base on the three-dimensional structure of MLC.

The gray color, the three-dimensional structure of allergens. The other color, the reported epitopes of allergens on the diagram.

C) Mapping of predicted disulfide bonds of AK by LC-MS/MS.

D) Mapping of modified amino acid residues of MLC by LC-MS/MS.

The gray color, the three-dimensional structure of allergens. The red color, the modified lysine residues. The green color, the modified arginine residues. The blue color, the modified cysteine residues. The dotted lines in yellow and blue represent disulfide bonds.

Figure S1

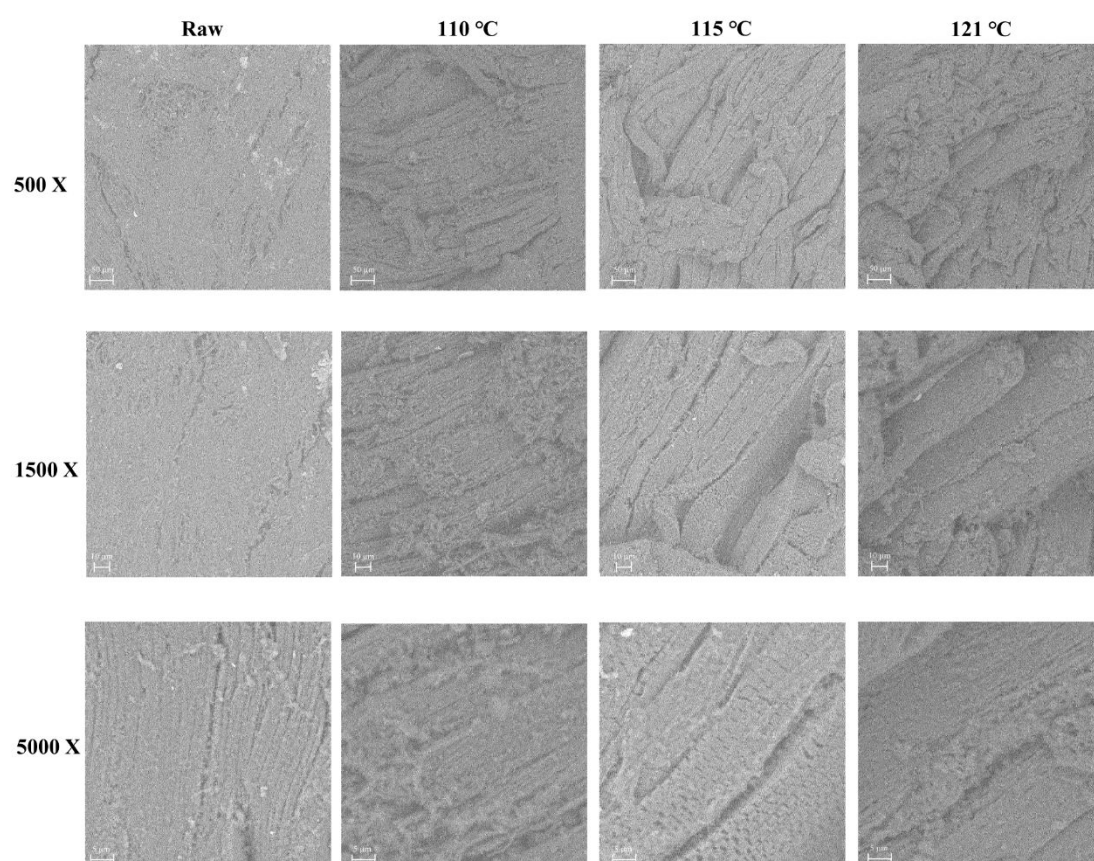


Figure S2

