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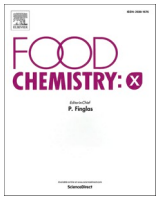

Effects of ultrasound-assisted high temperature-pressure treatment on the structure and allergenicity of tropomyosin from clam (*Mactra veneriformis*)

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ABSTRACT

Tropomyosin (TM) is the major allergen in clams. This study aimed to evaluate the effects of ultrasound-assisted high temperature-pressure treatment on the structure and allergenicity of TM from clams. The results showed that the combined treatment significantly affected the structure of TM—converting the α-helix to β-sheet and random coil, and decreasing the sulfhydryl group content, surface hydrophobicity, and particle size. These structural changes caused the unfolding of the protein, disrupting and modifying the allergenic epitopes. The significant reduction in the allergenicity of TM was approximately 68.1% when treated with combined processing ($P < 0.05$). Notably, an increase in the content of the relevant amino acids and a smaller particle size accelerated the penetration of the enzyme into the protein matrix, resulting in strengthening the gastrointestinal digestibility of TM. These results prove that ultrasound-assisted high temperature-pressure treatment has great potential in reducing allergenicity, benefiting the development of hypoallergenic clam products.

1. Introduction

Clam (*Mactra veneriformis*) is a popular shellfish due to its high-quality protein content and physiologically active compounds such as polyunsaturated fatty acids and minerals (Fu, Wang, Zhu, & Wang, 2019; Zhang et al., 2022a). The annual production of clam in China is as high as 4.29 million tons, and its cultivation is increasing at a rate of approximately 1.7%. However, Shellfish are declared by the Food and Agriculture Organization of the United Nations and the World Health Organization as one of the eight highly allergic foods (Prester, 2016). Reports have suggested that allergic reactions resulting from shellfish consumption affect 10.3% of the total population, causing an increasing public health concern worldwide (Wai et al., 2020). Some consumers are highly allergic to clams, which can trigger severe allergic reactions including wheezing, hives, or even life-threatening anaphylaxis (Cheng, Wang, & Sun, 2022). The major allergen in clams was identified as tropomyosin (TM), 35–38 kDa (Gonzalez-Estrada, Silvers, Klein, Zell, Wang, & Lang, 2017). Therefore, reducing the allergenicity or immunoreactivity of TM at its source is beneficial to clam-allergic individuals.

TM, as a food matrix component, activates the immune system to trigger allergic symptoms after processing and digestion (Liu et al., 2021a). The allergenicity of TM depends on the retention of its native structural epitope recognized by IgE or IgG (Khan et al., 2019). Processing techniques (i.e., thermal or non-thermal treatment) can disrupt conformational or linear epitopes by inducing conformational changes (Rahaman, Vasiljevic, & Ramchandran, 2016). Thermal treatment is the conventional and widely used food processing technique, including microwave, boiling, steaming, and high temperature-pressure (HTP). In particular, HTP, commonly known as industrial sterilization, can effectively reduce the allergenicity of certain foods. Cabanillas et al. (2015) found that peanuts treated with HTP at 121 °C for 15 min were less allergenic than those boiled for 60 min. Non-thermal treatments, including ultrasound, high hydrostatic pressure, gamma irradiation, and enzymatic hydrolysis, have also been used in an attempt to reduce TM allergenicity (Lu, Cheng, Jiang, Lin, & Lu, 2023). Among these novel processing techniques, ultrasound shows great potential in reducing allergenicity due to its acoustic cavitation. During sonication, cavitation bubbles occur. Once the bubbles reach a critical size, they violently collapse, producing high pressures (1000 atm) and temperatures (5000 K), which are sufficient to alter the conformation of TM, especially the destruction or masking of the epitopes existing on its molecular surface (Nayak, Li, Ahmed, & Lin, 2017). Dong, Wang and Raghavan (2020) found the allergenicity of TM decreased with the increase of high-intensity ultrasound (HIU) treatment time. In another study, Zhang, Zhang, Chen and Zhou (2018) treated TM with ultrasound and observed a considerable decrease in allergenicity, which they attributed to the

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degradation of TM under HIU. However, TM allergenicity cannot be eliminated completely by a single technology (Cheng et al., 2022). Combining ultrasound and HTP treatment might provide a solution that minimizes the allergenic severity of TM.

A common feature of food allergens is resistance to digestion. TM is an particularly stable myofibrillar protein (Lu et al., 2023). The presence of firm α-helix, hydrophobic domains, or disulfide bonds in TM helps it to resist gastrointestinal digestion and to maintain allergenic epitopes, thus stabilizing its sensitizing potential (Sun, Liu, Liu, Wang, Liu, & Lin, 2022). Processing technique-induced conformational changes of TM both at a micro and macro level may further impact digestion stability as well as its presentation to the immune system, ultimately affecting its allergenicity (Rahaman et al., 2016). However, changes in digestibility and allergenicity of TM treated with ultrasound-assisted HTP are unclear. Therefore, understanding the link between structure and allergenicity of TM after combined treatment can help to improve digestibility and mitigate immune response.

The main objective of the study was to investigate the effects of ultrasound-assisted HTP treatment on the structure and allergenicity of TM from *Mactra veneriformis*. Amino acid composition, circular dichroism, sulfhydryl group content, surface hydrophobicity, particle size, and zeta potential were used to analyze the structural changes. The allergenicity of TM was evaluated using ELISA and SDS-PAGE. A simulated gastrointestinal digestion test was used to assess the digestion stability and *in vitro* digestibility of TM before and after processing. The results would reveal the underlying mechanism of combined treatment in reducing allergenicity and lay a theoretical foundation for the development of hypoallergenic clam foods.

2. Materials and methods

2.1. Materials

Clam (*Mactra veneriforimis*) was purchased from a local market in Baoding, Hebei, China. The selected clams were of uniform size (12–14 g). 8-anilino-1-naphthalenesulphonic acid (ANS) and 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pepsin (500 U/mg protein), trypsin (250 U/mg protein), and ethylenediaminetetraacetic acid (EDTA) were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). The sandwich ELISA kit used to detect the allergenicity of TM was purchased from Indoor Biotechnologies, Inc. (Charlottesville, Virginia, USA). The primary antibody was a rabbit anti-shrimp tropomyosin antibody. All other chemicals were of analytical grade.

2.2. Purification of TM

TM was purified following the method described by Zhang et al. (2018). All procedures were performed at 4 °C unless otherwise stated. The fresh clam muscles were minced and stirred with 50% pre-cooled acetone for 6 h. The mixture was centrifuged at 10,000 × *g* for 15 min, and the precipitate was re-suspended in the acetone. The final residue was dried overnight on a filter paper. The dried powder was suspended in 0.01 mol/L phosphate buffer saline (PBS, pH 7.4), stirred for 4 h, and then centrifuged. All the extracts were combined after the crude TM was extracted twice. After adjusting the pH to 4.5 with 1 mol/L HCl, the solution was held for 2 h and then centrifuged. The resulting sediment was dissolved in PBS and regulated to pH 7.6 with 1 mol/L NaOH, followed by fractionation with ammonium sulfate to 41% saturation. The precipitate was dialyzed for 48 h in deionized water, then boiled for 10 min. The supernatant solution was filtered, then lyophilized using a vacuum freeze drier (LGJ-10C, Foring Technology Development Co., Ltd, Beijing, China), and finally stored at −80 °C.

2.3. Ultrasound-assisted HTP treatments

TM was dissolved in 0.01 mol/L PBS to a concentration of 1 mg/mL. 50 mL of TM solution was used in all treatments. Ultrasound treatment of TM solutions was carried out at 500 W (22 KHz) for 30 min, using an ultrasonic processor (JY99-IIDN, Scientz Biotechnology Co., Ltd, Ningbo, Zhejiang, China), and TM sample was processed in an ice water bath to keep the temperature below 20 °C. HTP treatment of TM solutions was processed at 0.14 MPa and 121 °C for 15 min in an autoclave (LDZX-50KBS, Shanghai Shenan Medical Equipment Co., Ltd, Shanghai, China). After HTP treatment, TM sample was cooled to room temperature (25 ± 2 °C) and then placed at 4 °C. Four treatments were used: (A) untreated TM (**Control**), (B) TM treated with high temperature-pressure (**HTP**), (C) TM treated with ultrasound at 500 W (**U500**), (D) TM treated with ultrasound followed by HTP (**UH500**).

2.4. Structure analysis

2.4.1. Amino acid composition

Amino acid content was determined according to the method with slight modifications (Zhang et al., 2018). TM sample (1 mg/mL) was mixed with sodium carbonate buffered salt solution (pH 9.0) and 3% 2,4-dinitrochlorobenzeneacetonitrile solution in a capped glass tube. The mixture was maintained at 95 °C for 150 min in darkness. Then, the pH was adjusted to neutrality with 10% glacial acetic acid. The solution was sonicated to remove air bubbles and passed through an organic membrane for further analysis using liquid chromatography (LC-2030, SHIMADZU Co., Ltd., Tokyo, Japan).

2.4.2. Circular dichroism (CD) spectrum

The CD spectropolarimeter (Chirascan V.4.7.0.194, Applied Photophysics Ltd., Leatherhead, Surrey, UK) was applied to determine changes in the secondary structure of the TM. Protein samples were diluted to 0.1 mg/mL. Diluted samples were scanned at 100 nm/min from 190 nm to 260 nm with a 1 nm bandwidth. The secondary structure compositions were analyzed using the Chirascan software.

2.4.3. Sulfhydryl (SH) group content

The SH group content of TM samples was analyzed following previous works (Yongsawatdigul & Park, 2003). For the total SH content, TM solution was mixed with buffer solution (8 mol/L urea, 10 mmol/L EDTA, 50 mmol/L KH_2PO_4, 0.6 mol/L KCl, pH 8.0). After adding 20 μL of 4 mg/mL DTNB, the mixture was incubated at 25 ℃ for 20 min. Absorbance values were measured at 412 nm to calculate the SH group contents using the molar extinction coefficient (13600 L/mol • cm). For the free SH group, the reaction mixtures were incubated in the absence of urea.

2.4.4. Surface hydrophobicity (H_0)

TM samples (4 mL, 0.05–0.35 mg/mL) were mixed with ANS solution (20 μL, 8.0 mmol/L) and incubated in darkness for 20 min. A fluorescence spectrophotometer (F-4600, Hitachi, Tokyo, Japan) was used to determine the fluorescence intensity of the mixture. The H_0 value was calculated from the protein concentration versus fluorescence intensity plot.

2.4.5. Particle size and zeta potential

TM samples were detected using a Nanoparticle zeta potential analyzer (Nano ZS900, Malvern Instruments Limited, Worcestershire, UK), following a method described by Yu et al. (2018). Deionized water was used to dilute the TM samples to 0.5 mg/mL. The particle size and zeta potential of TM were measured at room temperature (25 ± 2 ℃).

2.5. Allergenicity analysis

2.5.1. Sodium-dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

TM sample was thoroughly mixed with 5 × loading buffer at a volume ratio of 4:1, and the electrophoresis sample was heated for 5 min. Volumes of 5 μL and 10 μL of marker (10–250 kDa) and each sample, respectively, were loaded per lane. Electrophoresis was performed on a 12% separation gel for 30 min at 80 V, followed by 2 h at 120 V. After running, the prefabricated gel was stained with protein staining buffer, and then decolorized in deionized water until the protein bands were clear. The gels were scanned with a chemiluminescence imaging system (Tanon 4600, Tanon Science & Technology Co., Ltd, Shanghai, China).

2.5.2. Enzyme-linked immunosorbent assay (ELISA)

Changes in allergen TM content during processing were analyzed using a double-antibody sandwich ELISA kit. TM samples and standards were added to the microtiter plate and incubated (37 °C, 1 h). After washing the plate with wash buffer for 5 times, polyclonal antibody/peroxidase-conjugated goat anti-rabbit IgG mixture was added. Plates were incubated and then washed again. Finally, the system was monitored by adding TMB. When the OD_{450} of standard 1 reached 0.08–0.09, the stop solution was added. The absorbance of each treatment group was detected at 450 nm, and concentrations for each sample were calculated using a standard curve obtained by the TM allergen standard. The reduction rate of allergen TM was calculated using the following formula:

$$\text{Reduction rate } (\%) = \frac{(C - C_0)}{C_0} \times 100$$

Where C_0 and C represent the concentration of the Control and treatment group, respectively.

2.6. Simulated gastrointestinal digestion stability assay

Simulated gastrointestinal digestion of the TM sample was carried out as described by Liu et al. (2019) with some modifications. The TM sample was divided into aliquots of 10 mL (1 mg/mL) in preparation for the experiment. Digestion was carried out at 37 ℃. Pepsin was added (1:50 pepsin to protein, w/w) at pH 2.0. At various time points (0, 5, 15, 30, 45 and 60 min), 200 μL of the reaction solution was taken out and the digestion was stopped by adding 60 μL of 0.2 mol/L Na_2CO_3. The pH of the resulting solution was adjusted to 7.5, then trypsin was added (1:200 trypsin to protein, w/w). At various time points (0, 1, 5, 7, 15, 30, 60, 120 and 180 min), 200 μL of the solution was taken out and immediately quenched by heating for 10 min. The enzymatic hydrolysate was separated using 12% SDS-PAGE gels. The total protein content of the TM sample before and after gastrointestinal digestion was measured by the bicinchoninic acid method. The *in vitro* protein digestibility (IVPD) of the TM sample was obtained using the following equation:

$$\text{IVPD } (\%) = \frac{(P_0 - P_1)}{P_0} \times 100$$

Where P_0 is the initial protein content and P_1 is the final undigested protein.

2.7. Statistical analysis

All experiments were performed in triplicate, and the data were expressed as mean ± standard deviation. The SPSS software (26.0, SPSS Inc., Chicago, USA) was used to determine the significant differences between the samples with one-way analyses of variance (ANOVA) and Duncan's multiple range test ($P < 0.05$). Principal component analysis (PCA) was conducted using the Origin software (version 2018, Microcal Inc., MA, USA).

Table 1
Changes in amino acid composition of TM treated with ultrasound-assisted HTP.

Amino acid (mg/100 mL)	Treatment			
	Control	HTP	U500	UH500
Met[1]	0.23 ± 0.07[b]	0.55 ± 0.12[a]	0.49 ± 0.08[a]	0.28 ± 0.10[b]
Glu	13.52 ± 0.13[c]	14.66 ± 0.28[a]	14.15 ± 0.40[b]	13.59 ± 0.18[c]
Tyr	2.68 ± 0.23[b]	2.81 ± 0.15[b]	3.64 ± 0.23[a]	3.07 ± 0.58[ab]
Cys	0.30 ± 0.06[a]	0.20 ± 0.13[a]	0.21 ± 0.10[a]	0.25 ± 0.12[a]
Asp	7.26 ± 0.17[a]	7.89 ± 0.43[a]	7.88 ± 0.21[a]	7.57 ± 0.57[a]
His	0.64 ± 0.11[a]	0.69 ± 0.19[a]	0.77 ± 0.19[a]	0.57 ± 0.12[a]
Ser	3.34 ± 0.13[a]	3.68 ± 0.08[a]	3.54 ± 0.26[a]	3.34 ± 0.40[a]
Arg	5.05 ± 0.47[a]	5.61 ± 0.23[a]	5.24 ± 0.41[a]	5.04 ± 0.53[a]
Gly	1.20 ± 0.14[a]	1.22 ± 0.41[a]	1.39 ± 0.24[a]	1.44 ± 0.30[a]
Thr	2.97 ± 0.62[a]	3.54 ± 0.12[a]	3.03 ± 0.25[a]	2.85 ± 0.58[a]
Pro[1]	1.07 ± 0.24[a]	1.14 ± 0.36[a]	1.14 ± 0.37[a]	1.12 ± 0.32[a]
Ala[1]	3.80 ± 0.70[a]	4.14 ± 0.10[a]	3.98 ± 0.46[a]	3.82 ± 0.36[a]
Val[1]	3.61 ± 0.23[a]	3.89 ± 0.31[a]	3.81 ± 0.39[a]	3.62 ± 0.40[a]
Ile[1]	3.30 ± 0.83[a]	3.51 ± 0.27[a]	3.50 ± 0.26[a]	3.34 ± 0.30[a]
Leu[1]	7.81 ± 0.17[a]	8.33 ± 0.31[a]	8.21 ± 0.33[a]	7.87 ± 0.43[a]
Phe[1]	1.39 ± 0.17[a]	1.47 ± 0.35[a]	1.48 ± 0.13[a]	1.44 ± 0.23[a]
Lys	6.22 ± 0.18[a]	6.78 ± 0.42[a]	7.00 ± 0.69[a]	6.47 ± 0.28[a]
Total amino acid	64.41 ± 2.94[a]	70.13 ± 0.96[a]	69.47 ± 2.95[a]	65.67 ± 4.49[a]
Non-polar amino acids (%)	32.92 ± 1.23[a]	32.83 ± 0.75[a]	32.56 ± 0.59[a]	32.72 ± 0.81[a]

Note: Different lowercase letters (a–c) in the same line indicate significant differences at $P < 0.05$. "1" represents non-polar amino acids.

3. Results and discussion

3.1. Effect of ultrasound-assisted HTP treatment on TM structure

3.1.1. Amino acid composition analysis of TM

Changes in the amino acid composition of TM could reflect the alteration in its conformation. The amino acids content of TM samples treated with ultrasound-assisted HTP was shown in Table 1. There was an increasing trend in the content of most free amino acids in the treatment group. Among them, the content of methionine (Met), glutamic acid (Glu) and tyrosine (Tyr) was significantly increased in the HTP and U500 ($P < 0.05$). An increase in total amino acids was observed in the TM treated samples, which may be due to the release of some amino acids from the protein destroyed by HTP and ultrasonication. However, the cysteine (Cys) content and the proportion of non-polar amino acids were decreased following treatment. High intensity ultrasound could induce the excitation of water to form hydrogen and hydroxyl radicals, which then react with oxygen to produce oxides (Wang & Xu, 2012). Notably, Cys was subject to oxidative modification in radical oxidation. HTP treatment was easy to transfer heat, causing denaturation, aggregation and oxidation within or between the TMs (Liu et al., 2021a). Oxidation of Cys resulted in the formation of disulfide bonds and mixed disulfides like glutathione. In addition, the linear epitopes of TM were peptide segments composed of some consecutive amino acids, which mainly existed in the primary structure and was important in the allergic reaction. Previous experiments (Reese et al., 2005) showed that

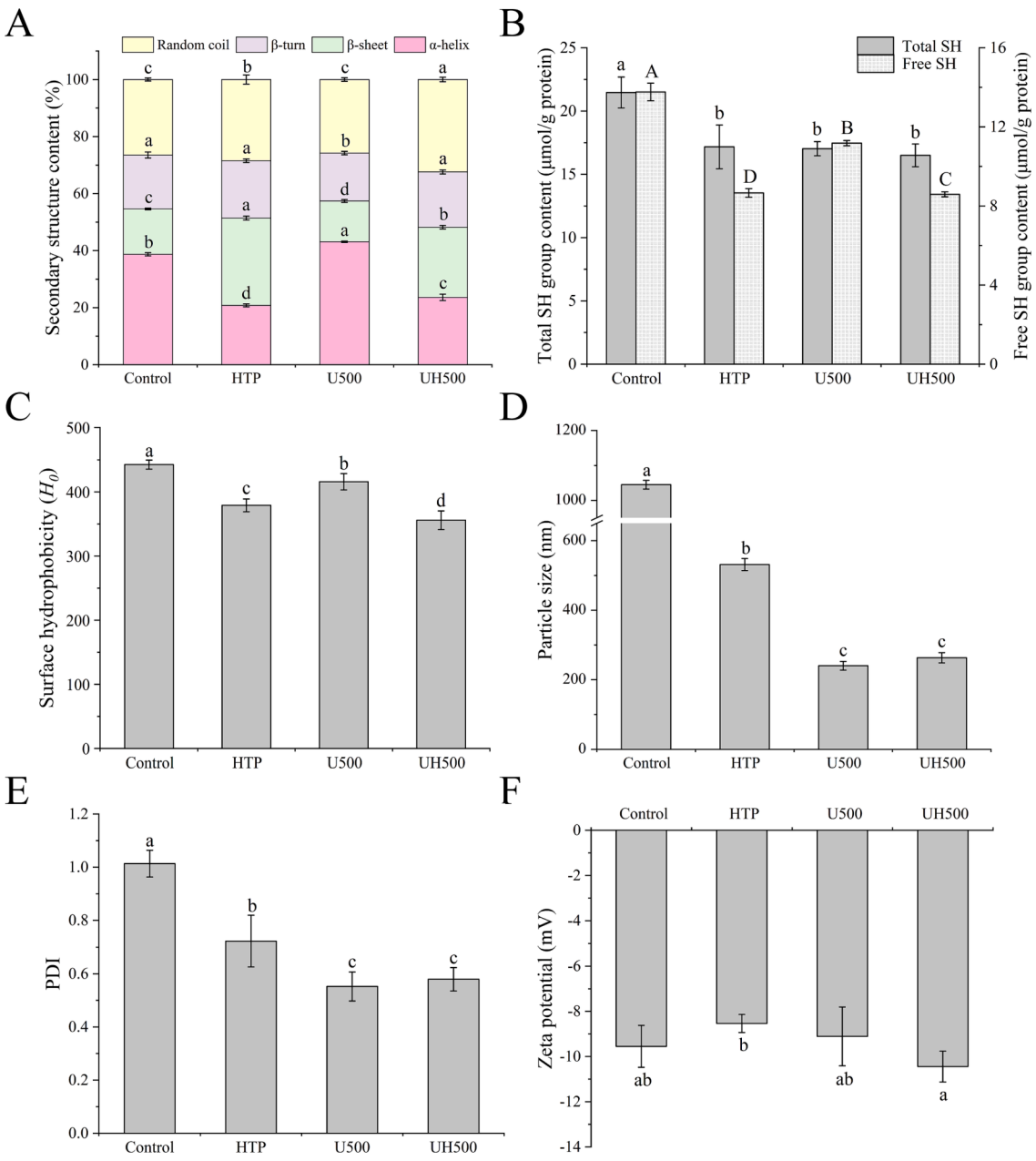

Fig. 1. Changes in secondary structure content (A), sulfhydryl group content (B), surface hydrophobicity (C), particle size (D), PDI (E), and zeta potential (F) of TM treated with ultrasound-assisted HTP. Different uppercase (A–D) and lowercase (a–d) letters indicate significant differences at $P < 0.05$.

processing or site-directed mutagenesis had no significant effect on allergen linear epitopes and could not eliminate their allergenicity completely.

3.1.2. *Secondary structure analysis of TM*

CD spectroscopy was performed to analyze the secondary structure changes of the TM samples. As shown in Fig. 1**A**, the relative contents of α-helix, β-sheet, β-turn and random coil in the control group were 38.7 ± 0.56%, 15.9 ± 0.30%, 18.9 ± 0.56% and 26.5 ± 0.26%, respectively, indicating that α-helix was the main configuration. These findings are consistent with the results mentioned by Jin, Deng, Qian, Zhang, Liu and Zhao (2015). The α-helix in the HTP was significantly decreased (17.9%) and was converted to β-sheet and random coil ($P < 0.05$). In the U500 group, the α-helix increased significantly by 4.4% ($P < 0.05$). TM samples treated with UH500 produced similar results as HTP treatment. In general, ultrasound-assisted HTP treatment transformed the secondary structure from ordered and rigid to disordered and flexible. Previous studies demonstrated that a reduction in the proportion of α-helix led to the masking of epitopes and lower allergenicity in peanut *Ara h 1* (Blanc et al., 2011) and soy protein extracts (Pi, Sun, Guo, Chen, Cheng, & Guo, 2022) after HTP treatment. The increase in the proportion of α-helix after ultrasonication indicated that ultrasonication promoted the α-helix formation, disrupted β-sheet, β-turn, and random coil configurations, while reducing interactions between amino acids in the three regions. Yang et al. (2017) found that disrupting the hydrogen bonding between peptide chains alters the spatial structure of protein molecules. Dong et al. (2020) demonstrated a similar change of the secondary structure in promoting the elimination of shrimp allergenicity during ultrasonication.

For the TM samples that were initially treated with ultrasound, followed by HTP, the ultrasound treatment was beneficial for the reconstruction of the internal hydrogen bonds, and the subsequent HTP caused the protein molecules to form irregular coils (Zhang, Zhao, Li, Wang, Hou, & Jiang, 2022), enhancing the conformational changes of

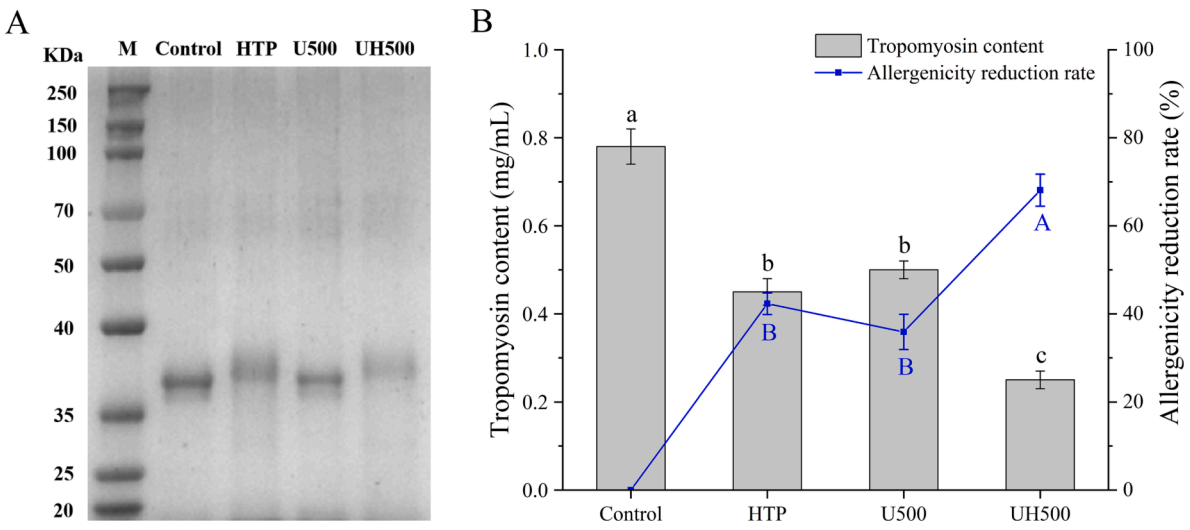

Fig. 2. SDS-PAGE profile (A) and TM content (B) of clam TM treated with ultrasound-assisted HTP. Different uppercase (A–B) and lowercase (a–c) letters indicate significant differences at $P < 0.05$.

the TM. Because the conformational epitopes of allergens were discontinuous peptides in the spatial structure of properly folded proteins, these changes of TM were important reasons for variations in allergenicity observed.

3.1.3. Tertiary structure analysis of TM

Sulfhydryl (SH) group content can indirectly reflect changes in protein tertiary structure. Fig. 1**B** shows that the total SH group content in the control group was 21.5 ± 1.22 μmol/g protein. After three treatments, the total SH content decreased significantly ($P < 0.05$), but there was no significant difference among HTP, U500, and UH500 groups. Meanwhile, the UH500 group had the lowest free SH content, which was 37.7% lower than the control group. Similarly, Zhang et al. (2022b) discovered a decrease in SH content of shrimp surimi gels following ultrasonic treatment. The decrease may be related to the oxidation of SH groups into disulfide bonds. Each helix of the TM contained one Cys at position 81 (Motoyama, Ishizaki, Nagashima, & Shiomi, 2006). Free SH content was associated with decreased Cys content in the amino acid composition. The change in free SH groups was due to the fact that ultrasound can generate highly reactive free radicals from protein solution, causing the oxidation of sensitive free SH groups, as well as the oxidation effects of temperature and pressure. Compared with ultrasound treatment at 500 W, HTP treatment significantly decreased the free SH group content, indicating that the oxidative modification effect of HTP was stronger. More disulfide bonds were formed by the exposed free SH groups in the tertiary structure of TM after the combined treatment. The ultrasound-assisted HTP treatment could build up the protein's three-dimensional network structure, in which disulfide bonds played a basic role. Previous studies reported that loss of a high level of spatial organization reduced the allergenicity of proteins (Costa et al., 2022; Yao, Jia, Lu, & Li, 2022).

Surface hydrophobicity (H_0) can be expressed indirectly by measuring the amount of ANS and hydrophobic groups. As is shown in Fig. 1**C,** the H_0 of HTP, U500, and UH500 decreased by 14.3%, 6.1% and 19.6%, respectively. High intensity ultrasound could alter the polar groups of protein molecules and cause reaggregation through high shear and cavitation physical fields, leading to the burial of hydrophobic amino acid residues (Zhang et al., 2022b). Furthermore, the molecular structure of TM was destroyed due to the pressure and thermal energy of HTP, and the hydrophobic region was also changed. The results are similar to previous reports that thermal treatment reduced the H_0 of *Ara h 1* (Tian, Rao, Zhang, Tao, & Xue, 2018). In conclusion, the combined treatment disrupted the tertiary structure of TM, and protein denaturation, protein unfolding, and protein molecule reaggregation jointly contributed to the variation in H_0, thereby damaging and masking the hydrophobic region. Studies demonstrated that the masking of epitopes induced by changes in H_0 was responsible for the reduction in allergenicity.

3.1.4. Particle size and zeta potential analysis of TM

Particle size and the polydispersity index (PDI) were key parameters that indirectly affected digestion and consequent sensitization of the immune system. Particle size distribution can reflect the degree of protein aggregation or degradation, and the PDI was correlated with particle size uniformity. As shown in Fig. 1**D,** TM was present in an aggregated state with an average particle size of 1045 ± 12.53 nm. The U500 group had the largest decrease in particle size, with 240.07 ± 12.58 nm ($P < 0.05$), followed by the UH500 and HTP groups with particle sizes of 263.17 ± 14.78 nm and 531.37 ± 17.18 nm, respectively. The corresponding PDI increased in the following order: the U500 group, UH500 group, HTP group and the control group (Fig. 1**E**), which demonstrated that the effect of ultrasound on particle size was more significant after the combined treatment. The cavitation effect produced by ultrasound generated turbulence and high shear energy, which provided enough energy for the collision of TM molecules to disrupt the aggregated state of proteins and make TM particle fragmentation increase. Similarly, X. Zhao et al. (2022) found that ultrasonic power of 300–500 W could dissociate soy protein into small molecules. Ding, Tian, Wang, Deng, Mao and Sang (2021) reported that the particle size of scallop protein decreased significantly with increasing ultrasonic power (0–600 W). At the same time, the HTP treatment also reduced the average particle size of TM. This finding indicated that the thermal effect and pressure had disrupted the aggregated state of the protein and the degree of homogeneity had improved compared to the control group. In summary, UH500 treatment reduced the particle size and dispersion of TM by destroying the chemical bonds and intermolecular interactions. Small particles could enlarge the exposed area, increasing their availability for hydrolysis by protease.

The change in zeta potential can reflect the degree of surface charge and system stability. As shown in Fig. 1**F,** all TM samples exhibited a net negative charge, indicating that more negative amino acids on the protein surface. After ultrasound-assisted HTP treatment, the absolute value of zeta potential was increased. A higher absolute value indicated better system stability (Morel, Dehlon, Autran, Leygue, & Bar-L'Helgouac'h, 2000). It can be explained by changes in the structure and conformation of TM in the UH500 group, with more charges

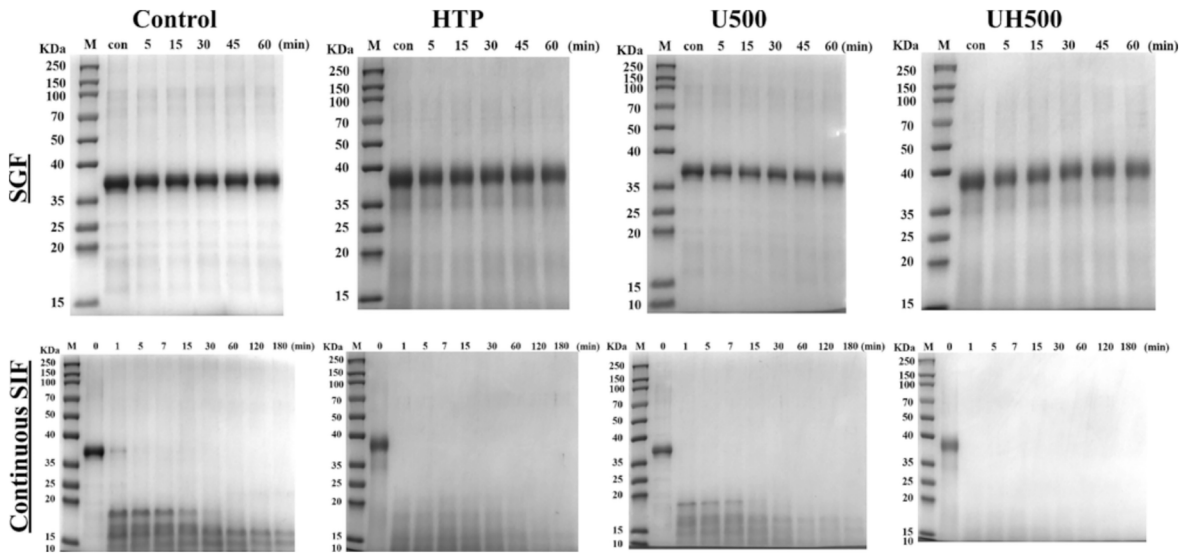

Fig. 3. The digestion stability of processed TM in simulated gastric fluids (SGF) digestion and continuous simulated intestinal fluid (Continuous SIF) digestion, respectively, assessed by SDS–PAGE. Lane M, protein marker.

accumulating on the protein molecular chains. The UH500 treatment led to strong electrostatic repulsions between the protein molecules. Additionally, the enhanced electrostatic repulsion could avoid and delay the aggregation of TM molecules to maintain the stability of the small particles and facilitate the digestive degradation of TM.

3.2. Effect of ultrasound-assisted HTP treatment on TM allergenicity

3.2.1. Changes in clam TM treated with ultrasound-assisted HTP

SDS-PAGE was used as a visualization method to analyze the changes in TM after ultrasound-assisted HTP treatment. As presented in Fig. 2**A**, the molecular weight of TM, the main allergen found in clams, was about 36 kDa. As processing proceeded, the protein bands of TM decreased in intensity, but no new protein bands appeared. The TM bands in the HTP and UH500 groups have shown a diffuse trend. A previous study had shown that HTP can loosen the structure of allergenic proteins and make the electrophoretic bands indistinguishable (Yu, Cao, Cai, Weng, Su, & Liu, 2011). In the study, there was protein degradation during the treatment process, resulting in the conversion of part of the protein into low molecular peptides, which reduced the allergenicity. The intensity of the bands in order from strong to weak were U500, HTP, and UH500. Thus, the combined treatment had the optimal effect on TM, resulting in a significant reduction in TM content.

3.2.2. Changes in allergenicity of clam TM treated with ultrasound-assisted HTP

Sandwich ELISA was used to evaluate the degree of reduction of TM allergenicity by processing treatment. As shown in Fig. 2**B**, TM exhibited a significant decline in immune response after the ultrasound and HTP treatments. The allergenicity of the U500, HTP and UH500 groups was reduced by 35.9%, 42.3% and 68.1%, respectively. And the combined treatment reduced TM content to the lowest level (0.25 ± 0.02 mg/mL). The results showed that ultrasound and HTP had a synergistic effect in reducing TM allergenicity. The decrease was associated with the conformational alteration of TM, especially the destruction or concealment of the allergenic epitopes existing on the protein surface (Venkatachalam et al., 2008). The ultrasound-induced hyposensitization was primarily attributed to the cavitation effect and excessive agitation generated by microstreaming, which may destroy the Van der Waals interactions and hydrogen bonds in polypeptides, leading to TM modification (Nayak et al., 2017). In a similar study, J. Zhao et al. (2022) discovered a reduction in allergenicity in shrimp extracts treated by HTP, with an abatement rate of 40.1%. Pi et al. (2022) found that autoclaving reduced the allergenicity of soybeans by 82%–83%. Higher temperature and pressure affected IgE-binding epitopes of allergens through protein-chemical crosslinking, which changed the recognition of allergens by IgE, and thereby reducing the allergenicity.

In addition, TM allergenicity was closely related to the changes in the secondary and tertiary structures (Dong, Wang, & Raghavan, 2021). In this study, the results revealed that UH500 was an effective method to reduce TM allergenicity. There was a significant reduction in α-helix content, SH content, and H_0, as well as an increase in random coil content. Thus, the structures of TM were loosened, which might alter the conformational epitopes of the allergen TM. Meanwhile, both ultrasound and HTP treatment had the potential to promote the elimination of allergen TM, but ultrasound caused the least damage to the original physicochemical properties of TM.

3.3. Effect of ultrasound-assisted HTP treatment on the digestibility of TM

Gastrointestinal digestion and intestinal permeability were important factors in assessing whether TM can cause allergic reactions in individuals. Throughout the digestion process, the protein was enzymatically digested using pepsin and trypsin, thus affecting the allergenicity of the digestive products. As shown in Fig. 3, the initial TM was at about 36 kDa. TM still existed in the digested products of the samples from each treatment group, suggesting a high digestive resistance to gastric digestion. Previously, seven heat-stable/indigestible amino acids of crab TM were discovered and proven to be linear epitopes related to IgE binding activity (Liu et al., 2021a). Fig. 3 showed that the linear epitopes of TM treated with ultrasound and HTP were hardly digested by SGF, so the allergenicity of linear epitopes was still maintained.

In the continuous simulated intestinal fluid (Continuous SIF) digestion, TM was susceptible to digestion by trypsin. The untreated TM band narrowed at 1 min, producing fragments of approximately 16, 18 and 19 kDa, and two fragments still existed at 180 min. For HTP treatment, the degradation bands of TM were diffused and difficult to distinguish. In the U500 group, degraded fragments of approximately 14 and 16 kDa were observed. Notably, the fastest degradation was observed for TM treated with ultrasound-assisted HTP, and the digested fraction was invisible after 60 min. This result indicated that the digestion resistance of TM after combined treatment was significantly reduced. This significant reduction in digestion resistance may be due to the cavitation

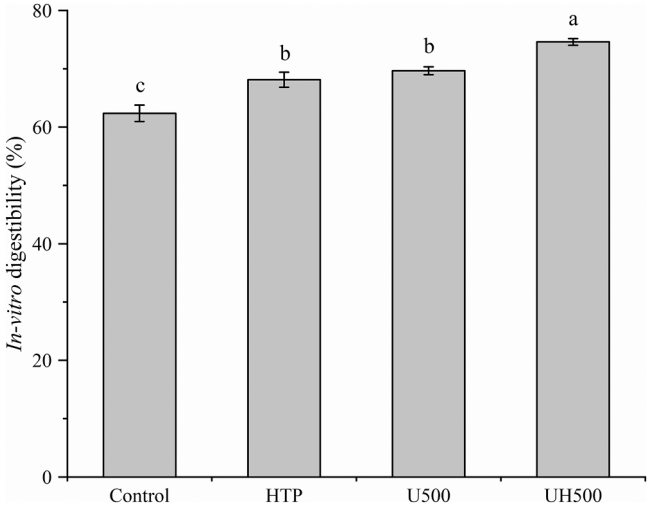

Fig. 4. *In vitro* digestibility of TM treated with ultrasound-assisted HTP. Different lowercase (a–c) letters indicate significant differences at $P < 0.05$.

effect of ultrasound modifying the conformation of the protein, changing the sensitivity of the protein to trypsin by exposing the cleavage site. Furthermore, HTP treatment destroyed the structure of TM significantly, especially the α-helix. Liu et al. (2021b) found that there were heat and digestion stable epitope peptides in α helix, which would be available for the reaction with antibodies. HTP treatment increased the susceptibility of TM to protease hydrolysis. In summary, combined treatment changed the structure of TM and promoted its digestion, with lower allergenicity of TM and its digestion products.

Differences in digestion patterns were observed between SGF and Continuous SIF as a result of different protease cleavage sites. Pepsin had a tendency to break peptide bonds next to Phe and Tyr residues, whereas trypsin had hydrolysis sites next to Lys and Arg (Mikita & Padlan, 2007). Table 1 revealed that TM had lower Phe and Tyr content and higher Lys and Arg content. Therefore, TM was resistant to pepsin, but sensitive to trypsin. The multiple proteolytic effects of trypsin can effectively hydrolyze TM to an extent and significantly reduce its allergenicity.

The combined effect of ultrasound and HTP treatment on the digestibility of TM was demonstrated in Fig. 4. HTP, U500, and UH500 processing can significantly enhance the *in vitro* digestibility of TM. The digestibility of UH500 was significantly increased to 74.6%, and the combined effect of ultrasound and HTP increased the digestibility by 12.3% ($P < 0.05$). In a similar study, Ding et al. (2021) treated scallop mantle protein with ultrasound and found that the *in vitro* digestibility increased from 70.3% to 86.3%. Another study involving autoclaved shrimp extracts showed that digested fractions were invisible within 30 min of SGF digestion and 20 min of SIF digestion (Mikita et al., 2007). This suggested that heat treatment can significantly increase the digestibility in shrimp, possibly due to heat-induced structural changes that exposed more cleavage sites and increased the susceptibility of the protein to proteolytic attack (Liu et al., 2019). In the combined treatment group, ultrasound had a greater effect on TM digestibility, which may be due to the increased number of binding sites with an enzyme hidden in TM following ultrasound shear force and cavitation damage. In addition, the smaller particle size of TM accelerated the penetration of the enzyme into the protein matrix and enabled more complete enzymatic digestion of protein.

3.4. Principal component analysis (PCA) of TM allergenicity

To further elucidate the factors that contributed to the hypoallergenic properties observed in the UH500 group, PCA was applied. Fig. 5 included most of the variance of all quantified variables; PC1 and PC2 accounted for 54.4% and 22.7% of the overall data variation, respectively. In this study, four groups were separated clearly under the PCA model, illustrating that ultrasound and HTP contributed significantly to the modification effect of TM. In the combined processing group, the TM content (allergenicity) can be best explained by PC1, which positively

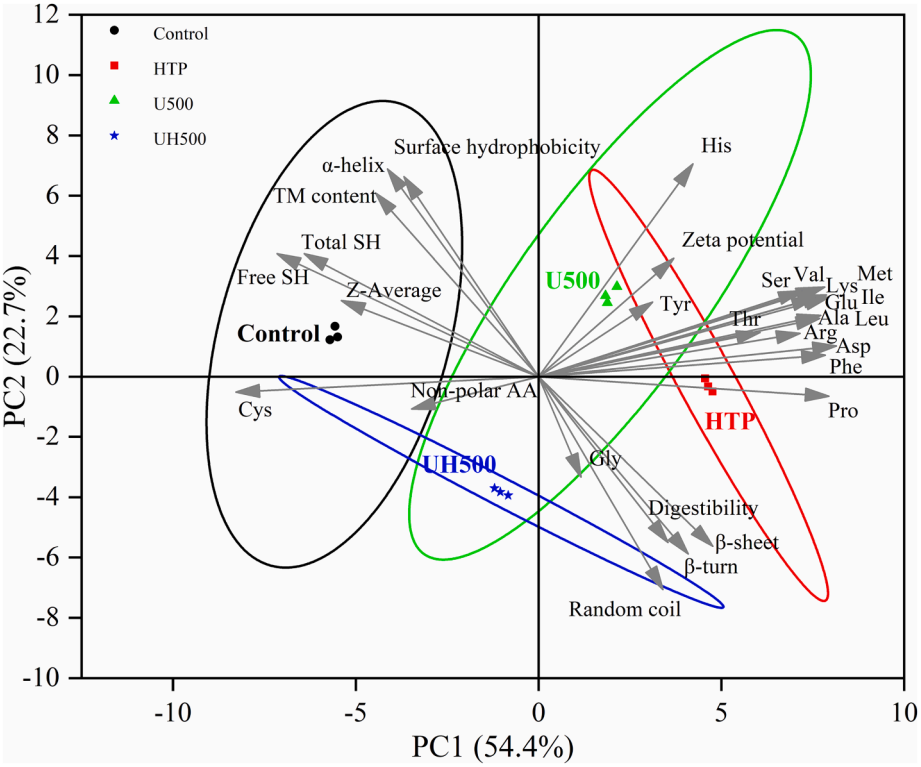

Fig. 5. Principal component analysis (PCA) biplot and component loadings (evaluated parameters).

correlated with Cys, α-helix, SH group content, H_0 and particle size, but negatively correlated with β-sheet, β-turn, random coil, zeta potential and *in vitro* digestibility. These findings demonstrated that differences in TM allergenicity can be explained by varying degrees of protein modification and TM digestion as a result of structural changes in treated TM, which destroyed some allergenic epitopes. The decrease in α-helix, SH content, H_0 and particle size, as well as the increase in random coil and *in vitro* digestibility, were beneficial for the reduction of allergenicity. These structural changes might lead to the unfolding of the protein, and IgE-binding epitopes might be disrupted and modified, exposing more protein hydrolytic cleavage sites, resulting in lower allergenicity and easier digestion.

4. Conclusions

This study systematically demonstrated the effects of ultrasound-assisted HTP treatment on the structure and allergenicity of TM from clams. In the combined processing treatment group, the TM allergenicity was positively correlated with Cys, α-helix, SH group, H_0 and particle size, but negatively correlated with random coil and *in vitro* digestibility. Ultrasound-assisted HTP treatment significantly affected the molecular conformation of TM, with more significant changes in the secondary and tertiary structures, while enhancing the gastrointestinal digestibility of TM. These changes might result in the destroying and masking of allergenic epitopes, thereby diminishing the allergenic risk of TM from clams and its digestion products. The present study provided a novel method for reducing the allergenicity of TM and laid a theoretical foundation for developing hypoallergenic clam products (such as canned clam meat) in the future.

CRediT authorship contribution statement

Yachun Chen: Conceptualization, Data curation, Writing – original draft. **Guifang Tian:** Software, Writing – review & editing, Visualization. **Liwen Wang:** Methodology, Formal analysis. **Yaxin Sang:** Validation, Resources, Supervision. **Jilu Sun:** 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.

Data availability

Data will be made available on request.

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