

Effects of dielectric barrier discharge cold plasma on structure, surface hydrophobicity and allergenic properties of shrimp tropomyosin

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ABSTRACT

Effects of dielectric barrier discharge (DBD) cold plasma (CP) on structure, surface hydrophobicity and allergenic properties of tropomyosin (TM) in shrimp were investigated in this study. Results showed that the molecular weight of TM increased and the protein concentration decreased with CP treatment time increased. The content of free amino acids was increased by 74.7 % and the distribution of aromatic amino acids was altered. The content of α -helix was decreased by 69 % and the surface hydrophobicity increased by 57.8 % after 20 min treatment. Allergenicity analysis showed that the IgE binding capacity decreased by 96 % after 20 min treatment, and the degranulation indexes of KU812 cells like the β -HEX release rate, the intracellular calcium ion intensity, the release of histamine and inflammatory cytokines (IL-4, TNF- α) were decreased by 32.5 %, 31.0 %, 37.3 %, 51.7 %, and 70.2 %, respectively. The current study confirmed that DBD CP could reduce the TM allergenicity through structural changes.

1. Introduction

Shrimp is nutritious seafood and rich in protein, minerals, polyunsaturated fatty acids and vitamins. However, shrimp is an allergy-prone food for allergy sufferers (Cheng et al., 2022; Ekezie et al., 2019; Li et al., 2021). Shrimp allergy belongs to type I hypersensitivity reactions mediated by immunoglobulin E (IgE) (Zhang et al., 2021), which often accompanies respiratory, digestive, and cutaneous symptoms, or even to death. It has been reported that the main allergen of shrimp is tropomyosin (TM), which is a dimer with an α -helical structure with a fibrillar protein having a molecular weight of 34–39 kDa (Faisal et al., 2019). TM is a high temperature-resistant allergenic protein that induces allergic reactions by binding with specific IgE epitopes in some patients. Therefore, thermal processing is inefficient for reducing the TM allergenicity in shrimp and may even increase the IgE binding capacity of TM in boiling water (Laly et al., 2019).

Reduction of allergenicity of aquatic products like shrimp using food processing technology is always a research hotspot. Nowadays, non-

thermal processing methods such as ultrasound, irradiation, and high-pressure treatment have been developed as good choices to decrease the allergenicity in aquatic products (Byun et al., 2000; Dong et al., 2020; Sankar & Panda, 2022). They can reduce the immune activity and digestive stability of allergens to a certain extent. However, they mainly act on non-covalent bonds like hydrogen bonds, electrostatic interactions, and hydrophobic interactions in protein molecules, rather than covalent bonds, and the reduction effect is not obvious (Pan et al., 2021; Zhu et al., 2022).

Differently, cold plasma (CP), as an innovative non-thermal processing technology, can not only modify non-covalent bonds of proteins, but also covalent bonds (Jiang et al., 2020). To be specific, plasma is regarded as the fourth state of matter, which is a gas mass containing electrons, ions, free radicals, excited atoms, molecules, other active particles and electromagnetic radiation like UV, with an approximate 10^{-9} ionization in typical atmospheric pressure (Moisan & Pelletier, 2012). Depending on the carrier gas, it can form various active oxidation systems. Moreover, other physical effects can be induced by plasma,

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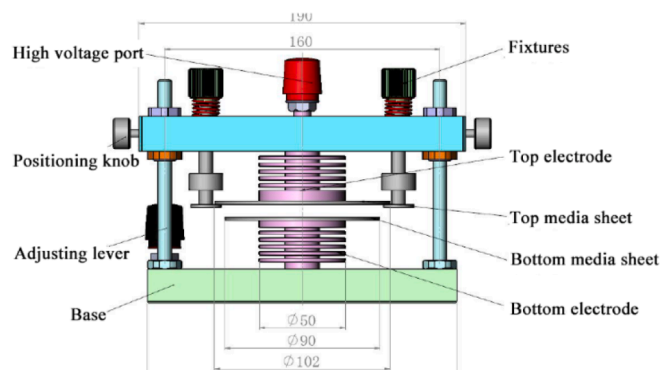
such as ultraviolet rays, and shock waves (Esua et al., 2021a; Esua et al., 2021b; Esua et al., 2022; Esua et al., 2022; Esua et al., 2022; Esua et al., 2022). Because of the advantages of low temperature, high efficiency, and no pollution, CP is increasingly applied in food-related industries, mostly including sterilization, toxin degradation, and pesticide residue elimination (Ali et al., 2021; Ali et al., 2021; Ali et al., 2022; Chen et al., 2020; Varilla, Marcone, & Annor, 2020). Recent studies have demonstrated that CP has the potential to the reduction of allergenicity of food allergens, like soy protein isolate, peanut protein, beta-lactoglobulin, α -casein and whey proteins (Ekezie et al., 2019; Ji et al., 2018; Liu et al., 2021; Meinschmidt et al., 2016; Ng et al., 2021).

As for the TM treatment using CP, very limited information is available. In our previous study, a cold argon plasma jet (CAPJ) was used to reduce the allergenicity of TM in king prawn (*Litopenaeus Vannamei*) and it was reported that IgE and IgG binding capacities were decreased by 17.6 % and 26.87 %, separately (Ekezie et al., 2019). In order to further improve the allergenicity reduction effectiveness, in this study, dielectric barrier discharge (DBD) CP was used to reduce the allergenicity of TM in shrimp. The underlying mechanism of allergenicity reduction and TM structural changes were explored and a cell model was constructed to assess the biological effects caused by changes in TM structure on the alteration of the allergenicity of TM.

2. Materials and methods

2.1. Materials and reagents

Shrimp (deceased) was obtained from a local supermarket (Guangzhou, China). Human peripheral blood basophilic leukaemia cells (KU812) were supplied by Warner Biotechnology Co., Ltd. (Wuhan, China). Human serum against shrimp (specific IgE level >0.35 kU/L) was obtained from PlasmaLab International Inc. (Everett, WA, USA), which was an FDA-licensed blood collection company. All human serum-use agreements were approved by the Ethics Committee of South China University of Technology (Guangzhou, China) under the International Ethical Guidelines for Biomedical Research Involving Human Subjects. Horseradish peroxidase (HRP)-conjugated Goat anti-rabbit IgG was obtained from Sangon Biotech (Shanghai) Co. Ltd. (Shanghai, China). HRP-conjugated goat anti-human IgE antibodies and Hank's Balanced Salt Solution (HBSS) was bought from Thermo Fisher Scientific Inc. (Invitrogen™, Carlsbad, CA, USA). Amino acid (AA) content detection kit and tetramethylbenzidine (TMB) substrate were bought from Solarbio Technology Co., Ltd. (Beijing, China). Bradford protein concentration detection kit was purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). Human histamine, IL-4, and TNF- α ELISA kits were obtained from Jiangsu Meimian Industry Co., Ltd. (Yancheng, China). Only chemical reagents of analytical grade were used in this study, except otherwise stated.



(a)



(b)



(c)

Fig. 1. Overview of DBD cold plasma. (a) plasma generator, (b) power supply, (c) plasma effect.

2.2. DBD CP treatment

TM was extracted from shrimp (*Fenneropenaeus Chinensis*) in the laboratory according to the protocol of Ekezie et al. (2019). Fig. 1 shows the DBD CP generator (DBD-50, Corona Lab, Nanjing, China), which consisted of a reactor and a power source. A high-voltage power supply (Fig. 1b) was connected between the top and bottom electrodes (Fig. 1a). When starting, the high-voltage current would break down the air between the top and bottom dielectric sheets, and the plasma with a glow-discharge-like effect (Fig. 1c) was produced, which was actually composed of many micro-discharges. The samples were processed in the discharge space between the top and bottom dielectric sheets. DBD plasma (input voltage 50 V, input current 1 A, applied voltage 50 kV) was used to treat 10 mL of TM solution (0.2 mg/mL, ultrapure water) with different times (0, 5, 10, 15, and 20 min). After that, the treated TM was lyophilized and redissolved with 2 mL of PBS (10 mM, pH 7.4) for further use.

2.3. Structure analysis of TM

The protein concentration was determined by the Bradford protein concentration detection kit (Beyotime Biotechnology Co., Ltd., Shanghai, China) as per specific procedures given in the manual.

SDS-PAGE analysis was applied to acquire protein molecular distributions of the TM samples, as per the description given in the study by Ekezie et al. (2019).

Free amino acid content was detected by an amino acid content detection kit (Solarbio Technology Co., Ltd., Beijing, China). The detection principle was that the α -amino group of amino acids could react with ninhydrin hydrate, resulting in a 570 nm characteristic absorption peak, thereby the amino acid content was obtained by the collection of the absorbance at 570 nm using a microplate reader (Multiscan 1 MK3, Thermo Fisher Scientific Inc., Waltham, MA, USA). Besides, the calculation method was given in the manual.

The alterations of the secondary structure of TM samples were detected by a circular dichroism spectrometer (Chirascan-plus, Applied Photophysics Ltd., Surrey, UK) according to the description of Ekezie et al. (2019). The fluorescence spectrum of the TM samples was obtained using a spectrofluorometer (RF-6000, Shimadzu Co., Kyoto, Japan) according to the report of Lv et al. (2021). UV spectroscopy of TM samples with the wavelength range of 200–600 nm was measured by a spectrophotometer (UV-1800, Shimadzu Co., Kyoto, Japan).

2.4. Determination of surface hydrophobicity

The surface hydrophobicity of TM samples was detected by 8-anilino-1-naphthalene sulphonic acid (ANS) fluorescent probe method as per the study by Song et al. (2015). Firstly, the protein concentration was diluted to a series of gradients of 0.05–1 mg/mL. Then, 2 mL of diluted samples with different concentrations was blended with 20 μ L of ANS solution (8 mM), with shaking for 3 min in the dark thereafter. The corresponding fluorescence intensity was obtained using a fluorescence spectrophotometer (RF-6000, Shimadzu Co., Kyoto, Japan).

2.5. Allergenicity analysis of TM

2.5.1. Indirect ELISA analysis

Indirect ELISA was used to detect the changes in the IgE binding capacity of TM by using the method of Zhenxing et al. (2006) with partial modifications. TM samples were diluted to 5 μ g/mL using carbonate buffer (50 mM, pH 9.6). During the washing procedure, the plate was washed 5 times (1 min for each time) with PBST (0.01 M PBS, 0.05 % Tween-20, pH 7.5). Human serum was diluted 20 times and HRP-anti-human IgE antibody was diluted 5000 times, respectively.

2.5.2. Dot blot analysis

Dot blot analysis is a visual expression of the IgE binding capacity of TM, which was elaborated by Lin et al. (2015). 3 μ L of 0.5 mg/mL TM sample was instilled into the surface of the Nitrocellulose (NC) membrane. Human serum was diluted 10 times and HRP-anti-human IgE antibody was diluted 500 times, respectively. Eventually, the membrane was scanned for grayscale analysis with a GS-800 densitometer (Model GS-800, Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.6. Cell model construction and analysis

2.6.1. Construction of sensitization model of KU812 cells

The sensitization model of KU812 cells was constructed according to the method of Zhu et al. (2019). KU812 cells were suspended in a complete RPMI 1640 medium (2 mM of L-glutamine, 100 U/mL of penicillin, 100 μ g/mL of streptomycin, 15 % of fetal bovine serum (FBS)) and subsequently cultured in a humidified incubator (Series 8000 DH, Thermo Fisher Scientific Inc., Waltham, MA, USA) with 5 % CO₂ at 37 °C. When the cells grew and proliferated to 2×10^6 cells/mL, they were washed once with sterile PBS solution (pH 7.4), and then resuspended ($>2 \times 10^6$ cells/mL) in a complete medium containing 10 % of shrimp allergy patient serum for activation.

2.6.2. Allergenic properties analysis of KU812 cells

After activating for 12 h, the cells were mixed gently and separated into different groups. TM under different plasma treatment conditions was added into each group to make the final protein concentration of 50 μ g/mL, and cultured at 37 °C for 1 h. Meanwhile, the addition of sterile PBS solution (pH 7.4) with equal volume was used as a blank control. After stimulating the activated cells with TM samples for 1 h, the cells and supernatant were collected by centrifugation (1500 rpm, 5 min) using a centrifuge (TGL-16 M, Cence Laboratory Instrument Development Co., Ltd., Changsha, China) separately for further experiments.

β -Hexosaminoglycosidase (β -HEX) release rate was measured as per the experimental protocol of Hoffmann et al. (1999). In the experimental groups, the supernatant was collected by direct centrifugation (1500 rpm, 5 min). In the control groups, the cells were treated with RIPA lysis buffer for 30 min and centrifuged (1500 rpm, 5 min) after lysis to collect the supernatant. In the blank group, the supernatant was replaced by an equal volume of sterile PBS (pH 7.4). 30 μ L of supernatant and 50 μ L of the substrate (40 μ M 4-nitrophenyl-2-acetylaminoglycopyranoside in citric acid-sodium citrate buffer) were mixed into a well of 96-well plate. After reacting for 1 h at 37 °C, 100 μ L of 0.2 M glycine solution (pH 10.7) was used to stop the reaction, and then the absorption value at 405 nm was obtained by a microplate reader (Multiscan 1 MK3, Thermo Fisher Scientific Inc., Waltham, MA, USA). According to the measured OD value, the β -HEX release rate was calculated with the following equation:

$$\beta - \text{HEX release rate (\%)} = \frac{\text{OD}_{\text{experimental groups}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{control groups}} - \text{OD}_{\text{blank}}} \times 100 \quad (1)$$

The calcium ion intensity was measured using the following methods. The stimulated cells were washed three times with complete RPMI 1640 medium (2 mM of L-glutamine, 100 U/mL of penicillin, 100 μ g/mL of streptomycin, and 15 % of FBS). After that, the cells were resuspended by fluorescent probe solution (1.5 μ M Fluo-4 AM in complete RPMI 1640 medium), and then cultured for 30 min in the dark at 37 °C. Thereafter, the cells were washed three times and resuspended with HBSS and the cells were cultured at 37 °C for 30 min in the dark. After appropriate shaking, a fluorescence spectrophotometer (RF-6000, Shimadzu Co., Kyoto, Japan) was used to detect the calcium ion intensity of cells with the 488 nm excitation wavelength and the 500–550 nm emission wavelength.

The content of released histamine and the content of cytokine (IL-4, TNF- α) released by the cells were detected using ELISA kits (Jiangsu Meimian Industry Co., Ltd., Yancheng, China), as per the manufacturer's

recommendations.

2.7. Statistical analysis

Statistical analysis of the data was carried out using Origin 2021 software (Origin Lab Co., MA, USA) and SPSS 19.0 software (IBM Co., NY, USA). One-way analysis of variance (ANOVA) was applied to analyze the significant differences among the means of the samples and represented in the form of mean $\pm$ standard deviation. $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Macroscopic characteristic analysis

As shown in Fig. 2a, with the prolongation of plasma treatment time, the volume of TM solutions gradually decreased. In addition, the white flocculent precipitate gradually changed to the yellow gelatinous precipitate (Fig. 2b). After the redissolving of freeze-dried protein, it was found that the TM redissolved solutions gradually changed from colourless and transparent to yellow and turbid. Besides, a small amount of undissolved yellow precipitate was observed floating in the solutions with 10–20 min treatment (Fig. 2c). Among them, the possible cause of the change in yellowness was the result of plasma oxidation of proteins by excluding Maillard reactions or any possible hypothesis of lipid peroxidation (Segat et al. 2015). Based on the above findings, it could be deduced that the phenomenon of the insoluble precipitate and colour changes was possibly associated with TM structure alterations.

3.2. Protein concentration analysis

The effect of plasma treatment on TM was further verified by protein concentration measurement. As shown in Fig. 3(a), the TM concentration decreased from 1.103 mg/mL (0 min) to 0.041 mg/mL (20 min), totally decreasing by 96.3 %. During treatment time of 0–15 min, the TM concentration decreased significantly with time increasing, while there was no significant difference in the TM concentration between 15 min and 20 min of plasma treatment ($p > 0.05$), which indicated that plasma treatment tended to be saturated. According to the change in TM concentration, it was surmised that plasma treatment might cause

agglomeration or degradation of TM.

In contrast to our results, one study found a general increase in the solubility of proteins after 80 Hz and 120 Hz atmospheric plasma treatment in comparison to the untreated control group (Zhang et al., 2021). Regarding the changes in solubility, an explanation was proposed by Ji et al. (2018). The hydrophilic active sites were exposed due to plasma-induced alterations in protein structure, which interacted with more water molecules, contributing to an increase in solubility, while some overcrowding of protein micelles caused by plasma treatment reduced the amount of hydrophilic active sites on the surface of the molecule, therefore reduced solubility. In addition, other physical process technologies, such as microwaves could also change the solubility of proteins. Dong et al. (2021) used the microwave to eliminate the allergenicity of shrimp and found that the total amount of soluble proteins was reduced by 30 %–75 % under different microwave processing conditions, which could be attributed to protein degradation and hydrolysis caused by the effect of friction in the microwave electromagnetic field.

3.3. Protein distribution analysis

SDS-PAGE is the most frequently used method to measure the molecular weight distribution of proteins. In this study, the addition of the reducing agent dithiothreitol (DTT) during the sample preparation would lead to the breaking of the disulfide bonds on the surface of the protein, thus corresponding SDS-PAGE results would exclude the situation of non-covalent cross-linking between protein molecules. As depicted in Fig. 3(b), on one hand, the TM bands tended to move up gradually, indicating that the molecular weight was gradually increasing, which was presumed to be the result of the partial covalent cross-linking of TM by plasma oxidation. In addition to the covalent cross-linking mentioned in this study, the previous study had shown that plasma treatment could also induce non-covalent cross-linking of TM (Ekezie et al., 2019). On the other hand, the intensity of bands gradually reduced, which was consistent with the gradual decrease of the TM concentration. This phenomenon might be caused by the formation of insoluble aggregates by protein interaction (Meinlschmidt et al., 2016). Combining the results of macroscopic characteristic analysis, it was conjectured that one of the reasons for the decrease in protein concentration was the aggregation and precipitation of TM. However, there was

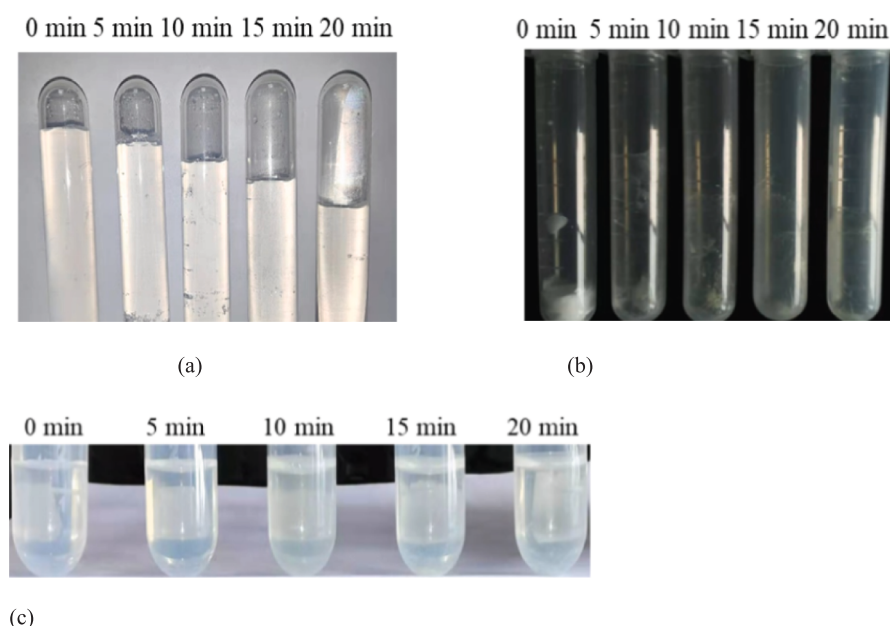
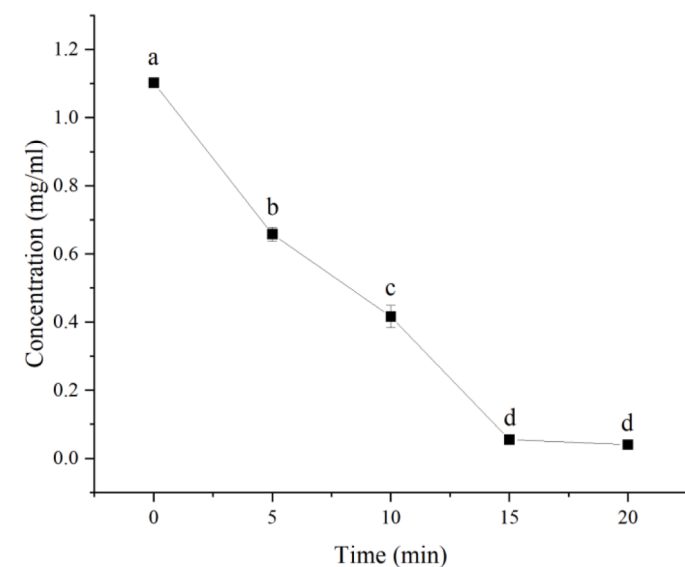
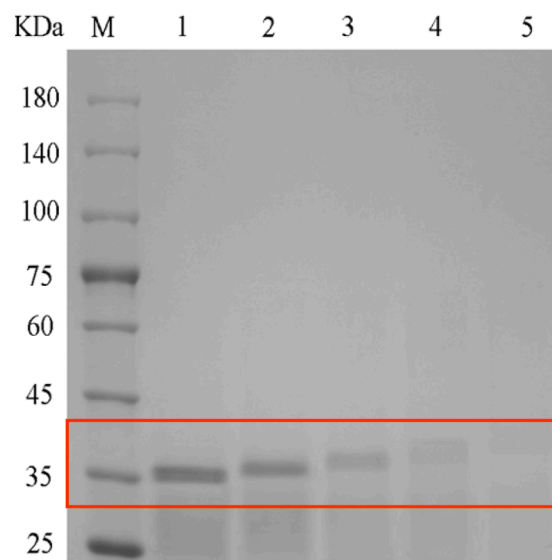


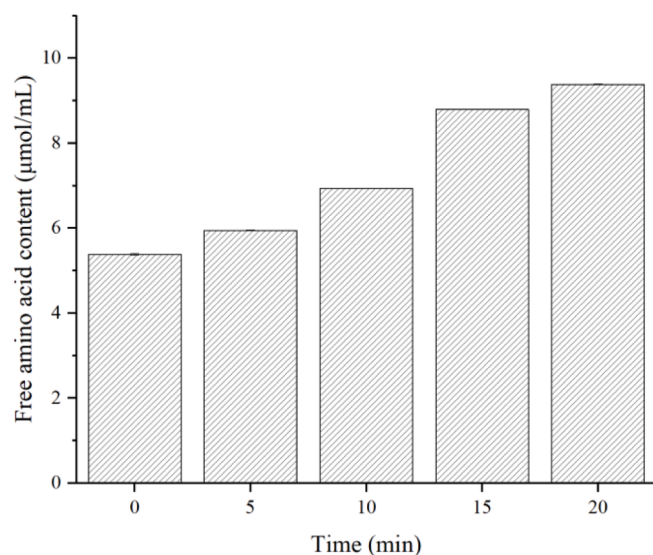
Fig. 2. Effects of cold plasma treatment on macro characteristics of TM. (a) TM solution state, (b) TM freeze-dried state, (c) TM re-solution state.



(a)



(b)



(c)

Fig. 3. (a) Effect of cold plasma treatment on TM concentration ($p < 0.05$), (b) SDS-PAGE results of cold plasma treatment on TM, M: Standard marker; 1: 0 min treatment; 2: 5 min treatment; 3: 10 min treatment; 4: 15 min treatment; 5: 20 min treatment; (c) effect of cold plasma treatment on free amino acid content of TM solution ($p < 0.05$).

another possibility of incremental molecular weight and band intensity. The increase in molecular weight was due to the introduction of oxygen atoms into amino acids of proteins by the oxidation of amino acid residues, while the lighter band was the result of damage to the protein polypeptide chain (Liu et al., 2021).

3.4. Free amino acid content analysis

The active site of plasma treatment on TM could be inferred by detecting the content of free amino acids in TM samples. Specifically, the free amino acids were derived from proteins, and the increased content of free amino acids indicated that the peptide bonds could be destroyed

by plasma treatment and the amino acid units were stripped out. It could be seen from Fig. 3(c) that the content of free amino acids gradually increased from 5.36 μmol/mL (0 min) to 9.37 μmol/mL (20 min), an increase of 74.7 %, showing that the high-energy active particles in the plasma could attack the more stable peptide bonds and partially break them, further verifying the potential of covalent modification of the plasma.

Similarly, Ekezie et al. (2019) found that CAPJ could increase the free amino acid content of TM solutions and the covalent modification ability of plasma treatment on TM was further proved. Moreover, the covalent modification ability of plasma was not only reflected in the destruction of covalent bonds, but also in inducing the formation of

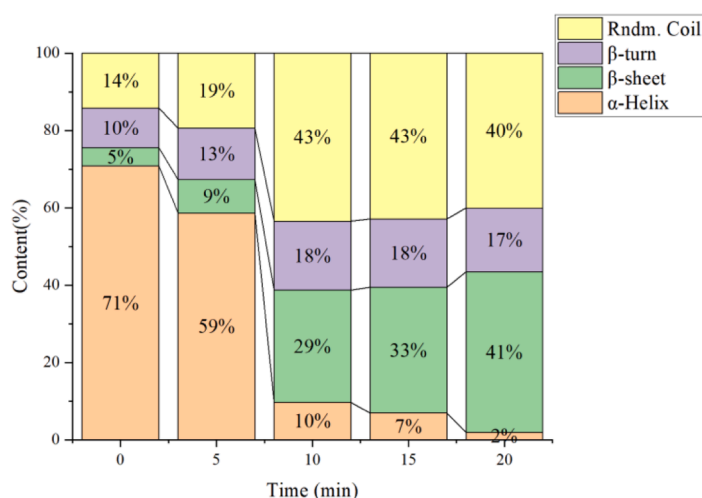
covalent bonds. Ng et al. (2021) found that after spark discharge (SD) and glow discharge (GD) treatment, the total amino acid content decreased with the treatment time because of amino acid oxidation. To some extent, ultrasound could also produce an oxidative effect similar to plasma treatment which helped in revealing the plasma oxidative effect. Zhang et al. (2018) explored the effect of ultrasonic treatment on the composition of protein amino acids and found that the content of Cys, Met and Lys decreased, surmising these sulfur-containing amino acids were oxidized by free radicals produced by ultrasound, resulting in the loss of them. All studies above demonstrated that the oxidative effect induced by plasma could act on the covalent bonds of target proteins, on the one hand, causing the oxidative loss of reducing amino acids, and on the other hand, increasing the content of free amino acids.

3.5. CD analysis

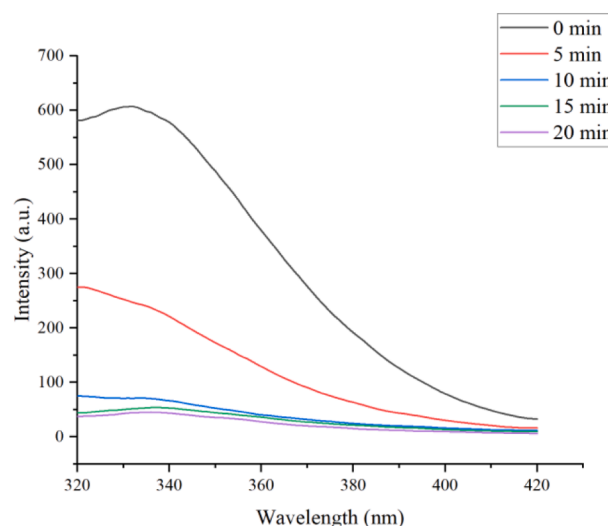
TM is a kind of protein formed by two peptide chains through the α -helix, and the conformational stability and allergenicity of TM are

closely related to the α -helix. As shown in Fig. 4(a), the proportion of α -helix reduced as time increased, while the content of β -sheet and random coil increased. When treated for 20 min, the proportion of α -helix of TM decreased by 69 %, while the content of β -sheet and random coil increased by 36 % and 26 %, separately. As it was assumed, the TM structure was expanded after plasma treatment, from the originally ordered state dominated by α -helix to the disordered state in which random coils accounted for a high proportion. Besides, the further formation of β -sheet, which might lead to the disappearance of partial β -turn and random-coil as connection structure, was also beneficial to the reaggregation of the TMs.

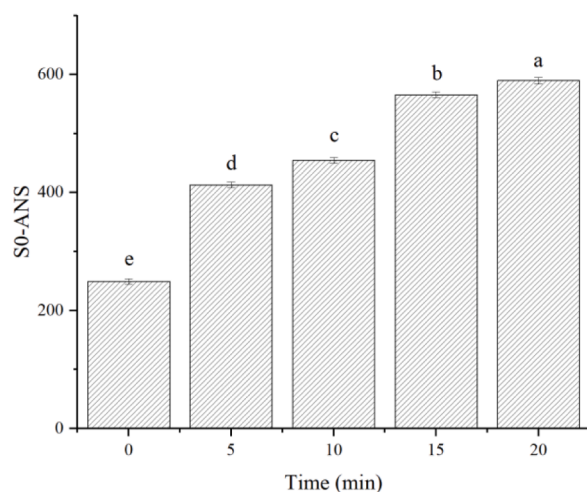
The relationship between secondary structure and TM allergenicity was revealed by some studies. Fu et al. (2019) attempted to reduce TM allergenicity by glycosylation and found that glycosylation induced the conversion of α -helix to β -sheet, accompanying decreased allergenicity, and further drew the conclusion that α -helix mediated the Maillard reaction to eliminate the allergenicity. Similarly, Ekezie et al. (2019) found that with the prolongation of CAPJ treatment time, the proportion



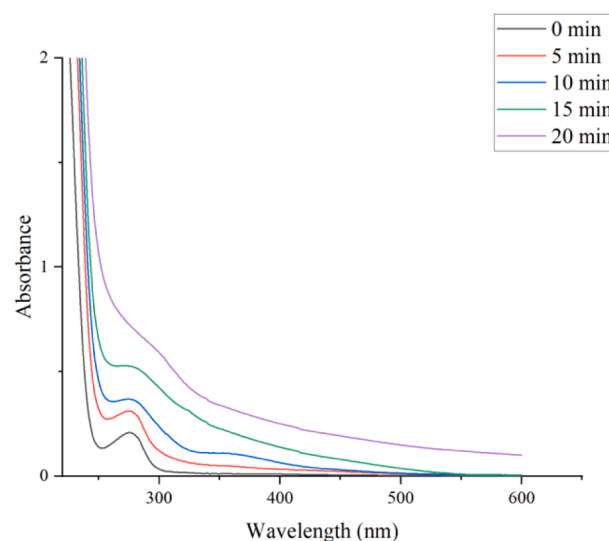
(a)



(b)



(c)



(d)

Fig. 4. Effect of cold plasma treatment on TM structure. (a) secondary structure, (b) fluorescence spectra, (c) surface hydrophobicity ($p < 0.05$), (d) ultraviolet spectrum.

of α -helix structure decreased, and others increased, followed by decreased allergenicity. These two studies were in accordance with the results of our study and demonstrated that the α -helix structure was connected with the allergenicity of TM.

3.6. Fluorescence spectroscopy analysis

Aromatic amino acids such as tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) residues in proteins emit fluorescence when excited by excitation light at 280 nm or 295 nm, which is called endogenous fluorescence. The intensity of endogenous fluorescence is affected by the distribution of aromatic amino acids, thus, the changes in endogenous fluorescence spectra are usually applied to reveal alterations of protein spatial structure. The results were shown in Fig. 4(b), after plasma treatment, the intensity of the maximum absorption peak gradually decreased, reflecting the gradual decrease in the distribution of aromatic amino acids on the surface of TM in the samples. Specifically, we speculated that plasma modification made TM cross-link and coagulate, resulting in a sharp decrease in soluble TM content, and a reduction in the overall aromatic amino acids content, showing a decrease in the intensity of the maximum absorption peak (607.5 to 44.9), in a combination of the results of SDS-PAGE and proteins concentration changes (Ekezie et al., 2019; Wu et al., 2022). Besides, the maximum absorption peak at 330 nm, underwent a red shift and gradually approached 338 nm, indicating that the polarity of the chromophore was changed, which was related to plasma oxidation (Ekezie et al., 2019; Pan et al., 2020; Pan et al., 2022).

Differently, Yuan et al. (2017) found that the endogenous fluorescence intensity of glycosylation-treated TM samples was about 85 % higher than that of untreated TM samples, in addition, glycosylation resulted in a blue-shift of the maximum emission wavelength (from 355 nm to 348 nm). The corresponding explanation was that the increase in the intensity of the maximum absorption peak was caused by unfolded protein and the binding of glucosamine to the site of other polypeptides, leading to inner Trp residues being exposed to the microenvironment. In the above results, the allergenicity of TM decreased to varying degrees, but the structural changes were not completely consistent, indicating that the allergenicity was not completely determined by the spatial structure.

3.7. Surface hydrophobicity analysis

In order to further probe into the conformational changes, the changes in TM surface hydrophobicity were explored. The distribution of hydrophobic groups on the surface of proteins can be reflected by surface hydrophobicity. Moreover, the exposure or embedding of hydrophobic groups of proteins might be caused by changes in protein structure, thereby affecting surface hydrophobicity. Hence, changes in surface hydrophobicity can be used to reveal structural changes. Fig. 4 (c) showed that the surface hydrophobicity increased from 249 at 0 min to 590 at 20 min, an increase of 57.8 %. The changes in surface hydrophobicity reflected that the TM was gradually unfolded with plasma treatment, leading to the gradual exposure of the internal hydrophobic groups (Ekezie et al., 2019). According to speculation, protein-protein interactions might be enhanced by the exposure of hydrophobic residues, resulting in reassociation or aggregation between unfolded proteins.

3.8. UV absorption analysis

The UV absorption spectrum is mainly affected by Trp, Try and Phe on the side chain of proteins (Tong et al., 2012), and the change in protein structure can be reflected by the change in the UV spectrum. From Fig. 4(d), it can be seen that as plasma treating, the UV absorption peak of TM red-shifted (from 276 nm to 293 nm) as a whole, and the UV absorption peak intensity gradually increased (from 0.209 to 0.629).

Interestingly, the characteristic peak shape tended to be incomplete, especially after treating for 20 min, the characteristic absorption peak around 280 nm was difficult to distinguish. According to the analysis, the red shift of the characteristic absorption peak was the result of the change in the characteristic amino acid microenvironment, while the increase in intensity was due to distribution changes of characteristic amino acids.

Similar results have been found by previous researchers, Ekezie et al. (2019) found that after continuous CAPJ treatment on TM, the intensity of the UV absorption peak gradually increased, but there was a difference that the UV absorption peak had a slight blue shift, showing that the effect of different plasma sources on characteristic amino acids was disparate. Moreover, chemical oxidation helped in revealing the plasma oxidation mechanism, Lv et al. (2016) reported that the UV absorption of TM treated with 4-hydroxy-2-nonene (HNE) decreased, indicating that the Tyr and Trp residues on the surface of TM molecules were modified. In addition, the UV absorption peaks of 0.01 mM and 0.1 mM HNE treating groups showed a clear blue shift, which manifested that the polarity of the microenvironment of amino acids was altered after oxidation, possibly because of peptide bond cleavage. However, no characteristic absorption peaks were found in higher concentrations of HNE, which might result from the denaturation of TM. In conclusion, changes in TM structure relied on species and intensities of oxidation to some extent.

3.9. Allergenicity analysis

In the previous section, we explored the effect of plasma treatment on TM structure, while we would reveal the effect of plasma treatment on TM allergenicity in this section, thereby speculating on the relationship between TM structure changes and allergenicity changes. IgE is an antibody that mediates type I hypersensitivity reactions, and its binding ability to allergens can reflect the allergenicity of allergens to a certain extent. Besides, methods commonly used to characterize IgE binding ability include ELISA, dot blot, immunoblotting, etc. As seen in Fig. 5(a), the IgE binding capacity of TM was decreased by plasma treatment and decreased by 96 % when treated for 20 min. Besides, dot blot is a visual reflection of the IgE binding capacity of TM, and the strength of IgE binding capacity is represented by the darkness of dots. Fig. 5(b) showed that as TM was treated, the colour of the dots gradually became lighter, especially at 15 min and 20 min, the colour was extremely light, which was in accordance with the quantitative ELISA results. ELISA and dot blot results illustrated the potential of plasma treatment to reduce TM allergenicity.

From the perspective of IgE binding capacity, CP showed better potential to reduce TM allergenicity compared with other techniques. Some studies showed that TM treated with polyphenols, glycosylation, and sonication respectively, and corresponding IgE binding capacity decreased by 25.1 %, 45.6 %, and 76 % (Lv et al., 2021; Lv et al., 2022; Dong et al., 2020). However, the treatment effect of the above studies was not as good as it of this study.

3.10. KU812 cell assay analysis

However, the strength of TM allergenicity cannot fully be reflected by the IgE binding ability. Therefore, it was tried to evaluate TM allergenicity changes by cytological means. In the process of type I hypersensitivity reactions, IgE-activated cells (mast cells, basophils) are stimulated by allergens contact, resulting in degranulation along with the release of histamine, β -HEX, cytokines and other biomarkers. Therefore, these cells are often used as cell models to study the occurrence of allergic reactions.

β -HEX is a glycosidase released by KU812 cells when degranulation occurs, and its release rate is used to reflect the degree of cell degranulation (Ahmed et al., 2020). As shown in Fig. 6(a), untreated TM could stimulate KU812 cells to release β -HEX, and the release rate reached

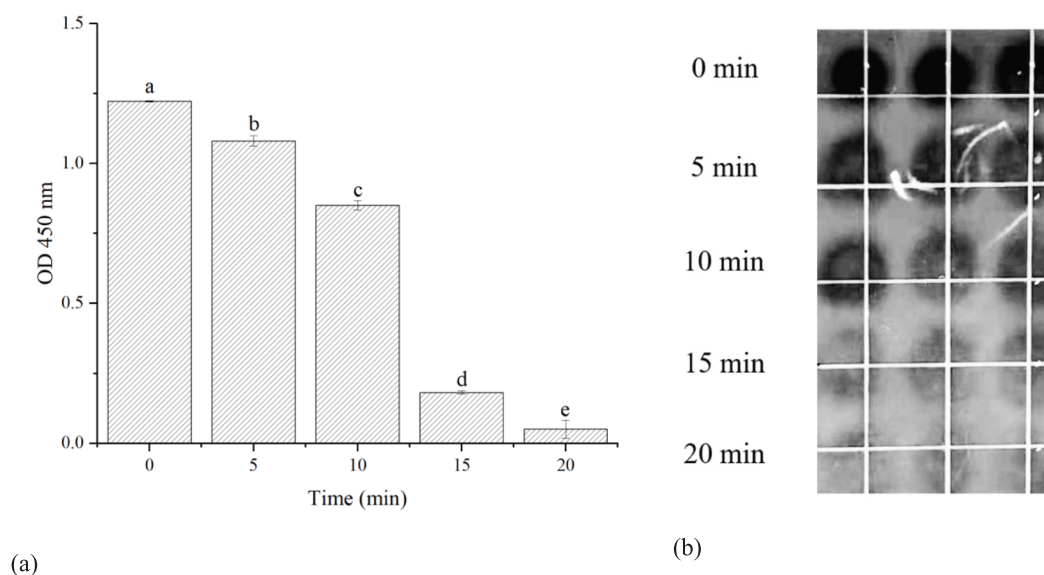


Fig. 5. Effect of cold plasma treatment on IgE binding ability of TM. (a) ELISA ($p < 0.05$), (b) dot blot.

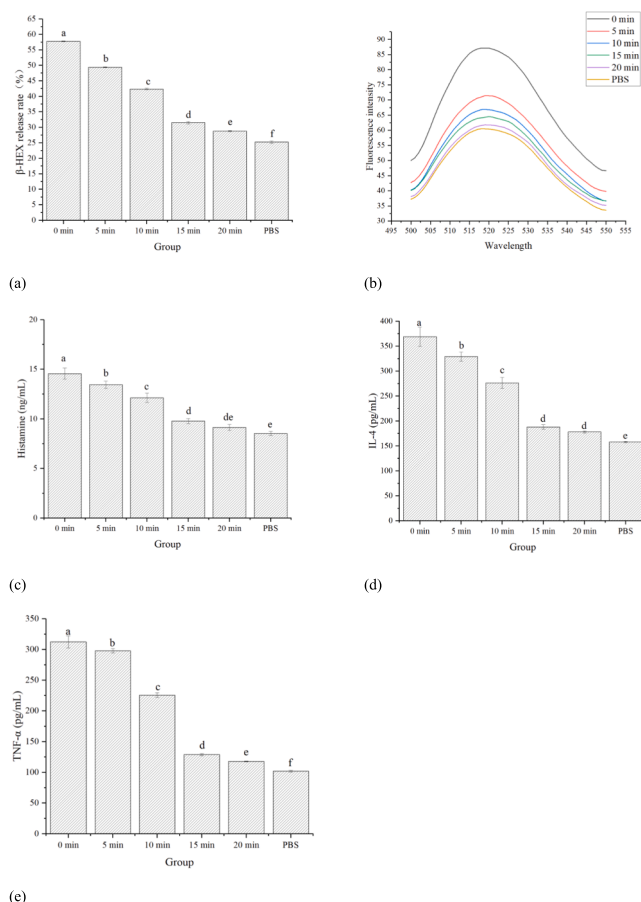


Fig. 6. Effect of cold plasma treatment on degranulation of KU812. (a) β-HEX ($p < 0.05$), (b) intracellular calcium ion strength ($p < 0.05$), (c) histamine ($p < 0.05$), (d) IL-4 ($p < 0.05$), (e) TNF-α ($p < 0.05$).

57.8 %. After plasma treatment, the release rate of β-HEX gradually reduced, and finally decreased to 25.3 % at 20 min, a decrease of 32.5 %.

When an allergic reaction occurs, basophils, as an important effector cell, initiate signalling phosphorylation of downstream pathways to

promote the influx of calcium ions. In general, the stronger the allergic reaction, the greater the intracellular calcium ion strength. As seen in Fig. 6(b), the intensity of the maximum absorption peak at 520 nm gradually reduced with the prolongation of treatment time, from 87 in the 0 min group to 60 in the 20 min group, which decreased by 31.0 %.

Histamine is one of the biomarkers released when degranulation occurs and is also used to reflect the degree of degranulation (Zhang et al., 2020). As shown in Fig. 6(c), with the prolongation of plasma treatment time, the release of histamine finally decreased from 14.5 ng/mL to 9.1 ng/mL, a decrease of 37.3 %, and there was no significant difference between the 20 min group and the PBS group.

In the process of the allergic reaction occurrence, cytokines such as IL-4, IL-13, and TNF-α are produced by KU812, which can induce the pathogenesis of various IgE-mediated allergic diseases, such as atopic dermatitis, atopic rhinitis and asthma (Song et al., 2016). IL-4 belongs to Th2-type lymphokines, which is closely related to the occurrence of type I hypersensitivity reaction. Its main function is to induce the transformation of B cell types and promote the proliferation of mast cells. As illustrated in Fig. 6(d), the release of IL-4 decreased from 368.9 pg/mL (0 min group) to 178.2 pg/mL (20 min group), which was reduced by 51.7 %. Moreover, the early and important inflammatory mediator produced in allergic reactions was TNF-α, which could stimulate neutrophils and lymphocytes, increase the permeability of vascular endothelial cells, and accelerate the entry of inflammatory cells into sites of inflammation. Fig. 6(e) showed that the release of TNF-α decreased from 312.5 pg/mL in the 0 min group to 102.0 pg/mL in the 20 min group, a decrease of 70.2 %.

In a word, β-HEX, histamine and other allergy markers release of TM-induced KU812 were hindered by CP, reflecting the decreased allergenicity of TM.

4. Conclusions

The effects of DBD CP treatment on the structure, surface hydrophobicity and potential allergenicity of TM in shrimp were investigated in this study. With plasma treatment time increased, the protein bands gradually up-shifted and became blurred. The protein concentration gradually decreased to 96.3 %. The content of free amino acids increased by 74.7 % after 20 min treatment. Plasma treatment affected the distribution of aromatic amino acids with the increased intensity of the absorption peak in the UV spectrum and a decrease in the fluorescence

spectrum. The content of α -helix reduced by 69 %, and the content of β -sheet and random coil increased by 36 % and 26 %. TM was unfolded and the internal hydrophobic groups were exposed on the TM surface, which was reflected by the increased surface hydrophobicity. The maximum reduction of IgE binding capacity of 96 % was obtained using plasma treatment with ELISA and dot blot analysis. The allergy markers such as the release rate of β -HEX, the intracellular calcium ion intensity, the release of histamine, and the release of inflammatory cytokines (IL-4, TNF- α) were decreased by 32.5 %, 31.0 %, 37.3 %, 51.7 %, and 70.2 % based on the KU812 cell assay analysis, confirming that plasma treatment could inhibit the occurrence of degranulation. The whole results confirmed that DBD CP showed great potential to eliminate the allergenicity of TM in shrimp by structural changes.

CRediT authorship contribution statement

Jun-Hu Cheng: Supervision, Validation, Funding acquisition, Resources. **Jilin Li:** Writing – original draft, Formal analysis, Investigation. **Da-Wen Sun:** Supervision, Funding acquisition, Resources, Writing – review & editing.

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

The authors are unable or have chosen not to specify which data has been used.

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