

Alteration of collagen thermal denaturation, structural and the abrogation of allergenicity in eel skin induced by ohmic heating

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ABSTRACT

This study aimed to evaluate the effect of ohmic heating (OH) on the thermal denaturation, structure, and allergenicity of collagen in fresh eel skin. The allergenicity of collagen decreased by approximately 70% at 50 °C as measured by simulated gastric fluid (SGF), simulated intestinal fluid (SIF) and ELISA in vitro. SDS-PAGE analysis showed that the molecular weight of collagen did not decrease, but the band strength decreased with an increase in the processing temperature. FTIR and SEM analyses showed that the secondary structure and microstructure of collagen also changed. The water retention, dielectric properties and amino acid content of collagen also decreased with increasing temperature. Compared to water bath heating (WH), OH required significantly less time and energy and reduced the allergenicity of fish skin collagen through protein unfolding and secondary structure changes, thus potentially reducing the allergenicity of eel.

1. Introduction

Food allergy, an abnormal immune response or allergic reaction upon consumption of certain foods, is one of major public health problem according to FAO Annual Survey 2020, as approximately 4% of the global population have food allergies to varying degrees. Moreover, food allergies are closely related to age because most children can develop tolerance to milk, soy, eggs, and wheat as they age. In contrast, allergies to aquatic products, peanuts, and nuts persist (Dong, Wang, & Raghavan, 2021). There is no effective treatment for food allergies, and the disease can only be managed by avoiding allergens or by treating symptoms (Pereira et al., 2018).

The Japanese eel (*Anguilla japonica*) resembles a long snake without scales and contains various amino acids, fatty acids, vitamins, and minerals required by the human body. The consumption of eel can effectively prevent cardiovascular diseases, promote metabolism in the body, improve liver function, help organ development, and prevent muscle tissue degeneration (Islam et al., 2020). Eel is a migratory fish that grows and fattens in freshwater, reproduces in seawater, and is

widely distributed in Japan, Korea, and coastal China (Rachmawati & Sistina, 2020). Eel is an important foreign exchange aquatic product in China. According to the FAO report, the production and export of eel (live, fresh, frozen, processed, and cooked) peaked in 1990 at approximately 207,073 tons and gradually declined to <170, 139 tons in 2019. China has been a major eel exporter of eel in the last decade, accounting for 64% of all exports. Over the past 20 years, Japan has become the largest importer of eels, accounting accounted for 70% of the global eel imports (Govindharaj, Roopavath, & Rath, 2019).

Although many countries mainly consume cooked eel, the Japanese are accustomed to consuming raw fish in the form of sashimi or sushi. Heat treatment of fish denatures fish collagen and forms gelatin, which is water-soluble, easily digested, and absorbed by enzymes in the gastrointestinal tract. However, the collagen in raw fish is insoluble in water, and form fibers through interchain hydrogen bonds with pyridinoline or deoxypyridinoline, makings digestion and absorption in the body difficult (Chikazawa, Hashimoto, Kobayashi, & Satoh, 2015). Hansen et al. (2004) conducted skin prick tests (SPTs), histamine release tests (HRTs), and food challenge studies using hydrolyzed fish gelatin. In

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the food challenge studies, none of the 30 fish-allergic patients reacted to hydrolyzed fish gelatin, but three and seven of the 30 patients tested positive for SPTs and HRTs of fish gelatin, respectively. In another study conducted by André, Cavagna, & André (2003), only three of 100 fish-allergic patients reacted to gelatin, indicating a low risk of collagen allergy. This is because the major immunoglobulin E (IgE) binding epitope of collagen is easily disrupted in gelatin, leaving patients without sensitization. However, it has been suggested that the T-cell epitope of collagen may be resistant to digestion by proteolytic enzymes. Therefore, eel collagen can induce allergic reactions in allergy-prone individuals (Kobayashi et al., 2016). In 2000, the sera of some patients with fish allergies contained proteins capable of interacting with IgE antibodies that specifically bind to type I collagen (Sakaguchi et al., 2000). Collagen from bigeye tuna was demonstrated to be a fish allergen by immune hybridization and inhibitory enzyme-linked immunosorbent assay (ELISA), and allergen common to many fish species. However, there was no immune cross-reactivity with collagen from other animals, confirming that collagen is a fish allergen (Huang et al., 2016).

Currently, heat treatment, ultra-high pressure, irradiation, ultrasound, enzymatic digestion, and fermentation are the most used methods for reducing the allergenicity of food allergens. Heat treatment is an important and widely used technique in the food industry. The heat treatment of foods is accompanied by various modification reactions, such as denaturation, hydrolysis of peptide bonds, restructuring of disulfide bonds, and interactions with other components, such as carbohydrates and lipids. In the case of proteins, heat treatment leads to the loss of secondary and tertiary structures, and non-covalent interactions. Thus, these reactions may affect allergenicity by disrupting or exposing conformational epitopes, reducing or enhancing immunoreactivity (Yan et al., 2013). However, improper heat treatment of foods can lead to uneven temperature distribution, which may result in incomplete sterilization, alteration of the food structure, and increased cooking losses. Ohmic heating (OH) is an emerging technology in food engineering that is widely used in the food industry and other fields owing to its rapid and uniform heating. The OH technique used a material that as a conductor in a circuit, and the heat generated by the its electrical conductivity is used for heating. According to Joule's law, material particles in the heating chamber vibrate upon excitation, generating energy and dissipating it as heat through friction (Jin et al., 2020). In recent years, OH has been used as a new heat treatment method for reducing the allergenicity of milk (Pereira et al., 2018) and beef (Tian et al., 2016). However, the reduction of fish allergenicity using OH technique has seldom been reported.

In this experiment, Japanese eel skin was used as the experimental material to extract fish skin collagen using pepsin combined with acid method. The denaturation temperature of collagen was first determined by differential scanning calorimetric (DSC), and then two different methods of OH and water bath heating (WH) were used to pretreat the collagen at variable temperature. Sodium dodecyl sulfonate-polyacrylamide gel electrophoresis analysis (SDS-PAGE), amino acid analyzer, fourier infrared transform spectroscopy (FTIR), and scanning electron microscopy (SEM) were subsequently used to demonstrate the effect of heat treatment on the structure of eel fish skin collagen. Amino acid analysis, dielectric properties, electrical conductivity, water retention, and oil absorption were subsequently used to demonstrate the effect of heat treatment on the physicochemical properties of eel skin collagen. The target proteins after heat treatment were subjected to ELISA with animal-specific antibodies and a simulated gastric digestion assay to confirm the degree of protein allergenicity abatement after the heat treatment.

Therefore, this study was performed to investigate the effect of OH on the thermal denaturation, structural properties, physicochemical properties, and allergenicity of collagen in fresh eel skin and to provide reference data for the potential applications of eel collagen in functional foods, cosmetics, and biomedical materials.

2. Materials and methods

2.1. Materials

The study was approved by the Animal Ethics Committee of Shanghai Ocean University. Ten fresh Japanese eels (*Anguilla japonica*) were purchased between August 15 and December 14, 2021, from the Luchao Port Seafood Market in the Pudong New Area, Shanghai, China. Eels were executed immediately after anesthesia with ice in the laboratory, and the dissected skin, flesh and bones were preserved separately in sealed bags and kept in a -20°C refrigerator for backup. The average length and weight of the selected eels were 82.3 ± 1.8 cm and 1850 ± 50 g.

In this study, sera from five patients allergic to eel were used for the evaluation of human sera for eel fish skin collagen allergenicity. Also, mixed sera from two patients who were not allergic to eel were used as negative controls. All sera were provided by Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, China. Allergic patients were identified by their presentation after eel consumption and their allergic medical history of allergic diseases. Prior to this, each serum owner was informed, agreed and provided consent for the experimental test.

2.2. Preparation of collagen

The collagen was extracted from the eel skins according to the Veeruraj, Arumugam, & Balasubramanian (2013) method with some modifications, and all procedures were performed at 4°C . The eel skins were cut into 1×1 cm pieces. To remove non-collagenous proteins, the fish skins were soaked in 0.1 M NaOH with a skin/solution ratio of 1:20 (w/v) for 36 h, and rinsed with ice water until neutral pH 7. The deproteinized skins were then defatted using 10% butyl alcohol with a solid/solvent ratio of 1:20 (w/v) for 24 h and rinsed with ice water. Subsequently, the rinsed skin was placed in 25% ethanol with a solid/solvent ratio of 1:20 for 24 h and rinsed with ice water. The fish skins were extracted with 0.5 M acetic acid containing 1% pepsin (greater than 3000 U, Shanghai Maclean Biotechnology Corporation, Shanghai, China) with a skin/solution ratio of 1:30 (w/v) of for 2 d. The extract was the centrifuged (10,000 g, 20 min) and the dialyze with 20 mM, phosphate buffer (pH 7.4) for 48 h. The precipitate was collected by centrifugation (10,000 g, 15 min), and the precipitate was dissolved in 0.5 M acetic acid. Then 4 M sodium chloride was added until a final concentration of 2.3 M to precipitate the collagen, which was collected by centrifugation (12000 g, 15 min). The precipitate was dissolved in 0.5 M acetic acid, and centrifuge to remove the insoluble precipitate. The supernatant was dialyzed with 0.1 M acetic acid for one day, and then dialyzed with distilled water to neutrality. Finally, the dialysate is freeze-dried and stored at -18°C for later use.

2.3. Differential scanning calorimeter (DSC)

The denaturation temperature of the collagen was measured with a DSC (DSC8500, PerkinElmer, Massachusetts, USA). The collagen (5–10 mg) was accurately weighed into an aluminum pan and sealed. Using an empty crucible as a control, the sample crucible was maintained at 10°C for 1 min, and then the collagen denaturation temperature was determined by scanning at a basic ramp rate of $5^{\circ}\text{C}/\text{min}$ in the range of 10 to 80°C . All measurements were performed at least in triplicate.

2.4. Temperature-changing treatment of collagen

From DSC, the denaturation temperature of collagen was determined and found to be 38.5°C . Therefore, the denaturation of collagen was implemented.

2.4.1. Ohmic heating treatment

The 4°C dialysate prepared in section 2.2 before freeze-drying was

used to rapidly fill an OH chamber (20 × 20 × 20 mm dimension and 5 mm thickness) made of polypropylene material and insulating tape, with titanium electrode plates (20 × 110 mm dimensions and 0.1 mm thickness) placed at both ends with a distance of 20 mm between them. OH was performed in the frequency range of 50 Hz at a voltage of 100 V. A thermocouple was placed in the center to monitor the temperature changes in real time. Using untreated proteins as a control group, the samples were heated from 4 °C to 30, 35, 40, 45, and 50 °C, which was halted immediately after reaching the indicated temperatures, and then time was measured and energy consumption was calculated. After the OH treatment was completed, the samples were cooled and placed in 90 mm diameter disposable petri dishes for freeze-drying.

2.4.2. Heat treatment in water bath

The 4 °C dialysate prepared prior to freeze-drying in Section 2.2, quickly remove 8 mL of liquid into a test tube and place the tube in a water bath (DF-101S, Shanghai Lichen Technology Corporation, Shanghai, China) containing 3 L of 4 °C to heat to the indicated temperature. Refer to 2.4.1 for the rest of the steps and conditions.

2.5. Study of the physicochemical properties of collagen

2.5.1. Sodium dodecyl sulfonate - polyacrylamide gel electrophoresis analysis (SDS-PAGE)

SDS-PAGE analysis was performed as previously described by Huang et al. (2018) with some modifications. The collagen (5 mg) was dissolved in 200 µL TE buffer (0.1 M Tris, 10 mM EDTA, pH 8.8), and 50 µL of 5 × sample buffer was added and mixed with a vortexer. The mixture was heated at 100 °C for 5 min and then cooled at 24–26 °C. Each sample (10 µL) was loaded onto a polyacrylamide gel (7.5%), and the 10 × of electrophoresis buffer was diluted to 1 ×. After loading, SDS-PAGE was performed in a vertical electrophoresis apparatus (DYCZ-25E, Beijing Liuyi Biotechnology Corporation, Beijing, China) at a voltage of 80 V for the first 20 min, followed by 100 V until the end of the analysis. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (Beijing Bomad Genetics Technology Corporation, Beijing, China) and decolorized with decolorizing solution until the bands were clear. Markers (Sichuan Aiki Biotechnology Corporation, Sichuan, China) with molecular weights ranging from 20 to 250 kDa were used to estimate the protein content.

2.5.2. Fourier infrared transform spectroscopy (FTIR)

The composition of the collagen secondary structure was analyzed by FTIR by mixing the freeze-dried collagen sample (3 mg) with dried potassium bromide (100 mg), and grinding it into a homogeneous powder using a mortar and pestle. The resulting powder was subjected to a pressure of about 1×10^7 Pa in a vacuum mold to produce a transparent disc of 13 × 1 mm. It was scanned on a FTIR spectrometer (Thermo IS 10, Thermo Nicolet Corporation, Wisconsin, USA) using a wavelength range of 4000–500 cm⁻¹, and the absorption intensity of each peak was calculated using the baseline method.

2.5.3. Microstructure

Microstructural changes in collagen before and after OH and WH were observed using a thermal field emission SEM (Hitachi SU5000, Hitachi High-Technologies Corporation, Chiyoda-ku, Japan). The heat-treated collagen samples were mounted and fixed on a standard SEM sample tray. Gold ions were applied and coated using an automated fine coater. All photographs were obtained at 1-Kx magnification.

2.5.4. Determination of total amino acid content

The total amino acid content of collagen in the eel skin was determined according to the method reported by Mesías, Wagner, George, & Morales (2016), was slight modifications. Pretreated collagen (5 mg) was added to the hydrolysis tube with 15 mL of 6 M hydrochloric acid solution and incubated with three drops of phenol. The hydrolysis tube

was vacuumed for 10 min before the cap was tightened, and the tube was placed in an electric blast oven (Shanghai Huitai Instrument Manufacturing Corporation, Shanghai, China) at 110 ± 1 °C. After 22 h of hydrolysis, the tube was removed and cooled at 24–26 °C, and the hydrolysate was filtered into a 50 mL volumetric flask and fixed to the scale with ultrapure water. After mixing, precisely 1 mL was removed, added to a test tube, and dried under reduced pressure at 40–50 °C, 0 Mpa using a reduced pressure desiccator (DZF-6090, Shanghai Shuangxu Electronics Corporation, Shanghai, China). The solution was dissolved 1–2 mL of sodium citrate pH 2.2 in a test tube, mixed well, aspirated through a 0.22 µm filter membrane, and then transferred to a feed bottle for measurement on an ultra-high-speed amino acid auto analyzer (LA-8080, Hitachi High-Technologies Corporation, Chiyoda-ku, Japan). The amino acid content was calculated according to equation:

$$X_i = \frac{C_s \times A_i \times F \times V \times M}{A_s \times m \times 10^9} \times 100$$

Where A_i is the peak area of amino acid i of the sample determination solution; A_s is the peak area of amino acid s of amino acid standard working solution; C_s is the content of amino acid s of the amino acid standard working solution (nM/mL); X_i is the content of amino acid i in the specimen (g/100 g); C_i is the content of amino acid i in the sample determination solution (nM/mL); F is the dilution factor; V is volume of the sample hydrolysis solution transfer volume fixation (mL); M is the molar mass of amino acid i (g/M); m is the sample weight (g); 10^9 is the factor for converting nanograms to grams, and 100 is the conversion factor.

The linearity range, limit of detection (LOD), limit of quantitation (LOQ), and intra and inter-day precision, spike recovery, and accuracy were considered in the method validation. The linearity range, LOD, and LOQ were determined by diluting the mixed standard stock solution (0.1 µmol/mL) with 0.02 M HCl to a concentration range of 1–500 µM. The intra and inter-day precisions were obtained by adding the mixed standard stock solution to the eel skin sample and detecting five replicates, while spike recovery was calculated by detecting high, medium, and low concentrations of the mixed standard stock solution (1–250 µM/mL) added to eel skin sample following equation:

$$\text{Spike recovery (\%)} = \frac{(\text{detected amount} - \text{original amount})}{\text{spiked amount}} \times 100$$

Correlation coefficients, linear range, LOQ, LOD, spike recovery, and precision of the mixed stock standard solution of amino acids.

2.5.5. Water retention and Oil-absorbing properties of collagen

Protein (0.05 g) was added to 5 mL of distilled water, stirred well, and centrifuged at 2000 g for 5 min. The supernatant was discarded, and the mass of the residue weighed. After placing it in an oven at 25 °C, the weight of the residue was measured every 20 min, and the water retention calculated according to equation:

$$Y(\%) = \frac{M_0 - M}{M_0 - 0.05} \times 100$$

Where Y is moisture residual rate (%); M_0 is mass of centrifuged residue, and M is mass of residue at each interval.

Oil absorption was determined based on a slightly modified method described by Yoshie-Stark, Wada, & Sche (2008). Two portions of 10 mL refined oil were added to 0.1 g of protein, mixed for 1 min, allowed to stand for 30 min, centrifuge at 2000 g for 15 min, and the oil absorbency calculated according to equation:

$$X(\%) = \frac{10 - V_f}{m}$$

Where X is oil absorption (%); V_f is volume of free oil, m : 0.1 (g).

2.5.6. Electrical conductivity

Immediately after the samples were treated with OH to the indicated temperatures, the conductivity was measured with a conductivity meter (Mettler Toledo FE30, Mettler Toledo Technology Corporation, Shanghai, China).

2.5.7. Dielectric properties analysis

The dielectric properties of collagen in eel skin were determined according to a slightly modified method described by Xiang et al. (2021). The dielectric properties of the collagen were measured using a coaxial probe (85070E, Agilent Technologies, CA, USA) connected to a performance network analyzer (PNA-L) (N5230C, Agilent Technologies, CA, USA). Prior to calibration, the test system was turned on and preheated in a stable environment for 2 h to eliminate errors caused by the equipment and environment. The coaxial probe was then calibrated using the air short-distilled water calibration procedure. After calibration, the dielectric properties of distilled water were measured to test its validity. This is because the dielectric properties of distilled water are relatively stable and are generally used to calibrate a dielectric property measurement system to ensure its validity (Zhang et al., 2017). The measurement frequency range was set 1 to 10 GHz, and the temperature range was 30–50 °C with a temperature interval of 5 °C. The untreated group was used as a control group. The sample temperature was tracked by thermocouples and the prepared collagen was poured into a 25 mL beaker for dielectric property measurements. The dielectric probe was placed approximately 1.5 cm from the surface of the liquid. The dielectric constants and dielectric loss factors were measured in triplicate for each solution.

2.6. Indirect ELISA

Indirect ELISA was performed using the method reported by Jin et al. (2015). The IgE-binding capacity (Immunoglobulin E-binding capacity) assay and the IgG-binding capacity (Immunoglobulin G-binding capacity) assay were used to evaluate and analyze collagen allergenicity.

2.6.1. IgE test

For the IgE test (using five serum samples from patients allergic to eel), the primary and secondary antibodies were 100 μ L human serum (1:20 v/v PBST dilution) and goat anti-human IgE-HRP (1:2500, v/v), respectively.

The experiment process was as follows: before and after OH and WH treatment, collagen was coated on a 96-well polystyrene plate (Corning Inc., USA) using a coating solution consisting of 0.29% (w/w) NaHCO₃ and 0.16% (w/w) Na₂CO₃. The pH was 9.6, and the protein coating volume is 5 μ g/ml per well. After coating overnight at 4 °C, each solution was wash 3 times for 30 s with PBST (pH 7.4, 136.75 mM NaCl, 2.68 mM KCl, 10.21 mM Na₂HPO₄, 1.76 mM KH₂PO₄, 0.05% Tween-20) on a plate washer (Mini shaker MH-1). Then block non-specific sites with PBST containing 5% skim milk (200 μ L per well) at 37 °C for 2 h. After washing the plate with PBST, 100 μ L of the human serum (1:20 v/v PBST dilution) was added and then incubated at 37 °C for 2 h. After washing with PBST, 100 μ L of the goat anti-human IgE-HRP (1:2500, v/v) solution was added, and then incubate at 37 °C for 2 h. Finally, 100 μ L of the TMB reagent was added for color development, the reaction stopped with 50 μ L of 2 M H₂SO₄, and measured the absorbance value at 450 nm (automatic microplate reader, Multiskan Go, Thermo Fisher Scientific Corporation, Ltd, USA).

2.6.2. Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)

For the IgG assay, the primary and secondary antibodies were 100 μ L human serum (1: 10,000 v/v of PBST dilution) and 100 μ L goat anti-human IgG-HRP PBST solution (1: 15,000, v/v), respectively.

Collagen-mimicking gastric juice digestion experiments were performed by referring to the method of Brodtkorb et al. (2019). The SGF was prepared following the United States Pharmacopoeia Standard

using a 0.32 mg/mL pepsin solution (dissolved in 2 mg/mL NaCl, and pH adjusted to 1.2). The total volume of the solution for the digestion reaction system was 1 mL, with a pepsin to test protein ratio of 1:100 (w/w) and a collagen concentration of 1 mg/mL, and the reaction was performed at 37 °C. The pepsin was preheated in a water bath for 10 min before the reaction, after the addition of collagen, 100 μ L of the reaction solution was removed at 0, 1, 2, 5, 15, 30, 60, and 90 min, respectively, and transferred to a 1.5 mL centrifuge tube, followed by the addition of 30 μ L of 0.2 M Na₂CO₃ to terminate the reaction. Hydrolysates were obtained for subsequent experiments after SGF digestion (R-SGF) of collagen in vitro.

Simulated gastrointestinal fluid was prepared following the United States Pharmacopoeia Standard using a 0.05 M KH₂PO₄ solution (pH 7.5) containing 0.10 mg/mL trypsin (386 U/mg proteins, proteins Shanghai Maclean Biologicals, Shanghai, China). The total volume of the solution for the digestion reaction system was 1 mL, with a trypsin to tested proteins ratio of 1:300 (w/w) and a collagen concentration of 1 mg/mL. The reactions were performed at 37 °C. Before the reaction, the trypsin solution was preheated in a water bath for 10 min. 100 μ L of the reaction solution was removed at 0, 1, 2, 5, 15, 30, and 60 min after the addition of and transferred to a 1.5 mL centrifuge tube, which was then placed in a boiling water bath for 5 min to terminate the reaction. Hydrolysates were obtained for subsequent experiments after SIF digestion (R-SIF) of collagen.

2.7. Statistical analysis

Analysis of variance (ANOVA) was performed using the SPSS 26.0 program, and the difference in the mean ($P < 0.05$) was determined by conducting multiple range tests. Use Origin program to draw (Origin 9.0, Origin Lab Corporation, USA).

3. Results and discussion

3.1. Heating time and energy consumption

In this study, 100 V of OH required 35, 43, 51, 60, and 68 s to increase the temperature from 4 °C to 30, 35, 40, 45, and 50 °C, respectively, and the corresponding energy consumption was 105, 129, 153, 180, and 204 J, respectively. The constant WH temperature using 3 L of water for the heating treatment, required 838, 931, 1017, 1096, and 1168 s to increase the temperature from 4 °C to 30, 35, 40, 45, and 50 °C, respectively. The corresponding energy consumption was 1.43×10^4 , 1.7×10^4 , 1.97×10^4 , 2.25×10^4 , and 2.52×10^4 J, respectively. Compared with conventional WH, OH treatment requires $19.2 \times$ less time and $121.6 \times$ less energy consumption to reduce eel allergenicity. This satisfies the national requirements for energy saving and emission reductions.

3.2. Collagen identification

Changes of molecular weight in the fish skin allergenic collagen before and after OH and WH were analyzed by SDS-PAGE. In Fig. 1A and 1B, the collagen treated by both OH and WH contained five bands, and the molecular weight of $\alpha 1$ was about 118 kDa; the molecular weight of $\alpha 2$ was lower than that of $\alpha 1$, and the color of the bands was slightly lighter than that of $\alpha 1$. The main component of eel skin collagen was found to be $\alpha 1$; the molecular weight of $\alpha 3$ was lower than $\alpha 1$, but higher than that of $\alpha 2$. The β band has a molecular weight greater than 200 kDa and is presumably a dimer of the $\alpha 1$ chain, and the γ band has a molecular weight of 50–60 kDa. These results are consistent with the published literature on collagen (Huang, Wang, Liang, Nie, & Huang, 2018). This suggests that there are no significant changes in the electrophoretic behavior of collagen with or without the use of OH or WH. Collagen remained present, and there were no changes in molecular weight due to protein denaturation. Similar results were observed for

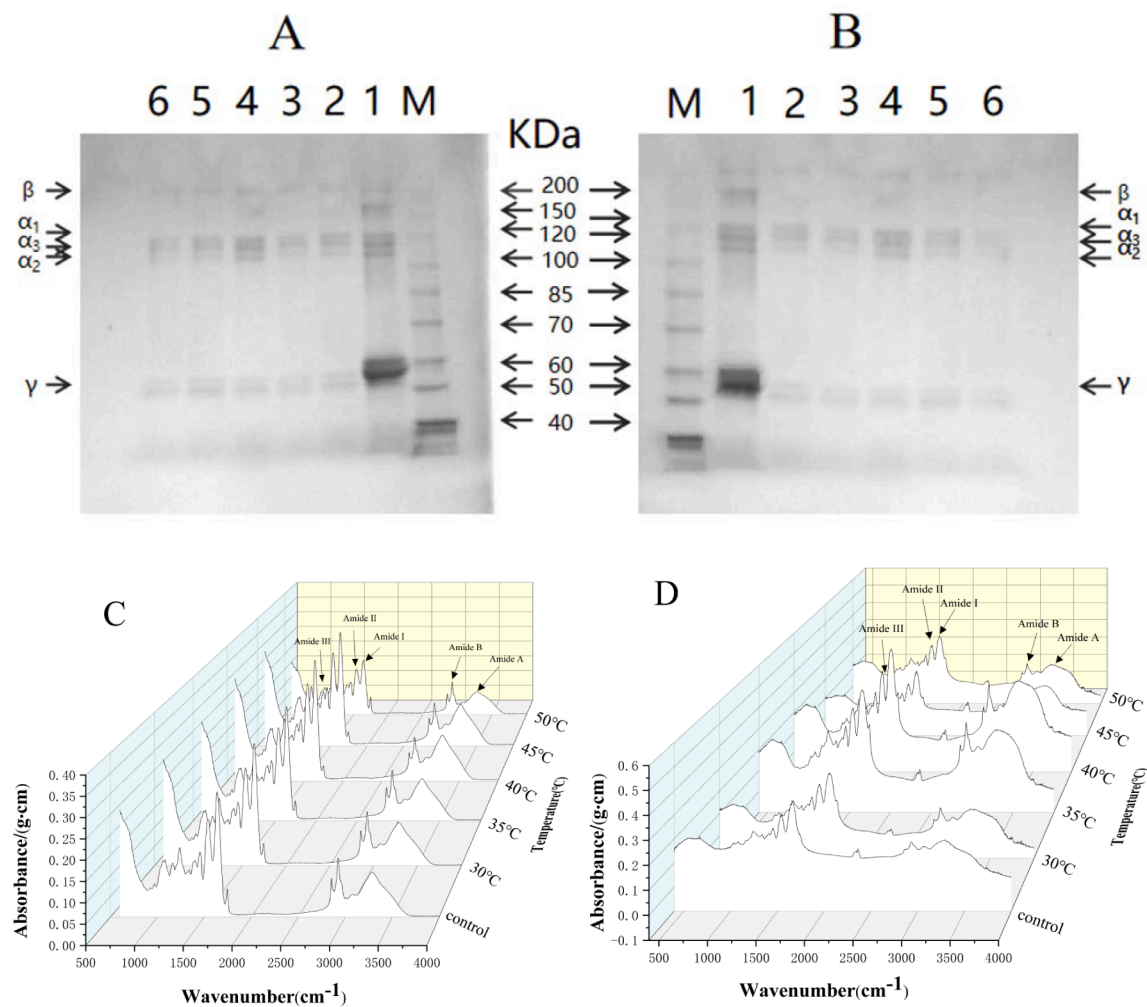


Fig. 1. SDS-PAGE (A, B), and FTIR 3D waterfall plots (C, D) of collagen at different heating temperatures after OH and WH treatment. SDS-PAGE analysis of: (A) WH, (B) OH. M: marker; 1: Control; 2: Heating from 4 °C to 30 °C; 3: Heating from 4 °C to 35 °C; 4: Heating from 4 °C to 40 °C; 5: Heating from 4 °C to 45 °C; 6: Heating from 4 °C to 50 °C. FTIR analysis of: (C) OH, (D) WH. The corresponding temperature changes are: Control; Heating from 4 °C to 30, 35, 40, 45, 50 °C.

shrimp proto-myosin (Dong, Wang, & Raghavan, 2021). However, the intensity of the corresponding $\alpha 1$ protein bands in electrophoresis decreased with increasing treatment temperature. Among all the OH treatments, the lowest intensity collagen bands were observed for the samples treated at 50 °C. This suggests the potential application of OH as a heat treatment method reduce eel skin allergenicity.

3.3. Analysis of the physicochemical properties of collagen

3.3.1. Effect of OH and WH on the secondary structure of collagen

In Fig. 1C and 1D, the secondary structure of collagen was detected by FTIR spectroscopy. The characteristic absorption peaks of collagen were due to amides A, B, I, II, and III, which were located at 3307, 2930, 1652, 1548, and 1238 cm^{-1} , respectively. The appearance of the Amide A area as a characteristic functional group of collagen is attributed to NH stretching, whereas the amide B area is due to CH_2 asymmetric stretching, amide I area to $\text{C}=\text{O}$ stretching, amide II area as NH bending and CN stretching, and amide III area to NH bending. The structure is similar to that of collagen extracted from tilapia, Parang-parang fish, and cod (Takeshi & Suzuki, 2000; Veeruraj, Arumugam, & Balasubramanian, 2013; Wijaya, Putriani, Safithri, & Tarman, 2020).

In Fig. 1C and 1D, the peak area and height of the corresponding IR absorption peak of amide A increases when the OH temperature increases from room temperature to 40 °C, but subsequently decreases from 40 to 50 °C as the protein completes denaturation at 40 °C. This is

similar to the results observed with WH. As the temperature increases, the hydrogen bonds in collagen gradually break. Through the thermal depolymerization process, the triple helix structure of collagen, consisting of hydrogen bonds, is transformed into the random helix structure of gelatin, and the physical properties are change accordingly (Wang et al., 2008). Therefore, OH treatment can accelerate the denaturation and folding process of proteins by changing their structures. In addition, prolonged high temperature OH can effectively increase the kinetic energy of protein molecules, leading to the vibration of polar groups, which further causes secondary structural changes.

3.3.2. Effect of OH and WH on the microstructure of collagen

The microstructure of OH and WH treated collagen was observed by SEM, and Fig. 2A shows that the surface of untreated collagen exhibits a relatively smooth lamellar structure (Tang et al., 2015). With an increase in temperature after OH heat treatment, the collagen surface produced folds and shown an irregular lamellar structure. When the temperature reached 40 °C, pores appeared on the collagen surface, and the number of pores increased with an increase in temperature. This may be because of the increased current density between the two polar plates with increasing OH temperature, resulting in the disruption of the microstructure of the protein into disordered lamellar structures and the generation of wrinkles and on the surface. In Fig. 2B, the lamellar structure was maintained before and after heat treatment with WH. However, folds were also generated on the of protein's surface with the

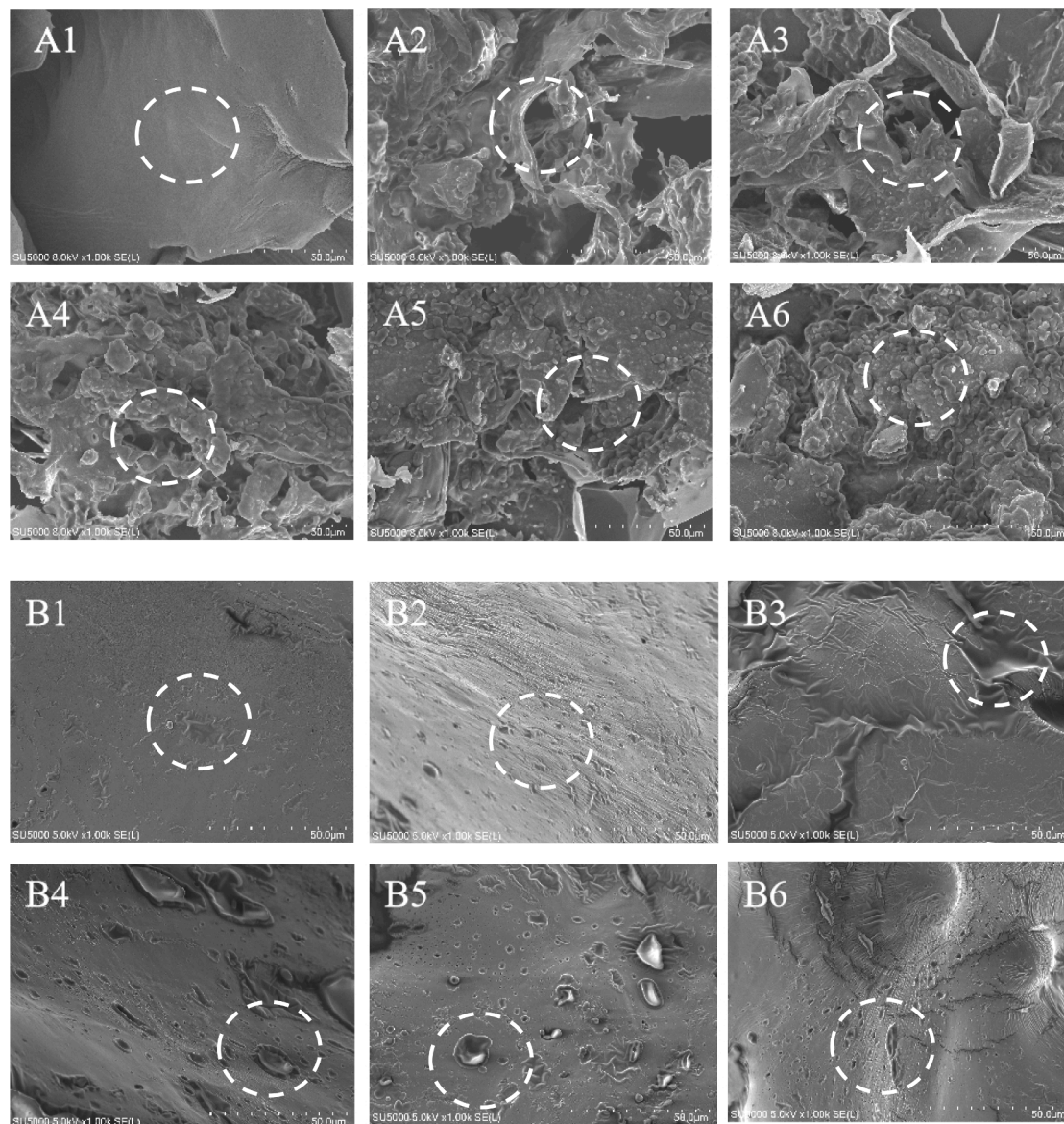


Fig. 2. Scanning electron microscopy patterns of collagen at different heating temperatures after OH and WH treatment. SEM analysis of: (A) OH, (B) WH. 1: Control; 2: Heating from 4 to 30 °C; 3: Heating from 4 to 35 °C; 4: Heating from 4 to 40 °C; 5: Heating from 4 to 45 °C; 6: Heating from 4 to 50 °C.

increase in the heating temperature. When the temperature exceeded 40 °C, voids were generated on the surface, and the number of voids on the surface increased with the increase of temperature.

3.3.3. Amino acid analysis of collagen at different heating temperatures after OH and WH treatment

Fig. 3A1 and B1 shows the amino acid composition of the eel skin collagen after OH and WH treatments. Glycine was the most abundant amino acid in collagen, accounting for 33.2–34.4% of the total amino acids. The eel skin collagen mainly comprised alanine, proline, glutamate, and hydroxyproline, whereas tryptophan and cysteine were not detected, similar to the collagen compositions found in other fish species (Bae et al., 2008; Nishimoto, Sakamoto, Mizuta, & Yoshinaka, 2005). From the collagen normalization plots in Fig. 3A2 and B2, the collagen amino acids in the WH-treated sample no particularly large fluctuations with increasing temperature. However, the amino acid contents of the collagen treated with OH at 30–35 °C, were significantly lower than those of the control group. After reaching the denaturation temperature, the amino acids content of each group increased again. However, at 50 °C, the content of several amino acids decreased significantly. This is

due to the composition change of nitrogenous substances as a result of the heating treatment. While, free amino acids are produced by the degradation of protein hydrolases and aminopeptidases, amino acids are converted to volatile compounds such as hydrocarbons, aldehydes, and amines by deamination and decarboxylation during heating (Zhang et al., 2018). In particular, at 50 °C, the amino acid content was decreased by 80.5% compared with that of the control group. In Fig. 3A4 and B4, the components are plotted in space after rotation for each amino acid principal component analysis after the influence of OH and WH. After OH treatment, non-polar amino acids were the major components, because of the increase in current density between the two polar plates with the increase in OH temperature. This causes the decomposition of the total polar amino acids by deamination and decarboxylation, resulting in a significant decrease in their content. In WH, however, no clear distinction the amino acids (Huang et al., 2016). In conclusion, OH was more effective than WH in reducing the amino acid content and changing the thermal stability of collagen.

The amino acid had good linear relationships in the range of 0–500 μM , with R^2 value was 0.9992 or higher. The LOQ value was between 0.56 and 2.21 μM , while the LOD value was between 0.18 and 0.73 μM .

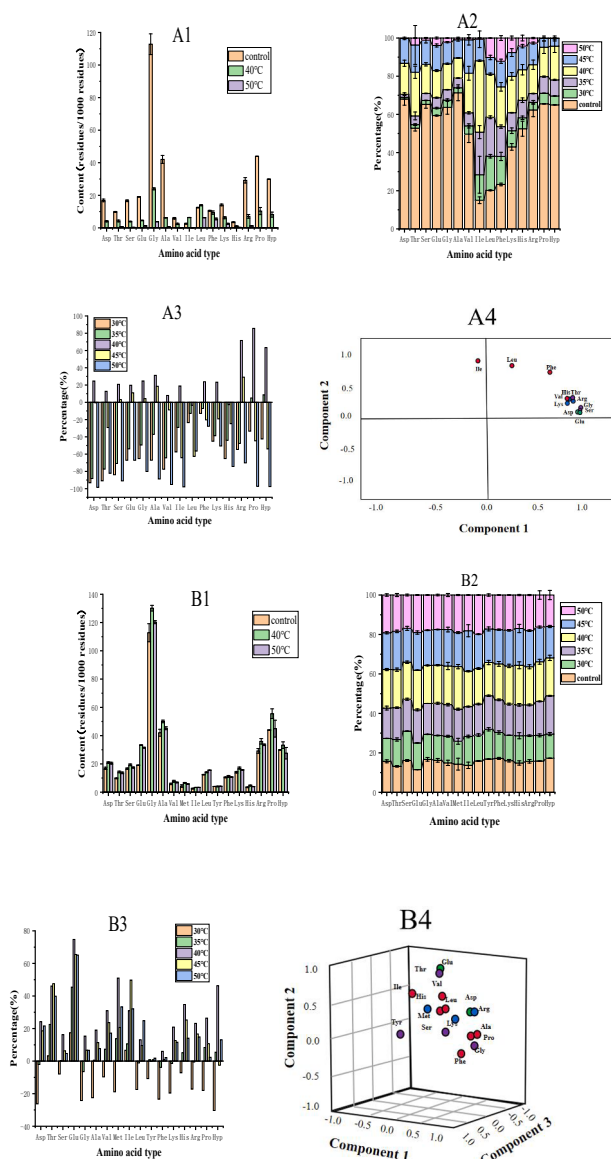


Fig. 3. Amino acid analysis profiles of OH and WH treated collagen at different heating temperatures. Amino acid analysis: A is OH, B is WH. 1: Total amino acid content of the control, 40, and 50 °C; 2: Normalized graph of total amino acid content; 3: Compared with the control group, the percentage of amino acid content change; 4: Component maps of the post-rotation space in amino acid principal component analysis. The red color is non-polar amino acids, the purple color is polar uncharged amino acids, the blue color is basic amino acids, and the green color is acidic amino acids.

The target analytes of intra and inter-day relative standard deviations of the target analytes were in the ranges 0.31–0.76% and 0.92–2.35%, and the spike recoveries were between 85.24 and 103.64% for all amino acids.

3.3.4. Water retention and oil absorption of collagen at different heating temperatures before and after OH and WH treatments

Because proteins are amphiphilic contain hydrophilic and hydrophobic groups, they possess two important functional properties: water retention and oil absorption (Liu, Li, Regenstein, & Peng, 2012). The water retention of proteins refers to their ability to retain water when subjected to external forces and is affected by concentration, ionic species, and the environment. The water retention is usually measured as the percentage of water remaining in the residue per gram of protein

after removal of absorbed water (Zeng et al., 2009). The oil uptake rate of a protein reflects its ability to adsorb oil and is usually measured as the volume of oil absorbed per gram of protein. The water retention and oil absorption rates are important indicators of the functional properties of proteins, and are closely related to the formation of flavor in foods and their overall sensory state (Ge, Wang, Li, Liu, & Qin, 2020).

The variation in the water retention of proteins with time under different heating conditions is shown in Fig. 4a and 4b. After 360 min, the residual water of proteins treated under both conditions was higher than 85%, proving that the proteins had better water retention in both cases. Furthermore, the water retention of proteins in the collagen group was slightly less than that in the WH group. This indicates that for eel skin collagen, the OH method is a slightly less destructive to the internal structure of the protein than the WH method, which is consistent with the observed by SEM (Samsudin, Halim, & Sarbon, 2018).

In Fig. 4c, the oil absorption rate of WH is higher than that of OH, which may be related to the following two points. On the one hand, the action of pepsin on the enzymatic cleavage site exposes more hydrophobic groups. Thus, an increase in temperature enhances the oil absorption of the hydrophobic group in soluble collagen. On the other hand, the absorption of protein oil is closely related to its structure. As the temperature increases, more bubble folds are generated on the protein surface after WH. The bubbles break with increasing temperature, which may also contribute to the higher oil absorption capacity of WH than that of OH (Bae et al., 2008).

3.3.5. Dielectric properties and electrical conductivity of OH- and WH-treated collagen at different heating temperatures

Immediately after heating, the dielectric properties were measured from 1 to 10 GHz to determine the basic characteristics of collagen treated at different heating temperatures. In Fig. 5A and 5B, the dielectric constant of the OH collagen group decreased with increasing temperature, and the dielectric loss increased with increasing temperature, consistent with the pattern observed in the WH group. During heating, only water loss due to protein denaturation affects the dielectric properties of fish (Liu, Fukuoka, & Sakai, 2012). Changes in the protein structure during fish protein denaturation did not directly affect the dielectric properties, as opposed to the effect of the moisture content on the dielectric properties of the meat. The collagen dielectric curves in the WH group were not smooth and were higher than those in the OH group. This is due to the increase in current density at both ends of the electrode with increasing heating temperature during OH and the dielectric imbalance between the dipole and ion loss due to the water loss after protein denaturation (Li, Li, Tang, Korai, & Jiao, 2019). Similar changes in dielectric properties were observed during the microwave heating of Antarctic krill (Zhang et al., 2017). The temperature-dependent behavior of the dielectric loss coefficient of Antarctic krill is thought to be determined by free water and dissolved ions (Ahmed et al., 2008). At lower frequencies, the dielectric loss coefficient increases continuously with temperature owing to the greater influence of ionic conductivity. At higher frequencies, the bipolar rotation of free water is the main contributor factor, and thus the dielectric loss coefficient decreases with increasing temperature.

In Fig. 5C, it can be seen that the conductivity decreases with the increase of heating temperature, this is because with the increase of OH time, the current density of the pole plate increases, which causes the structure of the protein to change, making it conductive by a factor of less, allowing the conductivity to decrease.

3.4. Effect of different heating temperatures on the allergenicity of collagen before and after OH and WH

The ELISA measurements were performed using sera from two non-allergic patients and five patients allergic to eel to assess the allergenicity of untreated and heat-treated collagen. In Table 1, irrespective of the differences between the individual sera, they showed different

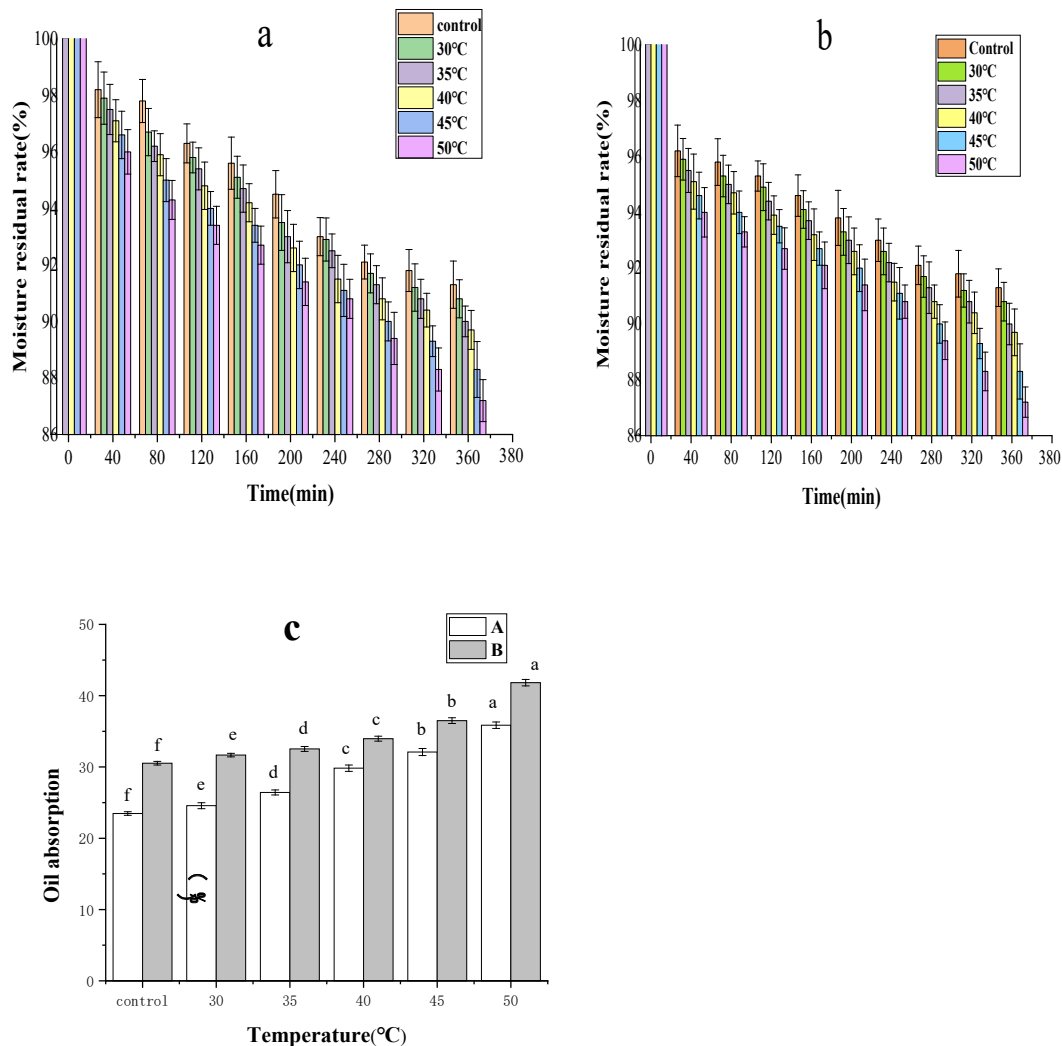


Fig. 4. Water retention test (a, b) and oil absorption (c) of fish skin collagen under OH and WH conditions. Water retention test analysis: a is OH, b is WH. Oil absorption analysis: A is OH, B is WH.

specificity for collagen at 30 °C. The IgE-binding control response was 0.18 ± 0.01 . The IgE-binding reactivity decreased significantly after high temperature treatment at 40 °C. When the temperature was increased to 50 °C, the IgE binding reactivity was further reduced. However, in most individuals, the IgE-binding reactivity at the high temperature treatment of 50 °C was indistinguishable from that at 45 °C, and decreased by 38–67% compared to that for untreated collagen. These results confirmed that both OH and WH significantly reduced the allergenicity of collagen, but OH was more effective than WH in reducing allergenicity.

The trend of IgG binding reactivity was consistent with that of IgE-binding reactivity. This suggests that OH reduces collagen allergenicity, in addition, the lowest binding capacity was obtained at 45–50 °C. The improved digestibility of eel collagen after OH treatment was further supported by the properties of the IgG-bound SGF and SIF hydrolysates after digestion. For the SGF and SIF hydrolysates, allergic reactions were reduced at temperatures reaching 40 °C. The lowest IgG responses for the pepsin and trypsin digestion products were obtained at 40–45 °C and 45–50 °C, respectively. In vivo studies including SPTs and histamine release assays, are required to further evaluate collagen sensitivity after OH treatment and digestion (Yu et al., 2011).

Previous studies have shown that OH induced structural changes in

allergens, such as denaturation, aggregation, and cross-linking, can be reflected in changes in IgE-responsive conformational epitopes (Dhineshkumar & Ramasamy, 2015). In the structural analysis of collagen, it was shown that at 40 °C, OH significantly altered the secondary and microscale structural properties of collagen and induction unfolding of the protein structure. This provides a direct explanation for the reduction of OH-induced allergenicity. However, the effect of OH on allergenicity proteins in other foods requires further investigation.

4. Conclusion

In this study, we investigated the effects of OH on the structure, thermal denaturation, physicochemical properties, and allergenicity of eel skin collagen. Compared with conventional WH, OH treatment requires $19.2 \times$ less time and $121.6 \times$ less energy consumption to reduce eel allergenicity. The molecular weight of the collagen remained unchanged both WH and OH, but the intensity of the electrophoresis strips became weaker with increasing heating time. Compared with WH, OH can increase the kinetic energy of protein molecules more effectively, leading to the vibration of polar groups and thereby changing the secondary structure. At the microscale, OH changed the collagen from a lamellar structure to a sheet-like structure, with fewer pores and folds on

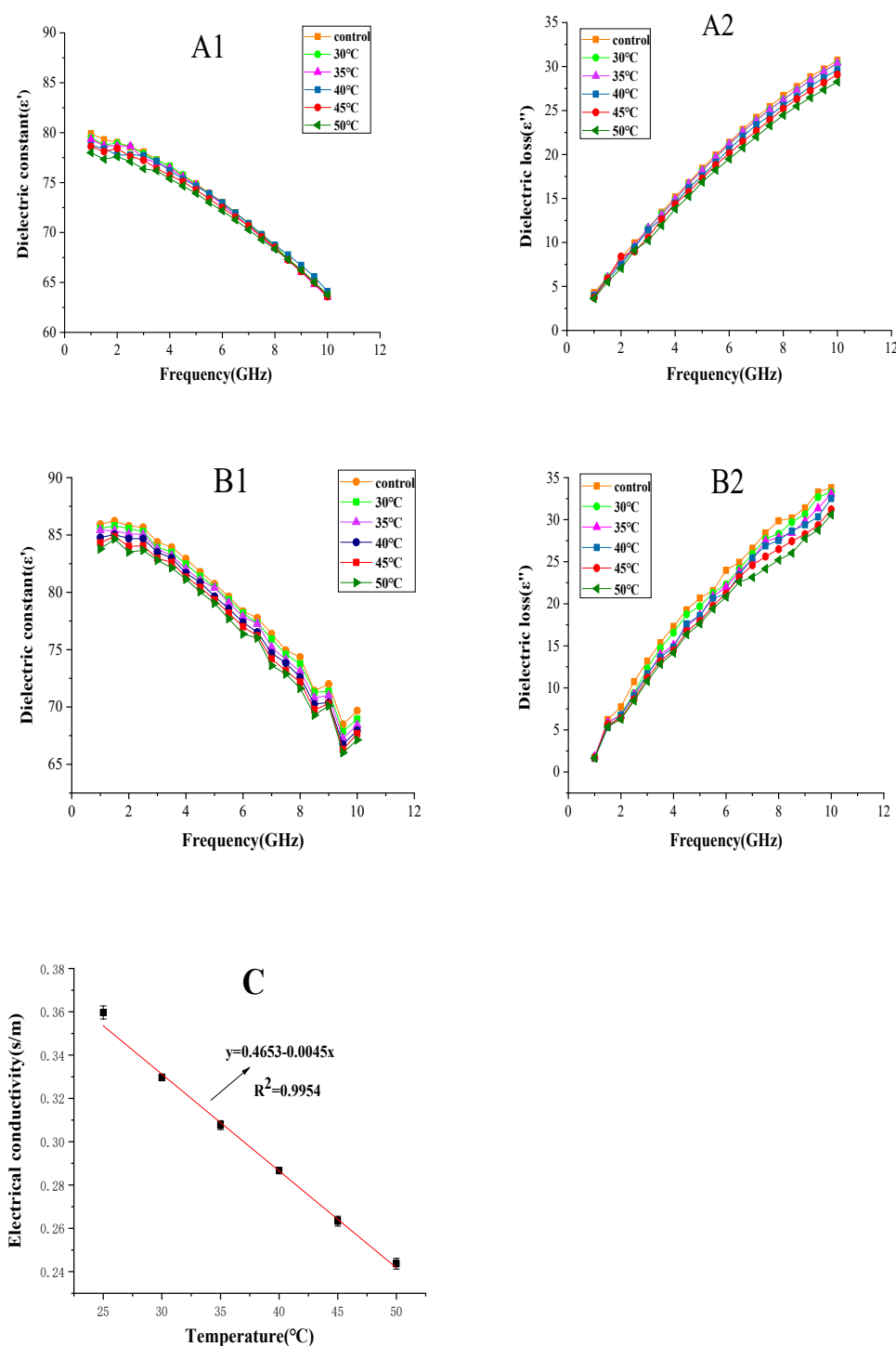


Fig. 5. Dielectric constant (ϵ') (A1, B1), dielectric loss factor (ϵ'') (A2, B2), and electrical conductivity (C) of fish skin collagen at different heating temperatures. Dielectric constant analysis: A is OH. B is WH. C is electrical conductivity.

the surface than those observed for samples treated by WH. The glycine content in the OH-treated collagen was decreased significantly compared with that in the control. The percentage of each amino acid increased after reaching the denaturation temperature. The percentage changes of each amino acid content in OH and WH differed from those in the control. The total polar amino acids were more readily decomposed by deamination and decarboxylation with OH treatment. Water

retention was decreased with increasing temperature under both OH and WH treatment, under the same heating conditions, water retention in the OH-treated sample was better than that in the sample treated by WH, while the opposite was true for oil absorption. During OH, with the increase in heating temperature, the increase in current density at both ends of the electrode and the loss of water after protein denaturation caused a dielectric imbalance between dipole and ion loss. Therefore,

Table 1

ELISA responses to collagen IgE and IgG under different conditions.

T(°C)	Serum IgE binding					Serum IgG binding		
	H1	H2	H3	H4	H5	H6	R-SGF	R-SIF
Control	0.47 ± 0.01 ^a	1.32 ± 0.05 ^a	1.23 ± 0.03 ^a	1.02 ± 0.05 ^a	0.99 ± 0.02 ^a	1.66 ± 0.04 ^a	1.47 ± 0.02 ^a	0.79 ± 0.04 ^a
30	0.37 ± 0.01 ^b	1.15 ± 0.04 ^b	0.96 ± 0.03 ^b	0.78 ± 0.01 ^b	0.84 ± 0.04 ^b	1.42 ± 0.02 ^b	1.26 ± 0.03 ^b	0.66 ± 0.03 ^b
35	0.34 ± 0.01 ^c	0.95 ± 0.03 ^c	0.70 ± 0.02 ^c	0.65 ± 0.03 ^c	0.69 ± 0.03 ^c	1.13 ± 0.03 ^c	1.13 ± 0.01 ^c	0.56 ± 0.02 ^c
40	0.29 ± 0.01 ^d	0.71 ± 0.02 ^d	0.55 ± 0.01 ^d	0.43 ± 0.04 ^d	0.49 ± 0.02 ^d	0.82 ± 0.03 ^d	0.93 ± 0.04 ^d	0.46 ± 0.02 ^d
45	0.28 ± 0.01 ^d	0.61 ± 0.02 ^e	0.38 ± 0.01 ^e	0.28 ± 0.01 ^e	0.37 ± 0.04 ^e	0.42 ± 0.03 ^e	0.76 ± 0.02 ^e	0.45 ± 0.02 ^d
50	0.28 ± 0.01 ^d	0.59 ± 0.02 ^f	0.37 ± 0.01 ^e	0.28 ± 0.01 ^e	0.37 ± 0.03 ^e	0.36 ± 0.01 ^f	0.74 ± 0.01 ^e	0.65 ± 0.02 ^b

Values were means ± SD. Values in the same column with different letters were significantly different ($p < 0.05$).

H1: indirect ELISA with human sera from non-allergic patients; H2–H6: indirect ELISA with human sera from five allergic patients. H2–WH; H3–H6–OH.

R-SGF: Indirect ELISA of the hydrolysate after simulated gastric fluid digestion (SGF) with goat anti-human IgG antibody.

R-SIF: The hydrolysate after simulated intestinal fluid digestion (SIF) was subjected to an indirect ELISA with goat anti-human IgG antibody.

the dielectric properties and conductivity decrease with an increase in the heating temperature. The decrease in collagen allergenicity in Japanese eel skin was negatively correlated with temperature ($P < 0.01$), and the IgE/IgG binding reaction of collagen was reduced by 70% at 50 °C. In conclusion, OH can effectively reduce the allergenicity of eel skin with reduced time and energy consumption relative to WH treatment.

However, the exact mechanism through which different OH treatment conditions reduce allergenicity remains unclear. Therefore, further studies are needed to identify the characterization sites in collagen and investigate their conformational changes during hydroxyl treatment. In addition, in vivo studies should be performed in future work to validate the current in vitro allergenicity studies.

CRediT authorship contribution statement

Xiaomin Li: Writing – original draft, Validation. **Yun Deng:** Resources, Conceptualization. **Weiqliang Qiu:** Project administration, Software. **Yuhui Feng:** Data curation, Software. **Yingshan Jin:** Methodology, Investigation. **Shanggui Deng:** Formal analysis, Software. **Ningping Tao:** Funding acquisition, Visualization. **Yinzhe Jin:** Supervision, Writing – review & editing, Project administration.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

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