Changes in Properties of White Shrimp (*Litopenaeus vannamei*) Protein during Thermal Denaturation

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Abstract Changes in white shrimp (*Litopenaeus vannamei*) protein during thermal denaturation were studied using Raman spectroscopy and isotopic H/D exchange. Denaturation of shrimp protein began after heating for 10 min at 50°C. A decrease in the percentage of α -helices accompanied by an increase in the percentage of β -sheets occurred while the total percentage of disordered structures increased. With extension of the exchange time, the relative intensity of the O-D bond increased, accompanied by a higher relative O-D bond intensity for heated shrimp, compared with unheated shrimp. H/D exchange revealed a higher rate of deuteration kinetics in heated shrimp than for unheated shrimp, especially during the first 2 h, consistent with water loss from denatured white shrimp protein. Physical property changes in muscle tissue can be caused by changes in hydrogen bonding and hydrophobicity during thermal processes.

Keywords: Raman spectroscopy, white shrimp, protein, structures, denaturation

Introduction

White shrimp (*Litopenaeus vannamei*) has one of the largest volumes of prawn farming production in the world. During processing and storage, efforts have been made to maintain the stability of shrimp muscle protein. Heating is the primary processing method for shrimp as a food. However, heat affects cooking loss and texture due to changes in proteins. Boiling time and the concentration of a salt solution used for boiling affect denaturation of proteins in shrimp, resulting in changes in shrinkage and texture (1). Based on a sensory evaluation, boiling time and the salt solution concentration were reported to significantly affect the texture and overall acceptance of dried shrimp products (2). A relationship between protein composition and property changes in marine-based foods during processing has been reported (3–5). Microstructure, especially the protein space structure, affects many physical properties of protein-enriched food products because the protein water holding capacity determines many physical properties of foods.

Protein structural changes occur during heating, drying, and high pressure freezing of food products. The water holding capacity and other physical properties of foods that determine mouth feel are affected by changes in protein structure. However, no information is available on the effect of heating on protein secondary structures that, in turn, affect many physical properties of shrimp.

Secondary structures of proteins, affect physical properties of

proteins and protein-rich food products. However, description of changes in protein structure without use of appropriate evaluation techniques is challenging. Recently, Raman spectroscopy has been used as a tool for quantification of molecular interactions and changes in proteins (6–9). However, no studies are known that report application of Raman spectroscopy for examination of protein structural changes produced in shrimp muscle tissue after thermal treatment.

Mouth feel and tenderness, which are mainly determined by the water holding capacity and protein denaturation, are important indices of shrimp product quality. Control of shrimp protein denaturation during heating processes is important. Therefore, relationships between protein structural changes and physical properties should be investigated. The objective of this work was to investigate the protein structural changes during thermal treatment based on isotopic H/D exchange of water and Raman spectroscopy in order to gain insights into structural and functional properties of shrimp proteins. A better understanding of shrimp protein structural changes that occur during thermal treatment can be helpful in understanding changes in quality of heated shrimp products.

Materials and Methods

Materials and chemicals Raw fresh white shrimp was purchased

from the Auchan Supermarket at Xuefu Road, Zhenjiang, China, in June of 2012. After washing with tap water, individual raw shrimp were deheaded, stripped, and kept in a polystyrene box filled with crushed ice prior to investigation. Each sample contained 100 ± 0.5 g shrimp. During preparation, the ambient temperature was not over 8°C . Deuterioxide was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Water loss Water loss was calculated from shrimp weight differences before and after heating at 50°C . Measurements were performed 6x and average values were reported.

$$\text{Water loss (\%)} = \frac{\text{Weight before heating} - \text{Weight after heating}}{\text{Weight before heating}} \times 100$$

Texture profile analysis Texture profile analysis was carried out following the method described by Niamnuy *et al.* (1) with modification. A texture analyzer (TA-XT2i; Stable Micro System Ltd., Surrey, UK) was used for evaluation of the hardness of heated shrimp. The 2nd segment from the head of shrimp was cut into an 8 mm square. The thickness of the square was the thickness of that censorious shrimp sample. A cylindrical compression probe (P/D.5, Stable Micro System Ltd.) was applied at a speed of 60 mm/min. Deformation of the shrimp sample thickness was 50% during being compressed. Maximum compressive stress was expressed as a hardness value (N) (10) and average values were calculated using 12 replicates for every tested shrimp sample.

Isotopic H/D exchange Isotopic H/D exchange was carried out as described by Ignacio *et al.* (11) with modification. Raw freshly prepared shrimp were heated at 50°C in water. Shrimp samples were taken out after 10 and 30 min. Raw fresh shrimp was used as a control. Approximately 100 g of shrimp was cut into small pieces and chopped using a blender (T18 ultra-turrax; IKA®-Werke GmbH & Co. KG, Staufen, Germany) in glass beaker for 2 min. The temperature was kept below 10°C .

Portions of minced shrimp were transferred to Spectra/Por Float-A-LyzerG2 dialysis tubes (3500-5000 MWCO; Spectrum, Irvine, CA, USA). Tubes were covered with excess deuterioxide (99.9% D, Sigma-Aldrich Co.) and maintained in a water bath at 15°C for 7 h. A tube was used for FT-Raman analysis with a 1 h interval. Deuteration kinetics was plotted as water fraction unexchanged as a function of H/D exchange time. This fraction was prescribed as the O-H/C-H peak area ratio, which was 1 when the exchange time was 0 h (11).

FT-Raman spectroscopy FT-Raman spectroscopy was carried out following the method of Ignacio *et al.* (11) with modification. Shrimp samples were transferred to a test plate. Raman spectra were excited using a 1,064 nm Nd:Yag laser line and recorded on a FT-Raman spectrometer (RFS 100/S; Bruker, Karlsruhe, Germany). Scattered radiation was collected at 180° from the source and frequency-

dependent scattering of the Raman spectra that occurs with the FT spectrometer (RFS 100/S; Bruker) was corrected based on point by point multiplication ($\gamma_{\text{laser}}/\gamma$)⁴. Raman spectra were resolved at a resolution of 4.0 $1/\text{cm}$. The temperature of shrimp samples was controlled using a thermostat at $15\text{--}20^{\circ}\text{C}$, and shrimp samples were illuminated with a laser at a power level of 300 mW. Two thousand scans were recorded for each shrimp sample, and the total scanning time for each spectrum was approximately 1 h.

The amide I band at approximately $1,650\text{ }1/\text{cm}$ was used for estimation of the secondary structural composition and was measured as reported previously (12,13). Normalization against the Raman band of phenylalanine near $1,003\text{ }1/\text{cm}$, which was considered to be invariable during conformational changes of proteins, was used as an internal standard for spectra. The $2935/1003\text{ }1/\text{cm}$ intensity ratio assigned as the CH_2 asymmetric and CH_3 symmetric stretching vibrations of aliphatic residues may be related to hydrophobic interactions. The $3220/3400\text{ }1/\text{cm}$ intensity ratio assigned as the symmetric and asymmetric OH band is related to water domain sizes (9). H/D exchange was determined based on a peak at approximately $2,500\text{ }1/\text{cm}$, which was responsible for O-D stretching.

Statistical analysis Three replicated experiments were conducted for each shrimp sample. Raman spectra were compared and evaluated using Nicolet Omnic 8.0 (Thermo Fisher, Waltham, MA, USA) and Opus 2.2 (Bruker) software. The statistical package used was Origin 8 (OriginLab Co., Northampton, MA, USA). The statistical package SPSS 18 for Windows (SPSS Inc., Chicago, IL, USA) was also used for a one-way analysis of variance (ANOVA). All analyses used a significance level of $p < 0.05$, such as physical properties. Changes of H/D exchange kinetics was established using regression analysis.

Results and Discussion

Physical changes in shrimp Most water in muscle tissue is held within myofibrils, which is the main component of muscle. During heat treatment, water loss significantly ($p < 0.05$) increased with time, compared with controls. Water loss increased from 2.49% in shrimp heated at 50°C for 5 min to 5.04% in shrimp heated at 50°C for 30 min. Hardness values also significantly ($p < 0.05$) increased with heat treatment from 13.92 N for raw shrimp to 19.78 N for shrimp heated for 15 min. The hardness value significantly ($p < 0.05$) decreased to 15.81 N for shrimp heated for 25 min, compared with controls. The hardness value of shrimp heated for 30 min significantly ($p < 0.05$) increased to 17.17 N, compared with controls.

Elasticity, cohesiveness, and chewiness values showed similar trends during the heating process caused by denaturation of myofibrillar protein, which constitutes most of shrimp muscle tissue. Aggregation caused by protein denaturation was induced by external and internal myofibrillar water loss. As a consequence, physical properties of shrimp, especially hardness and chewiness values,

Fig. 1. Amide I Raman spectra of raw fresh white shrimp (*Litopenaeus vannamei*) and shrimp heated at 50°C for different times. A: fresh raw shrimp muscle, B: shrimp heated for 10 min, C: shrimp heated for 20 min, D: shrimp heated for 30 min.

changed. Changes in protein water holding capacity and texture have been attributed to alterations in protein-water interactions (14,15). Protein denaturation and aggregation induced by heat caused shrinkage of both the filament lattice and collagen, and also caused exposure of hydrophobic aliphatic side chains of myofibrillar protein, which allowed new intra- and inter-protein interactions that induced a more dense protein structure (9,16). Hence, water was pressed out of muscle cells, leading to water loss. However, high water loss induced both a hardness increase in shrimp products and a decrease in weight. Both factors reduce product quality. In addition, soluble proteins that escape with water loss during the boiling process can produce a paste on cooking equipment causing difficulty in post-use cleaning and increasing production costs. Therefore, protein denaturation should be controlled based on adjustment of the cooking process to achieve a low level of water loss.

Protein secondary structures A band at approximately 1,655 1/cm was associated with secondary structures of protein conformation (12) and consisted of overlapped band components falling in ranges of 1,658-1,650 1/cm, 1,680-1,665 1/cm, and 1,665-1,660 1/cm attributable to α -helices, β -sheets, and random coils, respectively (17).

Amide I spectra from raw fresh shrimp and shrimp samples heated at 50°C for 10, 20, and 30 min were determined (Fig. 1). The amide I band revealed a shifting of the band maximum toward high

frequencies from raw fresh shrimp to heated shrimp. The longer the heating time, the greater the displacement of the maximum peak. During the initial 20 min of heating, the relative intensity of the amide I band significantly ($p<0.05$) increased with the heating time, compared with controls, but subsequently significantly ($p<0.05$) decreased, compared with shrimp heated for 20 min.

Analysis of protein secondary structures (Table 1) showed that raw fresh white shrimp possessed 36.62% α -helices, 21.82% β -sheets, 22.41% unordered structures, and 19.15% turns. Secondary structural percentages (Table 1) confirmed that the most pronounced changes that occurred in white shrimp muscle tissue heated at 50°C mainly involved a significant ($p<0.05$) increase of β -sheet components at the expense of α -helices with heating time, compared with controls. The helical structure percentage significantly ($p<0.05$) decreased by 55% after heating, compared with controls, while the β -sheet percentage significantly ($p<0.05$) increased by 65% and the unordered structural percentage significantly ($p<0.05$) increased by approximately 18%, compared with controls.

The percentage value of protein turn structures did not change. Herrero *et al.* (6) reported that a significant decrease in the percentage of α -helices in meat surimi after heating was accompanied by a significant increase in the β -sheet structural percentage. The decrease of the α -helices structural percentage was an indicator of protein denaturation and subsequent rearrangement to form a new 3 dimensional structure (18). Niwa (19) suggested a relationship between liberation of bound water from the random coil peptide carbonyl group and formation of β -sheets upon heating at a high temperature.

C-H stretching region Relative intensity values of the strongest C-H band located near 2,935 1/cm attributed to CH_2 asymmetric and CH_3 symmetric stretching vibrations of aliphatic residues (11) are shown in Fig. 2. The relative intensity of this band significantly ($p<0.05$) increased in heated shrimp with heating time (Table 2), compared with controls. The relative intensity of the band centered at 3,064 1/cm also significantly ($p<0.05$) increased, compared with controls, probably attributable to denaturation of proteins involving changes in the environment of aliphatic C-H groups and protein tertiary structures related to hydrophobic interactions (20,21). The spatial structure of native proteins maintained by hydrogen bonds and hydrophobic interactions was destroyed during heat treatment. As the heating time increased, proteins from the original curl ordered structure turned into a disordered loose stretch, which induced

Table 1. Percentage of secondary structural features in the amide I band of shrimp protein heated at 50°C

Secondary structure	Fresh Raw	Heated 10 min	Heated 20 min	Heated 30 min
α -Helices	36.62	30.74	24.42	16.57
β -Sheets	21.82	24.96	29.48	36.07
Regular structures	58.44	55.70	53.90	52.64
Unordered	22.41	21.60	30.46	26.54
Turns	19.15	22.70	15.64	20.82

Fig. 2. Raman spectra in the 2,200-3,500 1/cm stretching region of white shrimp muscle tissue. A: fresh raw shrimp muscle tissue, B: shrimp heated for 10 min, C: shrimp heated for 30 min.

exposure of a large number of aliphatic amino acid residues, valine, leucine, and isoleucine on the molecular surface and breakage of hydrogen bonds. As a result, the hydrophobicity of protein was enhanced. However, Nazlin *et al.* (22) reported that the main protein band 2,940 1/cm was attributable to aromatic and aliphatic amino acids and other amino acids, and the relative intensity of the band increased significantly when β -lactoglobulin was heated. Other features in the C-H stretching region that can be used for detection of hydrophobic interactions, denaturation, and conformational changes also changed during heat treatment of proteins. However, changes in hydrogen bonding and hydrophobicity of proteins caused by heat treatment probably altered protein secondary structures.

O-H stretching region Intramolecular vibrations of water included 2 O-H stretching bands (Fig. 2). The symmetric band of the γ_s (OH) maximum appeared at approximately 3,220 1/cm, and the asymmetric band of γ_{as} (OH) appeared at approximately 3,400 1/cm (23). The relative intensity of the symmetric band increased in shrimp samples heated at 50°C for 10 min. However, the relative intensity of this peak in shrimp samples decreased after heating for 30 min. An increase in the relative peak area of the band 3,065 1/cm for aromatic amino acids is also shown in Fig. 2. Arteaga (24) established a positive correlation between the position of the band 3,065 1/cm and the hydrophobicity value measured using fluorescent probe methods. The Raman spectral zone between 3,100 1/cm and 3,500 1/cm that contributed to symmetric and asymmetric O-H stretching

motions was related to structural changes in hydrogen-bound water. The 3,220/3,400 1/cm intensity ratios of the O-H band were positively correlated to the water domain size (23,25). In this study, 3,220/3,400 1/cm intensity ratios of the O-H band decreased with heat treatment, which indicated that the water holding capacity of white shrimp protein weakened during thermal denaturation.

The intensity of the O-H stretching band relative to the intensity of the C-H stretching band was a spectroscopic parameter used in this study. This relative intensity ratio, determined using Raman signals at approximately 3,220 1/cm and 2,935 1/cm ($I_{3,220\ 1/cm}/I_{2,935\ 1/cm}$) (Table 2) decreased gradually during heat treatment, indicating water loss due to protein denaturation.

H/D exchange The O-D stretching profiles of raw fresh shrimp (Fig. 3) and shrimp heated for 30 min at 50°C (Fig. 4) during H/D exchange were determined. For both, there was no band near 2,500 1/cm for O-D stretching when the exchange time was 0 h (26). However, a band centered 2,500 1/cm appeared with deuteration. Furthermore, the intensity of this peak increased with deuteration until a maximum value was reached after 5 h. The maximum intensity of this band for heated shrimp was higher than for raw fresh shrimp, suggesting that the amount of exchangeable H atom in heated shrimp protein increased with protein denaturation as a result of hydrophobic interaction enhancement. Therefore, the protein-bound water capacity decreased, and water loss increased during heat treatment.

H/D exchange in proteins was affected by the environment of the O-H group. The relative intensity of the O-D stretching band at 2,500 1/cm in the 3 shrimp samples that heated for 10, 20, and 30 min increased with deuteration, and the relative intensity of the O-D stretching band in heated shrimp samples was higher than for raw fresh shrimp and exhibited a time-effect relationship. The rate of H/D exchange in thermal denatured shrimp proteins was higher than for natural proteins indicating that more hydrogen atoms were exposed in denatured proteins. The increase in amounts of exchangeable hydrogen might be induced by exposure of hydrophobic amino acid residues during protein denaturation (13,27), suggesting that exposure of hydrophobic amino acid residues was the main reason for the protein bound water capacity decrease. As such, the more hydrophobic amino acid residues were exposed, the more water loss occurred. Finally, the weight and quality of the shrimp product decreased with a longer cooking time.

Fresh shrimp protein contains a high proportion α -helices and β -

Table 2. C-H stretching expressed as intensity normalized against the 1,004 1/cm peak, the relative 3,220/3,440 1/cm intensity ratio, the relative 3,220/2,935 1/cm intensity ratio (mean $\pm$ SEM) in fresh, raw white shrimp (*Litopenaeus vannamei*) heated for 10 min and 30 min

Shrimp Type	$I_{2,935/1,004}$ C-H stretching	$I_{3,220/3,400}$ O-H stretching	$I_{3,220/2,935}$
Fresh raw	1.53 $\pm$ 0.03 ^{c1)}	2.22 $\pm$ 0.02 ^a	0.91 $\pm$ 0.01 ^a
Heated 10 min	2.45 $\pm$ 0.02 ^b	1.95 $\pm$ 0.02 ^b	0.66 $\pm$ 0.01 ^b
Heated 30 min	3.23 $\pm$ 0.03 ^a	1.58 $\pm$ 0.02 ^c	0.39 $\pm$ 0.01 ^c

¹⁾Different letters for the same parameter in a column indicate significant differences ($p < 0.05$).

Fig. 3. Raman spectra H/D exchange for 0, 2, 4, and 6 h in fresh raw white shrimp (a: shrimp muscle tissue, b: shrimp heated for 30 min). A: 0 h, B: 2 h, C: 4 h, D: 6 h.

sheets. Hydrogen atoms are bound in the protein secondary structure, which makes exchange difficult. A decreasing trend in hydrogen atom binding in regular protein secondary structures was observed during thermal treatment (Table 1). In contrast, the percentage of unordered secondary structures increased, accompanied by enhancement of hydrophobic interactions. Consequently, exchangeable hydrogen in shrimp muscle protein was exposed. It has been demonstrated that the amount of quickly exchangeable hydrogen is less in regular protein, which contains a high proportion of secondary structural α -helices and β -sheets, compared with denatured protein (28). Leikin *et al.* (26) also reported that amides exchange protons at a much slower rate in collagen aggregates, particularly in well ordered, thick collagen films. Thus, the total α -helices and β -sheets percentage is inversely proportional to the content of hydrogen that can be quickly exchanged.

Deuteration kinetics expressed as a fraction of unconverted water O-H bonds vs. time were significantly ($p < 0.05$) different between fresh shrimp and heated shrimp, but no significant ($p > 0.05$) difference was observed between shrimp samples heated for 10 and 30 min. Kinetics were rapid in the initial 2 h for both fresh and heated shrimp, specifically rapid for the latter. However, kinetics of the 3 shrimp samples heated for 10, 20, and 30 min became less rapid after 2 h. Exchange, as in chemical reactions, was generally faster in the initial stage, then gradually reached equilibrium. The bonding energy of O-D was higher than for O-H, so the amount of O-D bonding gradually increased to eventual saturation at equilibrium.

The exchange rate fitted to linear equations was compared using regression analysis. The linear regression equation for exchange in fresh shrimp during the first 2 h was $y = 1.01 - 0.13x$ ($R^2 = 0.98$, x -exchange time). The linear equation for fresh shrimp deuterated over 2 h was $y = 0.85 - 0.05x$ ($R^2 = 0.97$). The slope of the equation for fresh shrimp during the first 2 h was lower than the slope for heated shrimp samples for the first 10 min ($y = 0.98 - 0.29x$; $R^2 = 0.97$) and 30 min ($y = 0.95 - 0.3x$; $R^2 = 0.84$). However, the slope of the equation for

Fig. 4. Raman spectra H/D exchange for 0, 2, 4, and 6 h in white shrimp heated for 30 min. A: 0 h, B: 2 h, C: 4 h, D: 6 h.

fresh shrimp deuterated over 2 h was higher than the slope for shrimp samples heated for 10 min ($y = 0.48 - 0.03x$, $R^2 = 0.96$) and heated for 30 min ($y = 0.47 - 0.03x$, $R^2 = 0.78$). H/D exchange kinetics showed that the hydrogen bonding strength of heated shrimp samples was lower than for fresh shrimp.

The water holding capacity of denatured protein is lower than for natural protein (9). The denatured protein structure became irregular and the total percentage of α -helices and β -sheets decreased, which resulted in an increase in the amount of exchangeable hydrogen. H/D exchange kinetics showed the extent of enhancement of hydrogen exposure caused by protein denaturation during thermal treatment. Water loss during heating of white shrimp was probably due to secondary structures and H/D exchange characteristics of protein. Exposure of hydrophobic aliphatic side chains of myofibrillar proteins enhanced changes in secondary structures, and the percentage of irregular structures increased during heat treatment. Consequently, aggregation of myofibrillar protein was enhanced and the water storage space of muscle decreased, thus inducing water loss. The degree of water loss should be reduced during heating processes to reduce the degree of protein denaturation in order to enhance the quality of cooked shrimp products.

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