Mobility and redistribution of waters within bighead carp (*Aristichthys nobilis*) heat-induced myosin gels

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**ABSTRACT**

Water-holding capacity is closely related to gel microstructure, and is a very important quality trait in surimi and surimi products. The changes in the secondary structure, gel microstructure, and the migration of water in bighead carp (*Aristichthys nobilis*) myosin gel induced by different temperatures (50–90°C) were investigated. The \(\alpha\)-helical structure of myosin decreased at temperatures of 40°C or higher. The fractal dimension of the gels increased at 40, 50, and 60°C, but decreased at temperatures over 60°C. The pore size of the gels increased with temperatures up to 50°C, decreased at 60°C, and then increased with temperatures up to 90°C again. The transverse relaxation times also varied; \(T_{21}\) remained constant at temperatures over 40°C; \(T_{22}\) decreased at temperatures lower than 50°C, increased at 60°C, and then decreased with temperatures up to 90°C; and \(T_{23}\) increased at temperatures lower than 50°C and then remained constant until 90°C. Principal component analysis showed that the proportion of \(T_{22}\) water (PT\(_{22}\)) was inversely correlated with the unfolding of myosin, whereas directly correlated with the pore size. The proportion of \(T_{23}\) water (PT\(_{23}\)) was positively correlated with the fractal dimensions of the gels, whereas negatively correlated with the pore size. The migration of the secondary layer of water was mainly caused by hydrophobic force and the physical space formed by the myosin backbone, and the migration of water within the third layer was mainly caused by capillary pressure. Therefore, the mobility and redistribution of waters depend on the water retention mechanism, which is determined by the physical structure of gels. This study provides further information about the relationship between the NMR data, gel microstructure, water mobility, and distribution.

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**Introduction**

Water-holding capacity (WHC) of surimi and surimi products is related to the microstructure of the gels, which are considered as a series of interconnected capillary tubes.\textsuperscript{[1]} Therefore, WHC was suggested to be mediated primarily by capillary pressure.\textsuperscript{[2,3]} However, the physical structures within gels are mainly influenced by temperature and the heating duration.\textsuperscript{[4–8]} During heating, proteins unfold, aggregate, and eventually form a three-dimensional network.\textsuperscript{[9,10]} The peptide backbone becomes extensively hydrated and the mobility of the water reduces when protein unfolding.\textsuperscript{[10]} Furthermore, the water molecules near the hydrophobic groups that were exposed when the protein unfolded arrange themselves into hydrogen-bonded clathrates, which are “ice-like” structures. As a result, the mobility of water molecules decreases, and the system becomes less random.\textsuperscript{[11]} A good equilibrium between the unfolding and aggregation of myosin is important for the formation of a gel.
with a good microstructure. Protein aggregation prior to unfolding leads to a coarse microstructure with larger pores and thus a lower capillary pressure, which, in turn, results in a lower WHC, and vice versa.\cite{12} Therefore, WHC is closely correlated with the unfolding and aggregation of the protein. However, how waters are incorporated into the protein matrix is still not completely understood, although most researchers believe in the prevailing hypothesis of capillary pressure following the Young–Laplace equation.\cite{1–3}

Low-field nuclear magnetic resonance (LF-NMR) spectroscopy has been used to study the relationship between the mobility of water and the food microstructure.\cite{13–15} The NMR spin-spin relaxation ($T_2$) is an important index representing the status of the water molecules.\cite{16} However, the three-dimensional network of the gel system was so complex that the relationship between the NMR data and the gels microstructure is not clear.

In this article, we investigated the moisture distribution and migration within bighead carp myosin heat-induced gel at various temperatures, as different temperatures caused changes in the unfolding and aggregation of the proteins. Therefore, we can get gels with different physical structure for to further understand the relationships between water migration, myosin secondary structures, and the three-dimensional network of the gels. Moreover, the present study suggested the differences in water mobility mainly attributed to the different water holding mechanism(s) and physical structure among gels.

**Materials and methods**

**Materials and chemicals**

Fresh bighead carp weighting 1000 ± 50 g (a total of three fishes) were purchased from the Auchan supermarket (Zhenjiang, China) and placed in a plastic bag filled with oxygen. The fishes were transported to the laboratory on ice in a polystyrene box within 30 min. Adenosine triphosphate disodium was obtained from Aladdin (Aladdin Co., Shanghai, China). All other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All reagents and chemicals are analytical grade.

**Preparation of myosin protein**

Myosin from the dorsal muscle of the bighead carp was prepared as described by Liu et al.\cite{17} with some modifications. The fresh bighead carp was stunned by knife-blade, gutted, immediately beheaded, and then cut in half through the dorsal flesh. The dorsal muscle was separated from the skin and bone by hand before chopping. The muscle was then promptly mixed with 100 mM KCl, 20 mM Tris-HCl (pH 7.5) buffer in a 10:1 v/v ratio (solvent:muscle). The fish muscle was kept on ice at temperatures below 8°C during the extraction process. The minced muscle was homogenized using a Waring blender (T18, IKA Co., Staufen, Germany) at 1,2000 \( \times \) g for three intervals of 20 s each. The mixture was allowed to rest for 15 min at 4°C and then centrifuged at 3000 \( \times \) g for 5 min (4°C) (Avanti J-26XP, Beckman Coulter, Germany). The precipitate was diluted by five times with a buffer containing 450 mM KCl, 5 mM beta-mercaptoethanol, 200 mM Mg(CH$_3$COOH)$_2$, 1 mM [ethylenedinitrilo] tetraacetic acid (EGTA), and 20 mM Tris-maleic acid (pH 6.8). Adenosine triphosphate disodium was added to the solution at a final concentration of 10 mM. The buffer was allowed to rest for 90 min at 4°C and then centrifuged at 1,2000 \( \times \) g for 10 min at 4°C. The supernatant was slowly diluted by seven times with 1 mM KHCO$_3$ solution and kept at 4°C for 25 min prior to centrifugation at 1,2000 \( \times \) g for 10 min. The precipitate was resuspended in a 500 mM KCl, 5 mM beta-mercaptoethanol, 20 mM Tris-HCl (pH 7.5) buffer at a 1: 2.5 v/v ratio of precipitate:buffer and left to rest for 10 min at 4°C. The solution was then diluted by five times in 1 mM KHCO$_3$. MgCl$_2$ was added to the solution to a final concentration of 10 mM. After
homogenization, the suspension was stored overnight at 4°C and then centrifuged at 17,000 g for 25 min (4°C). The collected myosin was kept on ice for further analysis.

**Myosin determination**

The collected myosin was dissolved in 500 mM NaCl, 20 mM Tris-HCl (pH 7.0), and then was centrifuged at 9000×g for 10 min at 4°C. The supernatant solution was used to prepare gels. The concentration of myosin in the supernatant solution was over 25 mg·mL⁻¹, which was determined by the Bradford method. The purity of the myosin was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Myosin purity was also determined by densitometry (Gel-Pro Analyzer, Version 4.00, Media Cybernetics, Bethesda, MD, USA). The electrophoretic profile of the extracted myosin is shown in Fig. 1. The purity of myosin used in this work was greater than 90%, although a little actin existed.

**Raman spectroscopy analysis**

The myosin solution was heated at 40, 50, 60, 70, and 90°C for 30 min, respectively. Following heating, the samples were cooled in water and frozen in a deep freezer at −80°C for more than 4 h. The samples were then freezing dried for 30 h with vacuum freeze-drying equipment (Boyikang Co., Ltd., Beijing, China). The experiments of Raman spectra were performed using laser Raman microspectroscope (DXR, Thermo Co., Waltham, MA, USA) at an excitation wavelength of 532 nm. The spectral resolution was 2 cm⁻¹. The samples were placed on a slide focused at approximately 10 mw. The spectra were recorded within the 400–3500 cm⁻¹ range. The final spectral

![Figure 1. SDS-PAGE of myosin extracted from fish muscle. Lane 1 designates molecular weight markers, loaded with 5 µg, and lane 2 for protein collected with 20 µg. MHC: myosin heavy chain.](image-url)
data of each sample were obtained by averaging 40 scans. All spectra were collected with multipoint baseline correction and smoothing using Omnic 8.2.0 software (Thermo Fisher Scientific Inc., Waltham, MA, USA). The peak intensity and the range area from 1600 to 1700 cm$^{-1}$ were obtained by deconvolution, second derivative analysis, and fitting in Peak Fit 4.12 software (Sea Solve Software Inc., San Jose, CA, USA). The amide I band (approximately 1650 cm$^{-1}$) was used to estimate the secondary structure of the protein.\textsuperscript{20}

**Scanning electron microscopy (SEM)**

The samples were prepared according to the procedure described by Hayakawa et al.\textsuperscript{21} with some modifications. The collected myosin was put in a series of small beakers (2 cm diameter) and heated at 40, 50, 60, 70, and 90°C for 30 min. Heat-induced myosin gel samples (5 × 5 × 1 mm cubes) were fixed in 3% glutaraldehyde containing 0.1 M phosphate buffer (pH 7.0) for 4 h. The gel pieces were rinsed three times with 0.1 M phosphate buffer (pH 7.0) and fixed again in 1% osmium tetroxide for 24 h at 4°C. The samples were dehydrated in a series of graded concentrations of ethanol, soaked in isoamyl acetate for 15 min, and then dried with a critical point dryer (CPD-030, Baltec Co., Pfäffikon, Switzerland). The dried specimens were coated with 10 nm of gold and later observed with a scanning electron microscope (Quanta-200, FEI Co., Hillsboro, OR, USA) with an accelerating voltage of 10 kV. Fifteen SEM images were obtained from each sample for a pore-scale analysis.

**Analysis of the pore diameters from SEM images**

SEM images were analyzed using the publicly available software ImageJ 1.43 \( \mu \) (NIH, Bethesda, MD, USA). The black areas represent the network holes, whereas the white areas represent the protein aggregates based on the thresholded binary images. To minimize human bias, a 10 × 10 \( \mu \)m grid was overlaid on top of each micrograph, and only the pores situated at grid intersections were selected and measured.\footnote{1} The average equivalent pore diameter was calculated according to the following equation:\textsuperscript{22}

\[
d = \sqrt[4]{\frac{4A}{\pi}}\tag{1}
\]

\[
A = \frac{S \times K}{n}\tag{2}
\]

where \( d \) is the average equivalent pore diameter, \( A \) is the average area, \( n \) is the number of pore, \( S \) is the total area of the image, and \( K \) is the pore fraction.

**Fractal dimension (\( D_f \)) evaluation**

The fractal dimension of the thresholded binary images was calculated by the box-counting method using MATLAB software (version 7.11, The Math Works Inc., Natick, MA, USA) and by Eqs. (3) and (4).\textsuperscript{23}

\[
D = \frac{\log(N_r)}{\log(1/r)}\tag{3}
\]

\[
D_f = D + 1\tag{4}
\]

where \( N_r \) is the box’s number within a gel network at the given scale, and \( r \) is the corresponding scale. The evaluation of \( D_f \) is based on the two-dimensional space. Therefore, it is necessary to add an extra dimension to the \( D \) value to characterize the three-dimensional structure of a protein gel.\textsuperscript{24}
**LF-NMR measurement**

Following the method described by Han et al.\(^{[25]}\) with some modifications, myosin gel (1 g) was placed inside the transparent tube (12 mm in diameter), and then was measured on the Niumag Benchtop Pulsed NMR analyzer (Niumag PQ001, Niumag Electric Cooperation, Shanghai, China) operating at a proton resonance frequency of 22.6 MHz. The transverse relaxation time \(T_2\) was measured using the Carr–Purcell–Meiboom–Gill (CPMG) with 12,000 echo, 8 scans, 6 s between scans, and a half-echo time of 400 µs between pulses of 90 and 180.\(^{[26,27]}\) The low-field NMR relaxation curve was fitted to a multieponential curve according to the following equations with Multi-Exp Inv Analysis software (Niumag Electric Cooperation, Shanghai, China).

\[
A(t) = \sum A_{0i} \exp \left( -\frac{t}{T_{2i}} \right)
\]

(5)

where \(A(t)\) is the amplitude at time \(t\), \(t\) is the time of decay, \(A_{0i}\) is the amplitude of the component \(i\), and \(T_{2i}\) is the transversal relaxation time of the component \(i\).

**Statistical analysis**

All experiments were run in triplicate and the data are presented as the mean with the standard deviation. Data were subjected to analysis of variance (ANOVA) and the differences between treatments were determined by Duncan’s multiple range test. The significance of difference was established at \(p < 0.05\). All statistical analyses were performed using SPSS 18.0 (SPSS Inc, Chicago, IL, USA).

**Results and discussion**

**Changes in secondary structure as observed by Raman spectroscopy**

The Raman spectra of the bighead carp myosin heated at various temperatures (40–90°C) are shown in Fig. 2(a). The data from amide I, a fraction of various myosin secondary structures, are shown in Table 1. As shown in Fig. 2(b), the percentage of \(\alpha\)-helix tended to decrease as the temperature increased. When incubated at 40°C for 30 min, the \(\alpha\)-helix content of the bighead carp myosin decreased from 39.4% to 37.3%, indicating that the myosin is unfolding. When heated at 90°C for 30 min, the \(\alpha\)-helix content of the myosin significantly decreased by 38.6% \((p < 0.01)\). The results showed that the degree of myosin unfolding increased with the increasing temperature. The percentages of random coils and \(\beta\)-turns also markedly increased \((p < 0.05)\). Liu et al.\(^{[28]}\) reported that \(\alpha\)-helix of silver carp myosin gradually decreased with increasing temperature. Hydrogen bonds and hydrophobic interactions are the critical factors in maintaining the native helical conformation of the myosin. Moreover, the balance must be maintained between intermolecular hydrogen bonding and side chain charge, which arises from the kinetic motion of molecules, as determined by temperature. With temperature increasing, the large degree of random motion tends to diminish the hydrogen bonds formation. As a result, the helix will collapse.\(^{[29]}\) During the protein unfolding, the percentage of disordered secondary structures increased along with a concomitant exposure of hydrophobic amino acids, which then caused neighboring protein molecules to interact, resulting in aggregation of the protein molecules. The bound water and the secondary layer of water around the protein molecules arranged themselves into hydrogen-bonded clathrates, which are “ice-like” structures.\(^{[11]}\) However, the unfolding and aggregation processes varied under different heating conditions. Consequently, hydrophobic interactions and hydrogen bonds between myosin and water molecules also varied, which causes different mobilities and redistributes the waters within gels.
Microstructure of the bighead carp myosin gel

The gel’s macroscopic and microscopic structural attributes change with the alteration of heat treatment conditions,\(^{[30]}\) by changing the rates and mechanisms of denaturation and aggregation.\(^{[25]}\) Fig. 3(a) shows SEM micrographs of the bighead carp myosin gels at temperatures 40, 50, 60, 70, and 90°C (\(a_1\)–\(e_1\)) and their corresponding 8-bit binary images (\(a_2\)–\(e_2\)). Significant differences in the physical structure of gels depending on temperature were obtained. Initially, the size of the aggregates seemed to be small and then increase with increasing temperature. At 40°C, the gel was mainly composed of particle aggregates (Fig. 3(a)—\(a_1, a_2\)). At 50°C, the organized network structure appeared in the sample (Fig. 3(a)—\(b_1, b_2\)), but the surface of the gel was rough. Additionally, the interconnection between protein molecules increased, which is visible in the threshold images (Fig. 3(a)).

**Figure 2.** Raman spectra (a) and changes of \(\alpha\)-helix content of myosin (b) were profiled at different heating temperature. 
\(a, b, c, d, e\) represented the profiles at 40, 50, 60, 70, and 90°C, respectively.

\[ y = -2.8329x + 43.495 \\
R^2 = 0.9192 \]
Table 1. The changes of secondary structure percentage in amide I band at different heating temperatures.

| T°C | 0   | 40  | 50  | 60  | 70  | 90  |
|-----|-----|-----|-----|-----|-----|-----|
| α-Helix | 39.44 ± 2.93<sup>a</sup> | 37.30 ± 3.47<sup>b</sup> | 36.44 ± 2.97<sup>c</sup> | 33.47 ± 3.71<sup>d</sup> | 30.33 ± 1.47<sup>e</sup> | 24.22 ± 2.23<sup>f</sup> |
| Random Coil | 27.95 ± 3.78<sup>b</sup> | 33.57 ± 4.84<sup>d</sup> | 31.70 ± 4.04<sup>c</sup> | 26.53 ± 1.78<sup>a</sup> | 34.90 ± 8.80<sup>g</sup> | 39.46 ± 6.95<sup>f</sup> |
| β-Sheet | 22.05 ± 2.45<sup>c</sup> | 17.01 ± 2.31<sup>a</sup> | 20.65 ± 1.81<sup>b</sup> | 23.82 ± 5.92<sup>d</sup> | 24.69 ± 9.29<sup>c</sup> | 21.88 ± 2.09<sup>e</sup> |
| β-Turn | 10.56 ± 4.94<sup>a</sup> | 12.11 ± 4.41<sup>b</sup> | 11.20 ± 3.91<sup>ab</sup> | 16.19 ± 4.45<sup>d</sup> | 10.09 ± 3.47<sup>a</sup> | 14.44 ± 3.81<sup>c</sup> |

*(mean ± SD, n = 3) with Duncan method for multiple comparison. Same column with different capital letters shows significant difference between the groups (p < 0.01); with different small letters shows significant difference between groups (p < 0.05); with the same small letters indicates no significant difference between groups (p > 0.05).*
Figure 3. Typical SEM micrographs at 5000× magnification (a) and changes of fractal dimension and pore diameters (b) of myosin gel. The SEM images, a1–e1, and the binary thresholded images (at a grey level of 100), a2–e2 at 40, 50, 60, 70, and 90°C, respectively. Scale bar in SEM images represents 1 µm.
average pore diameter decreased from 0.105 µm to 0.080 µm as the temperature increased to 60°C and then increased markedly to approximately 0.128 µm when the temperature up to 90°C. Compared with the data at 60°C, bigger pore size and more free water were present at 40°C. Additionally, the gel that formed at 90°C exhibited the same value (Fig. 3(b)). The smallest average pore diameter was seen in the gel that was heat induced at 60°C, which might be caused by the modori from the endogenous protease in the fish muscle.\(^{[31,32]}\) At 60°C, part of the aggregates and covalent cross-linking within the gel might be destroyed by the endogenous protease, resulting in a smaller pore size. As discussed earlier, myosin unfolded with increasing temperatures, and more reactive groups, which was necessary for aggregation, exposed. Therefore, the reactive groups of myosin molecules reacted more adequately and led to the formation of the continuous gel matrix and eventually a smaller pore size.\(^{[33,34]}\)

Fractal analysis is an effective method for quantifying the changes in the microstructure of the gel.\(^{[35]}\) The fractal dimension (\(D_f\)) gives the degree of complexity of the protein aggregates. From the slopes of the plots, the values of \(D\) of the heat-induced gels were 1.841 (40°C), 1.863 (50°C), 1.890 (60°C), 1.875 (70°C), and 1.871 (90°C), and the corresponding values of \(D_f\) were 2.841, 2.863, 2.890, 2.875, and 2.871, respectively. The \(D_f\) values increased from 2.841 to 2.890 as the temperature increased from 40°C to 60°C, which indicated that the gels were weak-link because of a low concentration (25 mg·mL\(^{-1}\)). The results suggested a high degree of complexity and the formation of a more ordered microstructure for all samples.\(^{[25]}\) An increased degree of complexity for all the samples compared to the \(D_f\) of 40°C was obtained as was reported by others.\(^{[25,36]}\) As shown in Table 1, the unfolding of the α-helical structures increased as the temperature increased. Moreover, the formation of internal order failed to keep pace with the myosin unfolding, which led to a large amount of protein self-aggregation and a higher \(D_f\) value at 60°C. In addition, the modori of fish protein gel should be considered. The lower \(D_f\) values at 70°C (2.875) and 90°C (2.871) could be attributed to the rapid aggregation that disrupted the protein’s intermolecular interactions. The surface hydrophobicity increased as the protein unfolded at higher temperatures. Higher hydrophobicity means increased interactions among protein molecules, which culminated in larger aggregates. Therefore, the gel that formed at a high temperature was composed of numerous larger aggregates, resulting in a larger pore size. Capillarity pressure is negatively correlated with pore sizes within three-dimensional networks of gels.\(^{[1,2,12]}\) Consequently, the WHC of the heat-induced gels decreased with increasing temperature because the mean pore size within the gels increased. Similarly, Park et al.\(^{[37]}\) reported that the pore diameter within fish surimi gel increased during the heating of gels up to 90°C. Stevenson et al.\(^{[3]}\) also indicated that pores with smaller or similar diameters formed in Alaska Pollock gels at higher heating temperatures. These results indicated that the distribution of water within gels is significantly dependent on the structure of the three-dimensional network.\(^{[11,38]}\)

**LF-NMR relaxation**

Figure 4(a) shows the relaxation time distribution of the samples, which were prepared by heating at 40, 50, 60, 70, and 90°C for 30 min. As shown in this figure, every curve had three peaks, which represented the \(T_{21}\) relaxation time (1–10 ms), the \(T_{22}\) relaxation time (200–400 ms), and the \(T_{23}\) relaxation time (1000–2500 ms). The \(T_{21}\) relaxation time was the shortest, indicating that the hydrogen atoms that belonged to the waters of hydration were closely bound to the proteins within the gels. The \(T_{22}\) relaxation time indicated that the hydrogen atoms had low mobility and low homogeneity, proving that they were part of a second layer of water (semibound water) around the protein molecules. The \(T_{23}\) relaxation time was the longest, indicating that the hydrogen atoms belonged to a third layer of water (with higher mobility), which was easily lost during the heating process.\(^{[39,40]}\) High \(T_2\) values suggested long relaxation times, which indicated that the binding forces between bound hydrogen atoms and macromolecules were weak. In contrast, low \(T_2\) value suggested that the hydrogen atoms were bound to the macromolecules by stronger forces.\(^{[41]}\) In addition, \(T_2\) reflected the mobility of the waters contained in gel pores, which supported the hypothesis of
Figure 4. LF-NMR $T_2$ relaxation curves (a), $T_2$ relaxation time (b) and the corresponding water populations (c) of bighead carp myosin gels as a function of heating temperature for 30 min (mean ± SD, $n=3$). $PT_{21}$, $PT_{22}$, and $PT_{23}$ are proportions for $T_{21}$, $T_{22}$, and $T_{23}$, respectively.
capillarity. High \( T_2 \) values indicated the waters had high mobility in big pores, while low \( T_2 \) value suggested that the mobility of waters in small pores was very limited.

Figure 4(b,c) show the \( T_2 \) values and the corresponding waters populations in the bighead carp myosin gel (25 mg·mL\(^{-1}\)) heated at various temperatures (40, 50, 60, 70, and 90°C) for 30 min. The \( T_{21} \) relaxation times of the myosin gels heated-induced at 40, 50, 60, 70, and 90°C was approximately 4 ms. Meanwhile, the \( T_{22} \) relaxation time substantially decreased from 231.01 to 100.00 ms as the temperature increased (\( p < 0.05 \)); however, it abruptly returned to 200.00 ms at 60°C. The \( T_{23} \) relaxation time significantly increased from 932.60 to 2154.43 ms as the temperature increased to 50°C. The \( T_{22} \) relaxation time remained constant as the temperature further increased. The proportion of \( T_{21} \) water (\( PT_{21} \)) was approximately 0.82% and remained stable (\( F \) value = 0.218, \( p > 0.05 \)) during heating at various temperatures. The proportion of \( T_{22} \) water (\( PT_{22} \)) declined significantly, from 86.63% to 70.05%, as the temperature increased from 40°C to 60°C (\( p < 0.05 \) (Fig. 4(c))). However, \( PT_{22} \) increased at temperatures of 70°C or 90°C. Compared with the proportion of \( T_{22} \) water (\( PT_{22} \)), the proportion of \( T_{23} \) water (\( PT_{23} \)) noticeably increased from 13.84% to 28.85% as the temperature increased from 40°C to 60°C. However, the \( PT_{23} \) decreased when the samples were heated at 70°C or greater. Changes of individual water molecules for \( T_{21} \) cannot fully explain the changes of the protein structure, which should be clear from the \( T_2 \) data of all water molecules in gels. Stevenson et al.\(^3\) suggested that \( T_{21} \) remained constant across gel treatments, including slow ramp heating and rapid heating. However, the data were not consistent with a non-significant change in the \( T_{2b} \) (corresponding to \( T_{21} \) in this work) portion within the pork myofibrillar protein thermal gel.\(^1\) Fish myosin is less stable than terrestrial animal myosin.\(^4\) The intermolecular bonds in the bighead carp myosin were significantly weaker than those in pork myosin at temperatures below 60°C.\(^4\)

Furthermore, the modori phenomenon occurred during the formation of fish surimi gel at approximately 60°C. Therefore, the three-dimensional network of fish protein was destroyed at 60°C, resulting in a decrease in the pore size of the gel and the \( PT_{22} \). Consequently, the \( PT_{22} \) increased in the gels. However, the gels formed at 70°C or higher had more regular networks because the extent of myosin aggregation increased. As a result, the pore size of the gels increased (Fig. 3(b)), leading to an increase in \( PT_{22} \) and a decrease in \( PT_{23} \). The highly organized protein secondary structures (high \( \alpha \)-helix content) became irregular (increased random coils and \( \beta \)-turns) due to the unfolding of myosin at high temperature (Table 1), and the surface area and volume of myosin increased. Although the hydrophilic amino acids originally present on the surface of myosin were still attracted to the water molecules, the hydrophobic amino acids exposed by protein unfolding repelled both the bound water and the secondary layer of water. Therefore, the second layer of water, which was retained within the tertiary and/or quaternary structures of the protein, might have been extruded during protein unfolding.\(^4\) Meanwhile, the three-dimensional networks of the gels formed at higher temperatures (>70°C) were more regular than those of the gels formed at low temperatures (<60°C), which caused the hydrophobic pores of the protein to become buried and form smaller pores. Consequently, the secondary layer of water was further pushed out. The content of the secondary layer of water was correlated with the pore sizes within gels, and the pore size increased as temperature increased (Fig. 3). As shown in Figs. 3(b) and 4(c), the smaller the pore sizes, the less water was in the secondary layer. This result suggested that the secondary layer of water was not bound by capillary forces but was trapped by the myosin chain three-dimensional network. Although the three-dimensional network formed by the myosin backbone trapped the waters, if the volume of the pores was too small, the water would be lost to the third layer of water. Therefore, the secondary layer of water was trapped by the three-dimensional network but not by capillary pressure. Wu et al.\(^4\) suggested that a decreasing \( T_2 \) value generally indicated water loss, whereas an increasing \( T_2 \) values indicated water gain. Stevenson et al.\(^3\) asserted that larger values of \( T_{21} \) (corresponding to \( T_{22} \) in this work) were weakly associated with higher water-holding capacity. Therefore, the \( T_{22} \) data was indicative of the mobility and redistribution of the waters within heat-induced myosin gels. Moreover, the data were more accurately reflected the changes in protein secondary structures and the interaction between the water and protein in small pores.
The $T_{23}$ relaxation time did not correlate with the third layer of water in this work, which had higher mobility in big pores (Fig. 2(b)), when the gels formed at higher temperatures (>50°C). The changes in the water content in the third layer were consistent with the fractal dimension of the gels; however, they were negatively correlated with the changes in pore size within the gels. Gels most likely hold water via capillary pressure. Therefore, the results in this work indicate that water molecules in the third layer were immediately trapped within the regular three-dimensional network of the gel by capillary pressure. Furthermore, a small amount of water in the second layer could be squeezed out of the $T_{22}$ water pools into the $T_{23}$ water pools during the initial stages of myosin denaturation at approximately 42°C. However, the change in $T_{23}$ in this study did not represent a change in the properties of the water, which could have been caused by the relatively narrow range of pore sizes in the gels (Fig. 2(b)). The results of the third layer of water showed that the water-holding capacity were correlated with the pore size within the gels, which was negatively correlated to capillary pressure. The mobility and redistribution of water within the third layer were mainly affected by the pore sizes within the heat-induced myosin gels. This seems to prove the capillary theory that the WHC of the gels should decrease as the gel pore size increases. In practice, the WHC of surimi gels is usually expressed in terms of expressible waters and is calculated by subtracting the mass of the water expressed during centrifugation from the specimen weight. Therefore, in this work, the $PT_{23}$ could be considered a measure of the WHC within myosin gels. However, Bertram et al. suggested that WHC, which highly correlated with $T_{22}$, was mainly affected by the loosely bound extra-myofibrillar water.

**Principal component analysis of LF-NMR and fractal dimension data**

Principal component analysis (PCA) was performed to describe the relationships among the measured parameters (Fig. 5). These results show the first two principal components (PC) solutions of the PCA for WHC, NMR data and gel physical structure properties of bighead carp myosin during heat-induced gelation. The first two principal components explained 88.54% of the total variation, indicating a strong relationship between $T_2$ relaxation data and the gel microstructure properties. The first principal component (PC1), which described 70.38% of the variation, showed...
that $T_{21}$, $T_{23}$, $PT_{21}$, $PT_{23}$, and $D_f$ were positively correlated with each other, whereas $T_{22}$, $PT_{22}$ and the pore diameter were negatively related to $T_{21}$, $T_{23}$, $PT_{21}$, $PT_{23}$, and $D_f$ in PC1. The second component, which explained 18.16% of the variations, was positively correlated with $T_{22}$, $T_{23}$, $PT_{21}$, $PT_{23}$, and pore diameter and negatively correlated with $T_{21}$, $PT_{22}$, and $D_f$. A pronounced effect mediated by $D_f$ and pore size on the water content and mean $T_2$ relaxation times in the thermal-induced myosin gel was observed. These results suggested water-holding capacity is depended on micro- and macrostructural changes in the gel.

**Conclusion**

The unfolding and aggregation of myosin differed when the myosin molecules were heated at different temperatures, which resulted in varied secondary structures in the myosin and three-dimensional networks within the gels. Therefore, the distribution and mobility of the water molecules within the myosin gel were different at various temperatures. The $T_{22}$ relaxation time reflected not only the mobility of the second layer of water within the gels but also the change in the microstructure of the gels. In contrast, the $T_{23}$ relaxation time did not correlate with the content of water within the third layer of the gels but could be considered as a measure of the WHC for the gels. The results further explained the correlations between relaxation time distribution spectra and waters in gels were influenced by the gels microstructure. The second layer of water was mainly forced out by hydrophobic force, but some of the waters were physically trapped in the space formed by the myosin backbone, and the third layer of water was basically kept by capillary pressure in the gels. Therefore, the correlation between the NMR relaxation time and the WHC for gels depends on the water holding mechanism of the gel, which was determined by gels microstructure. Moreover, the present study provided further information to explain the water holding mechanism(s) associated with differences in water mobility and physical structure among gels.

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