Influence of salt substitute containing KCl, L-histidine and L-lysine on the secondary structure and gel properties of myosin

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\textbf{ABSTRACT}

The effect of a salt substitute (SS) containing L-lysine (Lys) and L-histidine (His) on secondary structure and gel properties of myosin from grass carp was examined. The results indicated that the \( \alpha \)-helix content of myosin treated with SS was 29.00\%, 29.03\% and 35.93\% more than that with NaCl at 0.4, 0.6 and 0.8 mol/L (\( P < 0.05 \)), respectively, suggesting that some fractions of the \( \beta \)-sheet, \( \beta \)-turn and random coil were transformed into \( \alpha \)-helix. A similar pattern of storage modulus (\( G' \)) was found between NaCl and SS treatments, and the \( G' \) of SS treatments at the end of gelation completion was higher than that of NaCl treatments. The salt substitute improved the gel strength and hardness of myosin at 0.4, 0.6 and 0.8 mol/L. The results indicated that the changes in secondary structure and gel properties of myosin may be mainly due to the L-lys and/or L-his in salt substitute.

\textbf{Influencia de un sustituto de sal que contiene KCl, L-histina y L-lisina en la estructura secundaria y las propiedades gelatinosas de la miosina}

\textbf{RESUMEN}

El presente estudio examinó el efecto que tiene un sustituto de la sal (SS) que contiene L-lisina (Lys) y L-histidina (His) en la estructura secundaria y las propiedades gelatinosas de la miosina extraída de carpas herbívoras (\textit{Ctenopharyngodon idella}). Los resultados dan cuenta de que el contenido de hélice-\( \alpha \) de la miosina tratada con SS es 29.00\%, 29.03\% y 35.93\% superior al del que contiene NaCl en concentraciones de 0.4, 0.6 y 0.8 mol/L (\( P < 0.05 \)), respectivamente. Ello sugiere que algunas fracciones de la lámina-\( \beta \), el giro-\( \beta \) y la espiral aleatoria se transformaron en una hélice-\( \alpha \). Además, entre los tratamientos con NaCl y SS se detectó un patrón similar en el módulo de almacenamiento (\( G' \)); el \( G' \) correspondiente a los tratamientos con SS al final de la gelificación fue más elevado que el de los tratamientos con NaCl. El sustituto de la sal mejoró la fuerza gelatinosa y la dureza de la miosina a 0.4, 0.6 y 0.8 mol/L. Estos resultados indican que los cambios en la estructura secundaria y las propiedades gelatinosas de la miosina pueden responder principalmente a la L-lys y/o la L-his en el sustituto de la sal.

\section{1. Introduction}

As a principal component of myofibrillar proteins, myosin was an asymmetrical protein with two globular heads attached to long \( \alpha \)-helical rod-like tail (Privalov, 1982). The structural characteristics and gel properties of myosin were affected by ionic environment, \( pH \) and temperature (Fu et al., 2012; Liu, Zhao, Xiong, Xie, & Qin, 2008; Martínez et al., 2014). Some salt species also influenced the structural properties and gelation of myosin. For instance, divergent metal ions such as calcium and magnesium have been shown to alter the structure and functionality of the protein (Nayak, Kenney, Slider, Head, & Killefer, 1998a, 1998b). These cations were also used as components of a salt substitute (Aliño, Grau, Fuentes, & Barat, 2010; Gou, Guerrero, Gelabert, & Arnau, 1996; Guárdia, Guerrero, Gelabert, Gou, & Arnau, 2008). In the course of developing a novel salt substitute, it was found that L-histidine and L-lysine had a significant effect on the saltiness of cuttlefish bone extract (Zhang et al., 2014). In our preliminary data, the cuttlefish bone extract was found to contain high levels of L-lysine and L-histidine and proved to have 27\% less sodium content than that in NaCl at the same degree of saltiness. It was suggested that L-lysine and L-histidine might contribute to salty taste and saltiness of cuttlefish bone extract. In addition, Hayakawa et al. (2009, 2012) found that myosin gel from chicken breast in low ionic strength solution (1 mmol/L KCl + 5 mmol/L L-histidine) showed a fine network. It was shown that the presentation of L-lysine and L-histidine not only produced a reduction in sodium content (53.79\% less) but also promoted physicochemical properties in dry-cured loin (Zhang, Zhang, Hui, Guo, & Peng, 2015). In Tahergorabi’s study (2012a, 2012b), L-lysine was used for masking the metallic-bitter aftertaste of KCl; the study also pointed out that a salt substitute with L-lysine caused good hardness, springiness and cohesiveness of Alaska pollock surimi products. However, there is little research about the influence of a salt substitute containing L-histidine and L-lysine on structural properties and gel properties of warm-
water fish myosin. Therefore, the objective of this study was to investigate the effect of salt substitute containing L-histidine and L-lysine on the secondary structure and gel properties of myosin from grass carp (*Ctenopharyngodon idella*).

2. Materials and methods

2.1. Fish samples and reagents

Live grass carp (*Ctenopharyngodon idella*) (1500 ± 50 g) were obtained in January, 2014 from a local fish market (Nanjing, Jiangsu, China) and taken to our laboratory in a plastic bag within 1800 s. The live were immediately gutted and washed with water. The dorsal muscle was separated manually from skin and bone and kept at 4°C not longer than 30 min for myosin preparation.

Sodium chloride (NaCl), sodium azide (NaN₃), Tris base, 2-mercaptoethanol (βME), ethylene glycolbis (β-aminooethyl) ether N, N, N', N'-tetracetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), magnesium acetate, adenosine triphosphate (ATP), magnesium chloride and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St Louis, MO). The other reagents were obtained from Sinopharm Chemical Regent Co., Ltd (Shanghai, China).

2.2. Myosin preparation

Myosin was extracted according to the method of Martone, Busconi, Folco, Trucco, and Sanchez (1986) with slight modifications. All steps were performed at 0–4°C to minimize proteolysis and protein denaturation. Fish meat was cut into pieces (1 cm × 1 cm × 1 cm), and finely chopped in a rotary screw mincer (Model X 70; Scharfen Gmbh & Co. Maschinenfabrik KG, Witten, Germany, 6000 rpm, 180 s). It was added with 10 volumes of buffer A (0.10 mol/L KCl, 1 mmol/L PMSF, 200 mg/L NaN₃, and 20 mmol/L Tris-HCl, pH 7.5). The mixture was kept at 0–4°C for 10 min with occasional stirring, washed muscle was recovered by centrifugation at 1000 g for 10 min. The pellet was suspended in 5 volumes of buffer B (0.45 mol/L KCl, 5 mmol/L βME, 0.2 mol/L Mg(CH₃COO)₂, 1 mmol/L EGTA, and 20 mmol/L Tris-HCl, pH 6.8), and ATP was added to a final concentration of 10 mmol/L. The mixture was kept at 0–4°C for 1 h with occasional stirring and centrifuged at 10000 g for 15 min. Supernatant was collected and added slowly with 25 volumes of 1 mM KHCO₃. After 15 min, precipitated myosin was collected by centrifugation at 12000 g (10 min), resuspended (10 min) gently with 5 volumes of buffer C (0.50 mol/L NaCl, 5 mmol/L βME, and 20 mmol/L Tris-HCl, pH 7.5), and added with 3 volumes of 1 mmol/L KHCO₃ and MgCl₂ to a final concentration of 10 mmol/L. The mixture was kept overnight prior to centrifugation at 23000 g for 15 min. Myosin was recovered as pellet and used immediately or stored at –20°C in glycerol. Myosin purity was checked using sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Figure 1 illustrated the electrophoretic profile of the extracted myosin. The purity of the myosin samples was greater than 90% as determined by densitometry (Gel Logic 200 Imaging System, Kodak Co., USA).

The protein content was determined by the Lowry’s method (1951) using bovine serum albumin (BSA) as a standard. The protein concentration was adjusted depending on the experiment.

2.3. Solubilization of myosin in NaCl or salt substitute (SS)

Myosin was solubilized in a solution of 0.5 M NaCl and the protein content was adjusted to 30 mg/ML. When solubilized, the myosin was equilibrated to the final concentration by overnight dialysis against solutions of 0.4 mol/L, 0.6 mol/L and 0.8 mol/L NaCl (1000 ML), respectively. Likewise, the myosin solubilized in 0.5 mol/L NaCl was equilibrated to the final concentration by overnight dialysis against salt substitute at the same concentration (0.4 mol/L, 0.6 mol/L and 0.8 mol/L NaCl, and with 0.4 mol/L, 0.6 mol/L and 0.8 mol/L salt substitute). The salt substitute (Rolyx Co., Ltd, Nanjing, China) contained the mixture of NaCl and KCl (910000 mg/Kg) and the mixture of L-his and L-lys (90000 mg/Kg).

2.4. Circular dichroism spectrum

The CD spectrum was measured using a Chriacan spectrometer (Applied Photophysics Co. Ltd., Surrey, United Kingdom). The myosin sample was transferred to a quartz cell with a 0.1 cm light-path length. Molecular ellipticity was measured in the range from 200 to 260 nm, the scan rate was 100 nm/min and the temperature was assessed at 20°C regulated with a control unit. Spectra were averaged over three scans and corrected for the solvent signal. Protein samples were diluted to 0.1 mg/ML with dialysis solutions. A mean residue weight of 110 g/mol has been assumed. The...

![Figure 1. SDS-PAGE of extracted myosin from grass carp. The marker and myosin were loaded with 20 μg of protein.](image-url)
percentages of α-helix, β-sheet, β-turn and random coil structures were determined using the protein secondary structure estimation program (CDNN method) provided with a Chirascan spectrometer. All treatments were tested in triplicate; mean values from these replicates were represented in the data reported.

2.5. Dynamic rheological property of myosin

Dynamic rheological property was measured using an Anton Paar Physica MCR301 rheometer. Measurements were conducted at a strain of 2% and a frequency of 0.1 Hz. The storage modulus (G') was determined with a temperature increasing from 25°C to 85°C at the heating rate of 1°C/min. A gap of 1 mm between parallel plates was used and the samples were surrounded by liquid paraffin to prevent evaporation. The storage modulus (G') (Pa) of three measurements was reported per treatment.

2.6. Preparation of heat-induced gels

Myosin solutions were placed in test tubes (inner diameter = 1.9 cm, length = 17 cm). The tubes were heated in a water bath at 40°C for 60 min and then at 90°C for 30 min. After being heated, the formed gels were cooled and stored at 2–4°C for 12 h before test.

2.7. Texture properties of gels

Prior to the gel strength measurement, gel samples were allowed to equilibrate at room temperature (25 ± 1°C) for 2 h, and then cut into pieces (2.0 cm in length). Gels were penetrated with a stainless steel probe (P/0.5s) attached to a Model TA-XT2i texture analyzer (Stable Micro Systems Ltd., Surry, U.K.) at a crosshead speed of 1 mm/s, pre- and post-test speed were 2 mm/s and 1 mm/s, respectively. The holding time between both compressions was 5 s and 50% compression was applied. The penetration force which was the peak force required to rupture the gels was expressed as the gel strength (Xiong & Brekke, 1991).

Hardness was measured by two-cycle compression using a texture analyzer TA-XT2i (Stable Micro Systems Ltd., Surry, U.K.) equipped with a flat-ended cylindrical plunger (P/50). Cylinder samples (2.0 cm in length) were compressed axially (50% compression), with a crosshead speed of 1 mm/s and pre- and post-test speed of 2 mm/s. The holding time between both compressions was 5 s (Cheret, Chapleau, Delbarre-Ladrat, Verrez-Bagnis, & Lamballerie, 2005).

2.8. Statistical analysis

The data were analyzed using repeated-measures ANOVA (SAS 8.2, SAS Inst. Inc. 2001). Significant differences (P < 0.05) were assessed using Duncan’s multiple range tests. All treatments were tested in triplicate. In addition, the maximum and minimum values of G' were selected from the data and these values were used to run the contrast analysis based on one-way ANOVA. For planed contrast analysis, the value of 0.4 mol/L of each treatment was compared with the combined values of 0.6 and 0.8 mol/L. Whereas, in the second level of contrast the value of 0.6 mol/L was compared with the values of 0.8 mol/L. Similar statistical procedure was followed for texture properties of gels.

3. Results and discussion

3.1. Circular dichroism spectrum

Myosin conformation was measured using circular dichroism (CD) (Figure 2). The CD spectrum exhibited two minima at around 208 and 222 nm, showing the predominant presence of α-helix structures (Greenfield, 1999). As shown in Figure 2, the α-helix content of myosin in 0.4 mol/L SS was 27.1% more than that of myosin in 0.4 mol/L NaCl (P < 0.05). A similar increase in α-helix content was also found in SS at both 0.6 mol/L and 0.8 mol/L. Contrary to the change of α-helix content, the β-sheet, β-turn and random coil fraction in SS treatment at 0.4 mol/L were 7.24, 4, and 12.3 % less, respectively, than that in the 0.4 mol/L NaCl treatment (P < 0.05). The similar tendencies were also found in 0.6 mol/L and 0.8 mol/L treatments. It was suggested that some fractions of the β-sheet, β-turn and random coil were transformed into α-helix. In NaCl treatments, there was no marked change in the content of each secondary structure (α-helix, β-sheet, β-turn and random coil) (P > 0.05). Similarly, each secondary structure content in SS treatments was also not significantly changed.

The results illustrated that the component lysine and histidine caused a change in myosin conformation. It was well known that the isoelectric point (pI) of lysine and histidine was 9.74 and 7.59, respectively (Gargaud, Amils, Cleaves, Viso, & Pinti, 2011; Zhang, Liao, Li, Nie, & Yao, 2005). Since myosin was treated at pH 6.5 ± 0.2, lysine and histidine were both positively charged and myosin gained a net negative charge. Hence, the lysine and histidine were likely able to bind to the charged residues of myosin due to electrostatic effect, which played an important role on stability of secondary structures of protein (Satoh, Nakaya, Ochiai, & Watabe, 2006). Electrostatic effect existed on the fully exposed surface of proteins, fully buried in the interior of the protein, or in a partially buried environment with varying degrees of hydrophobicity of the surrounding residues. It was generally affected in two ways: through non-specific screening and specific ion binding to the protein (Goto, Takahashi, & Fink, 1990). According to our results in CD spectrum, it could be hypothesized that binding of histidine and lysine with net positive charge would disrupt interhelical or intrahelical ion pairs leading to the transformation of myosin secondary structures. It was speculated that the secondary structures were more sensitive to the salt components than salt concentrations in the range 0.4–0.8 mol/L.

3.2. Dynamic rheological property of myosin

The storage modulus (G') of myosin treated with NaCl or with SS was illustrated in Figure 3. For SS treatments, the G' showed only marginal change until about 35°C followed by a marked increase, reaching the peak at about 40°C and then dropped down, which might be due to disentanglement and the increased mobility of the myosin molecules as result of breaking of protein-protein bonds (Chen, Dickinson, & Edwards, 1999). Upon further heating, G' increased again at about 50°C up to 85°C. The G' of NaCl treatments exhibited a similar pattern to that of SS treatments with increasing temperature.
The storage modulus (G') is a measure of gel’s ability to be non-permanently deformed, and it is often employed to characterize a transition from viscous protein paste to a solid (Tahergorabi & Jaczynski, 2012a). The G’ change rate was calculated by ΔG’ = (G’1 - G’0)/G’0, where G’1 is the G’ at 85°C and G’0 is the G’ at 25°C (Zhang, Wu, Jamali, Guo, & Peng, 2017). It was observed that the ΔG’ of SS treatments was 135.53%, 144.54% and 248.73% more than that of NaCl treatments at 0.4 mol/L, 0.6 mol/L and 0.8 mol/L, respectively. When the gelation was completed (80°C), the G’ of SS treatment at 0.4 mol/L were 88.03 Pa, which was 87.49% more than that of NaCl treatment, suggesting that the degree of protein cross-linking was higher in SS treatment. Similar results were found in 0.6 mol/L and 0.8 mol/L treatments. According to the report of Kim, Park, and Yoon (2005), the unfolded α-helix hydrophobic regions of myosin interacted with each other above 50°C and meanwhile the G’ increased rapidly with the temperature until the gelation was completed where G’ reached a plateau. In the present study, the salt substitute caused an increase in α-helix contents of myosin (Figure 2). It was suggested that more α-helix structures formed in the SS treatments prior to heating, resulting in more α-helix of myosin unfolding thereby increasing the hydrophobicity and a subsequent increase in G’ during heating. Based on contrast analysis (Table 1), it was suggested that the different concentrations of NaCl and SS treatments had a significant impact on the G’ at given maximum as well as at minimum values.

### 3.3. Gel strength and hardness of myosin heat-induced gels

In treatments with NaCl, gel strength of myosin from grass carp increased with concentrations from 0.4 to 0.6 (Figure 4). The result was in line with the study of Lin and Park (2010) and Ishioroshi, Jima, and Yasui (2010), who reported that the solubility of myosin was increased with increasing concentration up to 0.5 mol/L. The increased solubility was obvious covariation with gel strength (Fretheim, Egeland, & Samejima, 1985). However, no significant difference was found between 0.8 mol/L and 0.4 mol/L (P > 0.05). Correspondingly, Suzuki (1981) reported that the
gel-forming ability of myosin gradually decreased when the salt concentration was too high (>1 mol/L). It could be due to decreased solubility of myosin caused by the salting-out effect (Regenstein, 1984; Steffansson & Hultin, 1994).

In treatments with SS, gel strength of myosin from grass carp increased from 0.4 mol/L to 0.8 mol/L (P < 0.05). At 0.4 mol/L and 0.6 mol/L level, no significant difference was found for gel strength in SS treatments (P > 0.05). The gel strength of myosin in SS was 8.423 g.cm more than that in NaCl at 0.8 mol/L (P < 0.05). It was illustrated that different salt substitute concentration levels (i.e. 0.4 mol/L and 0.6 mol/L) did not cause a significant difference in gel strength of myosin, while L-histidine and L-lysine in the salt substitute increased the gel strength of myosin at 0.8 mol/L compared to the NaCl. The possible explanation was that the combined action of L-histidine and L-lysine could cause myosin to depolymerize, and consequently increase the solubility. It was suggested that the gel strength of myosin from grass carp was more sensitive to the salt components than concentrations.

In treatments with NaCl, the gel hardness of myosin from grass carp increased with salt concentrations, although no

![Figure 3. The storage modulus (G') of myosin with NaCl and SS.](image)

Figura 3. El módulo de almacenamiento (G') de la miosina con NaCl y SS.

| Table 1. Contrast analysis for dynamic rheological property of myosin and texture properties of gels. |
| Tabla 1. Análisis de contraste de la propiedad reológica dinámica de la miosina y las propiedades texturales de los geles. |

| Parameter      | Contrast level within treatment          | Significance for contrast test | NaCl treatment | SS treatment |
|----------------|------------------------------------------|-------------------------------|---------------|-------------|
| G' Maximum     | 0.4 mol/L verses 0.6 mol/L + 0.8 mol/L   | 0.000                         | 0.000         | 0.000       |
|                | 0.6 mol/L verses 0.8 mol/L               | 0.000                         | 0.000         |
| G' Minimum     | 0.4 mol/L verses 0.6 mol/L + 0.8 mol/L   | 0.000                         | 0.000         | 0.000       |
|                | 0.6 mol/L verses 0.8 mol/L               | 0.000                         |               |
| Gel Strength   | 0.4 mol/L verses 0.6 mol/L + 0.8 mol/L   | 0.030                         | 0.000         | 0.741       |
|                | 0.6 mol/L verses 0.8 mol/L               | 0.022                         | 0.001         |
| Hardness       | 0.4 mol/L verses 0.6 mol/L + 0.8 mol/L   | 0.000                         | 0.000         | 0.039       |
|                | 0.6 mol/L verses 0.8 mol/L               | 0.006                         |               |             |
significant difference was found between 0.6 mol/L and 0.8 mol/L (P > 0.05) (Figure 4). In treatments with SS, gel hardness increased from 0.4 mol/L to 0.6 mol/L and a decrease was found in hardness at 0.8 mol/L (P < 0.05). The gel hardness at 0.8 mol/L was 23.35 g fewer than that in SS at 0.6 mol/L (P < 0.05). This result was in agreement with the gel strength results. At 0.4 mol/L and 0.6 mol/L, the hardness of myosin gels in NaCl treatment was 21.423 g and 12.133 g more than that in SS, respectively (P < 0.05). At 0.8 mol/L level, there was no significant difference in hardness between NaCl and SS. On contrast, Tahergorabi et al. (2012b) reported that the hardness of surimi products was decreased when salt substitute containing L-lysine was added. The difference might be due to the combined action of L-histidine and L-lysine, which resulted in increased hardness of fish myosin. These results were in accordance with the circular dichroism spectrum and dynamic rheological property of myosin with NaCl or SS in the present study. Namely, the high α-helix contents had a positive role in increasing G’ of myosin contributing to the gel texture, which was supported by the report of Laniner, Carvajal, and Yongsawatdigul (2005), who pointed out that the well protein cross-linking resulted in stronger textural properties. Furthermore, Table 1 for contrast analysis showed that except 0.4 mol/L versus 0.6 mol/L + 0.8 mol/L, all other levels of NaCl and SS had a significant effect on texture properties of gels.

4. Conclusions

The combined action of L-histidine and L-lysine in salt substitute caused a transformation of β-sheet, β-turn and random coil to α-helix. The increased α-helix fraction prior to heating resulted in an increase in G’ and improved gel properties of myosin from grass carp. It was suggested that the secondary structure and gel property were more sensitive to the addition of L-lysine and/or L-histidine than salt concentrations in the range 0.4 mol/L-0.8 mol/L. Further studies need to elucidate the mechanisms of the secondary structure transformations resulting from L-histidine and L-lysine.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Fundamental Research Funds for the Central Universities [KYZZ201656]: National Natural Science Foundation of China [31601491].

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