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Effect of $CaCl_2$ on denaturation and aggregation of silver carp myosin during setting

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ABSTRACT

The effect of $CaCl_2$ on denaturation and aggregation of silver carp myosin incubated at 40 °C was investigated by circular dichroism spectroscopy, surface hydrophobicity (S_0-ANS), total sulfhydryl (SH) group content, zeta potential, turbidity, *z*-average diameter (d_z), and dynamic rheological analysis. During setting at 40 °C, both $CaCl_2$ and heating induced conformational changes of the fish myosin, and exposure of more hydrophobic amino acid residues and free SH groups, followed by myosin aggregation via hydrophobic interactions and disulfide bonds. Additionally, turbidity and d_z of myosin increased significantly with increasing $CaCl_2$ concentration, and the added $CaCl_2$ further increased the extent and rate of aggregation of myosin by promoting the formation of Ca bridges. Myosin with 60 mM $CaCl_2$ showed the maximal G' value and the highest rate of G' development. However, the G' value would decrease with an excessive amount of $CaCl_2$ (100 mM).

1. Introduction

The gel formation of fish proteins involves partial denaturation of protein, followed by irreversible aggregation, which results in a three dimensional network (Liu et al., 2010). Myosin is abundant in muscle protein and plays an essential role in gel formation in fish products. The gelation properties of myosin depend strongly on pH, temperature (Liu, Zhao, Xiong, Xie, & Qin, 2008) and divalent cations, such as calcium, magnesium and zinc (Arfat & Benjakul, 2013). Surimi gel strength can be enhanced by preincubation at a low temperature (⩽40 °C). This preincubation process is referred to as setting or suwari (Arfat & Benjakul, 2012; Sato et al., 2001). It has been generally accepted that the setting procedure is induced by endogenous transglutaminase (endo-TGase) (Yin & Park, 2014). The endo-TGase activity of surimi can be activated by calcium compounds to catalyze acyl transfer reaction between the γ-carboxyl amide carboxamide groups of glutamine and the ε-amino groups of lysine, forming stronger gel networks via ε-(γ-glutamyl)lysine cross-links (Benjakul, Visessanguan, & Pecharat, 2004; Shi, Luo, Shen, & Li, 2014). $CaCl_2$ has been widely applied to improve the gel strength of surimi products (Ding et al., 2011; Ramírez, Rodríguez-Sosa, Morales, & Vázquez, 2003). However, Ca^{2+} is also known as a destabilizing salt in the Hofmeister series. It may reduce the free energy required to transfer the non-polar groups into water, weaken the intramolecular hydrophobic interaction and promote the unfolding of proteins (Lertwittayanon, Benjakul, Maqsood, & Encarnacion, 2013). In this unfolding process, some functional groups, such as hydrophobic amino acid residues and sulfhydryl groups, are exposed, and the cross-linking of proteins is enhanced via hydrophobic interactions and disulfide bonds (Hemung & Yongsawatdigul, 2005; Yongsawatdigul & Sinsuwan, 2007). Additionally, when pH is far from the isoelectric point (pI) of the protein, Ca^{2+} may induce protein cross-linking via the salt bridges among negatively charged carboxyl groups (Arfat & Benjakul, 2012).

Silver carp (*Hypophthalmichthys molitrix*) is one of the most intensively cultured warm freshwater fish species in China, due to its fast growth rate, high production and low cost of cultivation (Liu, Zhao, Xiong, & Zhang, 2009). However, silver carp does not present good gel formation (Barrera, Ramırez, González-Cabriales, & Vázquez, 2002). In our previous study, the gel strength of silver carp surimi was improved by the addition of $CaCl_2$, to enhance the non-disulfide covalent bonds (Liu, Xiong, & Xie, 2006). However, the effect of $CaCl_2$ on denaturation and aggregation of silver carp myosin during setting remains to be elucidated.

The aim of this study was to investigate the effect of $CaCl_2$ on conformation and chemical interactions of silver carp myosin, as

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well as the relationships between heat-induced aggregation and rheological properties during setting at 40 °C, by circular dichroism spectroscopy, surface hydrophobicity (S_0-ANS), total sulfhydryl (SH) content, zeta potential, turbidity, *z*-average diameter (d_z), and dynamic rheological analyses.

2. Materials and methods

2.1. Materials

Live silver carp (1.5–2 kg) were obtained from the market of Huazhong Agricultural University and taken to the laboratory in a plastic bag without water within 20 min. The live fish were killed by blunt force trauma to the head and scaled, eviscerated, and washed with tap water. The dorsal muscle was separated manually from skin and bone, and kept at 4 °C for less than 1 h for myosin preparation. All chemicals used were of analytical grade.

2.2. Preparation of myosin

Myosin was prepared from the fresh silver carp as described by Ding, Liu, Rong, and Xiong (2014) with some modifications. All solutions used for the myosin preparation were kept at 4 °C to minimize protein denaturation. The muscle was minced with a food processor (BraunK600, Braun GmbH, Germany), and then mixed with 10 volumes of solution A (0.10 M KCl, 0.02% sodium azide and 20 mM Tris–HCl buffer, pH 7.5). The mixture was homogenized at 14,000 rpm for 1 min, using an S18N-19G dispersing element (T18, DIGITAL ULTRA-TURRAX, IKA, Staufen, Germany); then the homogenate was held at 4 °C for 15 min and centrifuged at 3000*g* for 5 min, using a high-speed refrigerated centrifuge (Avanti J-26 XP, Beckmen Coulter, Fullerton, CA, USA). The sediment was suspended with 5 volumes of solution B (0.45 M KCl, 5 mM β-mercaptoethanol, 0.2 M $Mg(COO)_2$, 1 mM ethyleneglycoltetraacetic acid (EGTA) and 20 mM Tris–HCl buffer, pH 6.8). After supplementation with adenosine triphosphate (ATP) to a final concentration of 5 mM, the mixture was kept at 4 °C for 60 min, and then centrifuged at 22,095*g* for 10 min. The resulting supernatant was diluted with 5 volumes of 1 mM $KHCO_3$ and kept at 4 °C for 15 min. Subsequently, the mixture was centrifuged at 22,095*g* for 10 min and the pellets were re-suspended with 2.5 volumes of solution C (0.5 M KCl, 5 mM β-mercaptoethanol and 20 mM Tris–HCl buffer, pH 7.5). The re-suspended pellets were incubated at 4 °C for 15 min and diluted with 2.5 volumes of 1 mM $KHCO_3$, which was then supplemented with $MgCl_2$ to a final concentration of 10 mM. After overnight incubation at 4 °C, the as-prepared solution was centrifuged at 22,095*g* for 10 min to obtain myosin. The myosin pellets were resuspended in 0.6 M NaCl (pH 6.8) (dissolved in ionized water) and then centrifuged at 5,000 g for 10 min. The supernatant was stored at 4 °C for use within 2 days. The protein concentration of the supernatant was determined by the Lowry method, using bovine serum albumin as a standard. The calcium content of the myosin was determined to be 0.51 μg/mg by the Lanbang Environment Engineering Co., Ltd (Wuhan, China).

2.3. Preparation of heated myosin solutions

Myosin solutions were diluted to 0.5 mg/ml with 0.6 M NaCl (pH 6.8) (dissolved in ionized water). Myosin solutions containing $CaCl_2$ at different concentrations (0, 20, 40, 60, and 100 mM) were incubated in the test tubes at 40 °C for various periods of time (0, 10, 20, 30, 40, 50 and 60 min). The final myosin solutions were kept at 4 °C for measurements of total SH content, turbidity, d_z, and zeta potential.

2.4. Circular dichroism

Circular dichroism (CD) analysis was performed using a JASCO spectropolarimeter (J-8100, Japan Spectroscopic Co., Ltd., and Tokyo, Japan). Myosin was dissolved in 0.45 M NaCl (pH 6.8) due to strong UV absorption of NaCl. Myosin solutions (0.02 mg/ml), containing $CaCl_2$ at five different concentrations (0, 20, 40, 60, and 100 mM) were incubated at 40 °C for 10, 20, 30, 40, 50 and 60 min, respectively. CD spectra of myosin treated by different $CaCl_2$ concentrations were obtained, using corresponding $CaCl_2$ concentrations for baseline. The changes of secondary structure were monitored by scanning solutions in the range of 198–250 nm in a 1 mm quartz cell. A mean molar residue weight of 110 g/mol was used to calculate the molar ellipticities of the fish myosin. Estimation of secondary structure composition was performed using the Jasco SSE-513 Protein Secondary Structure Estimation Program (Japan Spectroscopic Co., Ltd., and Tokyo, Japan).

2.5. Surface hydrophobicity

Surface hydrophobicity (S_0-ANS) of the fish myosin was determined as described by Yongsawatdigul and Park (2003), using 8-anilo-1-naphthalenesulphonic acid (ANS) as a probe. The myosin solutions (1 mg/ml) were diluted to 0.001, 0.005, 0.01, 0.04 and 0.06 mg/ml with $CaCl_2$ at various concentrations (0, 20, 40, 60 and 100 mM). Four ml of each protein solution were supplemented with 20 μl of 8 mM ANS in 0.1 M potassium phosphate buffer (pH 7.0) and mixed well, followed by heating at 40 °C for different periods of time (0, 10, 20, 30, 40, 50 and 60 min). Fluorescence intensity was measured by a fluorescence spectrophotometer (F-4600, Hitachi, Japan) at the excitation and emission wavelengths of 364 and 534 nm, respectively. The excitation and emission slits widths were set at 5.0 nm. Protein hydrophobicity was calculated from initial slope of plot of fluorescence intensity against protein concentration using linear regression analysis. The initial slope was defined as S_0-ANS.

2.6. Total sulfhydryl content

Total sulfhydryl (SH) content was measured according to the method of Yarnpakdee, Benjakul, Visessanguan, and Kijroongrojana (2009) with a slight modification. A 0.5 ml aliquot of myosin solution was supplemented with 5 ml of 0.2 M Tris–HCl buffer (pH 6.8) containing 8 M urea, 2% sodium dodecyl sulfate (SDS) and 10 mM ethylenediaminetetraacetic acid (EDTA). After incubation with 0.5 ml of 10 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) in 0.2 M Tris–HCl buffer (pH 6.8) at 40 °C for 30 min, absorbance at 412 nm was measured, using a spectrophotometer. Total SH content was calculated using a molar extinction coefficient of 13,600 M^{-1} cm^{-1}.

2.7. Turbidity

Turbidity was measured according to the method of Liu et al. (2011). All the samples were placed in a quartz cuvette (light path length of 10 mm). Turbidity was monitored at 320 nm, using a UV/vis spectrophotometer (Shimadzu, Kyoto, Japan).

2.8. Particle size and zeta potential

The zeta (ζ) potential and particle size of the fish myosin solutions were measured, using a ZetaPlus zeta potential analyzer (Zetasizer Nani series-Zen 3600, Malvern Instruments Ltd., Worcs, UK) at room temperature. The obtained mean particle size