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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).

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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

is referred to as "z-average" diameter (d_z) and the rate of d_z increment is calculated by the following equation:

$$\text{The rate of } d_z \text{ increment (nm/min)} = (d_{z2} - d_{z1})/60 \quad (1)$$

where d_{z2} and d_{z1} are the values at the final and the initial d_z (nm) during setting at 40 °C, respectively.

2.9. Dynamic rheology

Dynamic rheology of the silver carp myosin was measured according to the method of Ding et al. (2014) with some modifications. Myosin solutions were diluted to 12 mg/ml with 0.6 M NaCl (pH 6.8) containing $CaCl_2$ solutions at different concentrations (0, 20, 40, 60 and 100 mM). The temperature ramp tests were performed in a rheometer (AR 2000 ex, TA instruments, New Castle, DE), using parallel-plate geometry (40 mm plate diameter and 1 mm plate gap). The exposed rim of the sol was covered with silicon oil to prevent dehydration. The gels were formed by heating from 20 to 40 °C at 3 °C/min, followed by incubation at 40 °C for 1 h. During the thermal cycle, gelation was monitored at a constant frequency of 1 Hz and stress of 1 Pa.

The rate of G' development during setting was indicated by the slope of G' versus time, and was estimated according to Visessanguan, Benjakul, and Tanaka (2003) by the following equation:

$$\text{Rate of } G'_1 \text{ development (Pa/min)} = (G'_2 - G'_1)/60 \quad (2)$$

where G'_2 and G'_1 are final and initial storage modulus G' (Pa) during setting at 40 °C, respectively.

2.10. Statistical analysis

The α-helical content was determined using the protein secondary structure estimation programme (Yang's method). The data were analyzed using Excel 2010 and Statistical Analysis System software (SAS Institute Inc., Cary, NC, USA). Duncan's multiple range tests were used to evaluate the differences between treatments at $P < 0.05$. All the figures were drawn by Origin 8.0.

3. Results and discussion

3.1. Effect of $CaCl_2$ on α-helical content of silver carp myosin

Fig. 1 shows the α-helical content of myosin at various $CaCl_2$ concentrations during setting at 40 °C. When incubated at 40 °C for 60 min, the α-helical content of the myosin without $CaCl_2$ decreased from 77.5% to 62.4%, indicating the occurrence of the unfolding of the myosin. This might be related to the instability of hydrogen bonds, which resulted in a double helix-to-single coil transformation (Liu et al., 2008). In the presence of $CaCl_2$, the α-helical content exhibited a similar pattern to that of the myosin without $CaCl_2$, and decreased markedly with the increase of $CaCl_2$ concentration. Therefore, both thermal energy and destabilizing effect of $CaCl_2$ synergistically contributed to the unfolding of the myosin molecule. It was reported that Zn^{2+} could depress yellow stripe trevally actomyosin thermal stability (Arfat & Benjakul, 2012). The α-helical content of pelagic fish actomyosin decreased with an increase of Zn^{2+} concentration (Mathew, Shamasundar, Kumar, & Prakash, 2009). It was therefore reasonable to presume that Ca^{2+} was more likely to interact with the negatively charged residues of myosin molecules, and myosin with lower thermal stability could undergo denaturation, resulting in a more effective unfolding. Although unfolding was necessary for the exposure of reactive groups, chemical and physical properties of the unfolded

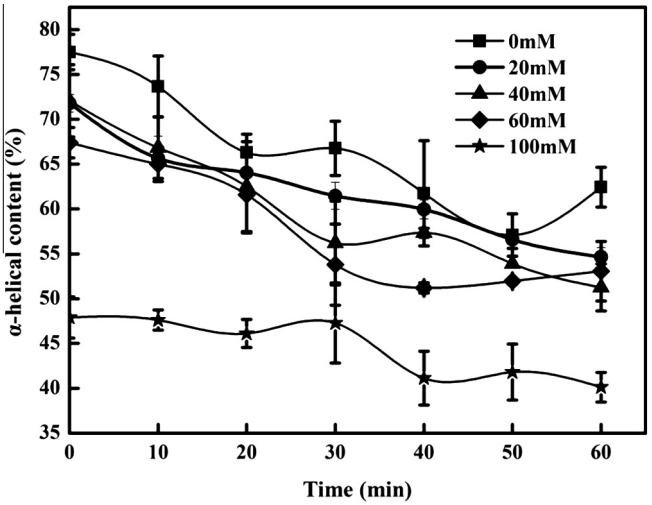


Fig. 1. Effect of $CaCl_2$ on α-helical content of silver carp myosin during setting at 40 °C.

domains seemed to be more beneficial for myosin aggregation (Benjakul, Visessanguan, Ishizaki, & Tanaka, 2001).

3.2. Effect of $CaCl_2$ on S_0-ANS of silver carp myosin

As shown in Table 1, during setting at 40 °C, the surface hydrophobicity (S_0-ANS) of the fish myosin without $CaCl_2$ increased, followed by a decrease, and reached a maximum at 50 min. Surface hydrophobicity is one of the most important surface-related properties in proteins. The increase in S_0-ANS was correlated with the change level of the secondary structure, implying that the exposure of hydrophobic amino acid resides might result from the unfolding of myosin molecules (Hemung, Li-Chan, & Yongsawatdigul, 2008; Wang, Luo, & Shen, 2013), which could promote hydrophobic interactions. Therefore, the formation of large aggregates was evident (Fig. 4), and hydrophobic cores might be buried in the interior of aggregates to inhibit the elevation of S_0-ANS (Wang, Li, Jiang, Qi, & Zhou, 2014). This explains why S_0-ANS decreased with the extension of incubation time. For myosin treated with 20, 40, 60 and 100 mM $CaCl_2$, S_0-ANS exhibited a pattern similar to that of the myosin without $CaCl_2$, and increased with increasing $CaCl_2$ concentration. However, the incubation time, for S_0-ANS to reach the peak, was brought forward from 50 min to 40, 30, 20 and 10 min, respectively (Table 1). According to the report of Jeyarajah and Allen (1994), calcium could increase the ANS binding sites and the fluorescence intensity by binding to the charged groups of β-lactoglobulin. Therefore, the increased ANS absorption may also result from the increased interactivity between ANS and myosin residues because of the $CaCl_2$. On the other hand, More $CaCl_2$ could promote the unravelling of hydrophobic domains on the myosin molecule at 40 °C. The exposed hydrophobic amino acid residues were more likely to undergo hydrophobic interactions (Yongsawatdigul & Sinsuwan, 2007). Thus, a greater extent of aggregation could be expected when a higher $CaCl_2$ content was incorporated (Fig. 4). This could explain why the incubation time (for S_0-ANS to reach the maximum) was reduced with increasing $CaCl_2$ concentration. Previous reports studied the effect of Ca^{2+} on S_0-ANS in tilapia actomyosin (Yongsawatdigul & Sinsuwan, 2007), threadfin bream myosin and actin (Hemung & Yongsawatdigul, 2005), and they both confirmed that the addition of 10–100 mM $CaCl_2$ induced conformational changes, and resulted in a notable increase in the S_0-ANS during incubation at 40 °C.

Table 1
Effect of $CaCl_2$ on surface hydrophobicity (S_0-ANS) of silver carp myosin during setting at 40 °C.

Setting time (min)	Surface hydrophobicity (S_0-ANS) $CaCl_2$ concentration (mM)				
	0	20	40	60	100
0	2222 ± 59.4 Fe	3444 ± 48.7 Fd	3551 ± 108 Ec	3803 ± 60.1 Fb	4013 ± 18.9 Ga
10	3243 ± 56.9 De	4777 ± 0.70 Dd	4905 ± 48.3 Dc	5081 ± 42.4 Eb	7752 ± 41 Aa
20	3373 ± 11.7 Ce	4822 ± 14.1 Cd	4915 ± 61.2 Dc	6774 ± 74.8 Aa	6538 ± 16.4 Eb
30	3147 ± 10.3 Ee	4917 ± 23.8 Bd	6304 ± 50.02 Aa	5673 ± 4.87 Cc	5861 ± 24.1 Db
40	3870 ± 15.7 Be	4994 ± 26.79 Ad	5271 ± 87 Cc	5764 ± 12.9 Bb	6144 ± 48.8 Ca
50	3981 ± 22.1 Ae	4513 ± 26.8 Ed	5288 ± 45.1 Cc	5799 ± 60.1 Bb	6525 ± 70.1 Ba
60	3884 ± 28.6 Bd	4768 ± 10.1 Fc	5618 ± 15.20 Ba	5710 ± 20.8 Da	4982 ± 7.70 Fb

Different capitals letters represent significant differences in the same column ($P < 0.05$).
Different lowercases letters represent significant differences in the same row ($P < 0.05$).

3.3. Effect of $CaCl_2$ on total SH content of silver carp myosin

Fig. 2 shows the total SH content of the fish myosin at various $CaCl_2$ concentrations during setting at 40 °C. After incubation at 40 °C for 60 min, the total SH content of myosin without $CaCl_2$ decreased from 5.02 mol/10^5 g protein to 4.12 mol/10^5 g protein, indicating the formation of disulfide bonds. For myosin in the presence of $CaCl_2$, the total SH content exhibited a trend similar to that of the control (myosin without $CaCl_2$). Additionally, the total SH content showed a continuous decreasing trend with increasing $CaCl_2$ concentration. The results indicated that both $CaCl_2$ and prolonged incubation time promoted the formation of disulfide bonds. The unfolding of myosin molecules led to the exposure of free SH groups, which subsequently underwent disulfide interchange, resulting in the formation of inter-molecular disulfide bonds from the oxidation of two cysteine thiol groups on adjacent protein chains (Lund, Lametsch, Hviid, Jensen, & Skibsted, 2007). A similar effect of $CaCl_2$ on the formation of disulfide bonds was also found in threadfin bream myosin (Hemung & Yongsawatdigul, 2005). It can therefore be presumed that disulfide bonds were partially responsible for the aggregation of the fish myosin incubated at 40 °C.

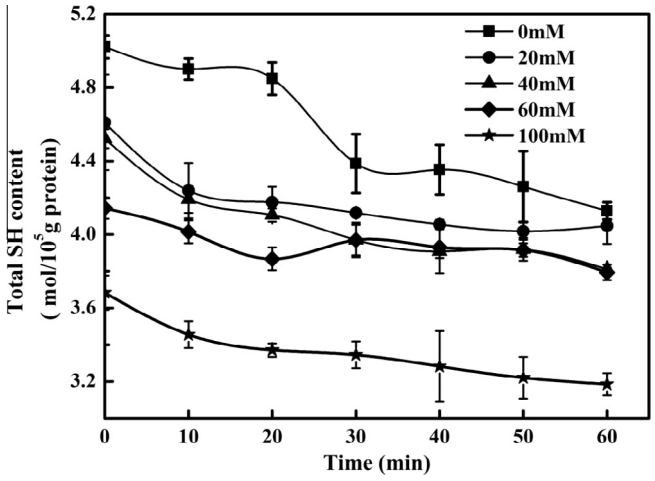


Fig. 2. Effect of $CaCl_2$ on total SH content of silver carp myosin during setting at 40 °C.

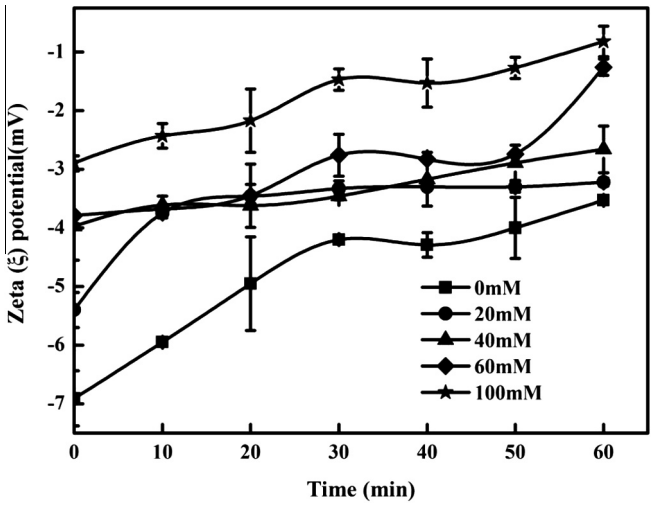


Fig. 3. Effect of $CaCl_2$ on zeta (ζ) potential (mV) of silver carp myosin during setting at 40 °C.

3.4. Effect of $CaCl_2$ on zeta potential of silver carp myosin

Fig. 3 lists the zeta potential (ζ) values of the fish myosin containing 0–100 mM $CaCl_2$ during setting at 40 °C. The negative charge was found to be the highest in the control, and became less negative with increasing $CaCl_2$ concentration and prolonged incubation time. The zeta potential of a protein particle is strongly pH-independent (Malhotra & Coupland, 2004), and the isoelectric point (pI) corresponds to the point where potential value is zero. The zeta potential increases in magnitude when pH is moved away from the pI (Singh, Benjakul, Maqsood, & Kishimura, 2011). The pH of a myosin solution with 0.6 M NaCl is 6.8, which is higher than its pI (about 5.5). Thus, the zeta potential of myosin in the present study was negative (−6.91 mV) (Fig. 3). The negative charge of proteins is governed by acidic amino acids, such as aspartic acid and glutamic acid, in myosin at a neutral pH (Arfat & Benjakul, 2012). When myosin with $CaCl_2$ was incubated at 40 °C, more negatively charged residues were exposed. Ca^{2+} might neutralize the negatively charged residues during setting, as indicated by the lowered negative charge (Fig. 3). A similar phenomenon was observed by Arfat and Benjakul (2012) in the incorporation of 100 μM $ZnSO_4$ or $ZnCl_2$, which induced a higher drop in negative charge of actomyosin. This behavior could be attributed to the interactions between negatively charged residues and Ca^{2+}, thereby inducing cross-linking via Ca bridges. The reinforced Ca bridges lowered the electrostatic repulsion forces between protein molecules (Chantarasuwan, Benjakul, & Visessanguan, 2011), and finally resulted in protein aggregation. The mobility of myosin solution might be limited, resulting from the aggregation of the protein to form larger, slower-moving colloidal objects (Brant, Lecoanet, & Wiesner, 2005). This might be another reason for reduced zeta potential values.

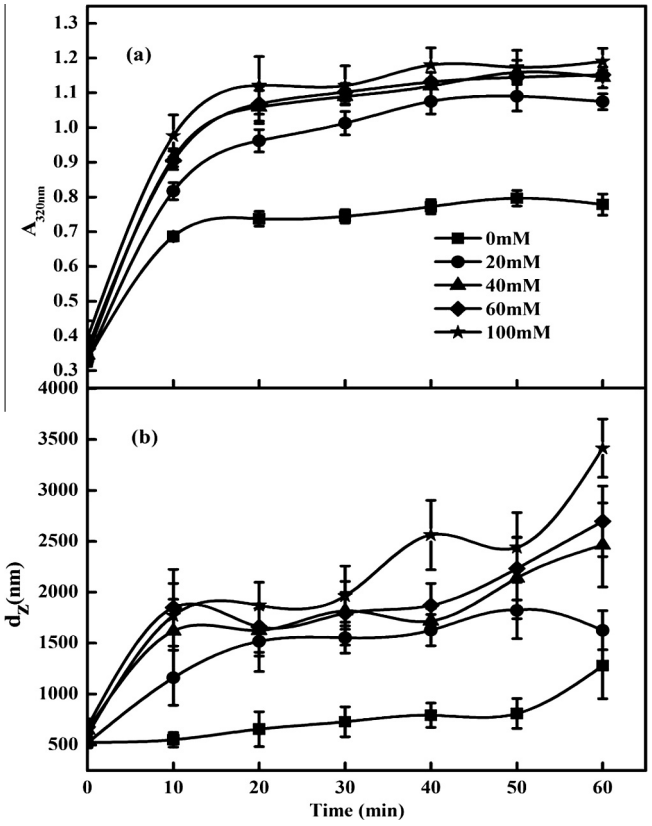


Fig. 4. Effect of $CaCl_2$ on turbidity (a) and d_z (b) of silver carp myosin during setting at 40 °C.

3.5. Effect of $CaCl_2$ on turbidity of silver carp myosin

Fig. 4a shows turbidity of the fish myosin with various $CaCl_2$ concentrations during setting at 40 °C. With the prolongation of setting at 40 °C, the turbidity of the fish myosin increased significantly within 10 min, followed by a slight increase. The turbidity of myosin with $CaCl_2$ exhibited a trend similar to that of myosin without $CaCl_2$, and increased with increasing $CaCl_2$ concentrations until 40 mM $CaCl_2$, followed by a plateau until 100 mM $CaCl_2$. A higher $CaCl_2$ concentration induced a greater extent of denaturation and aggregation. The increase in turbidity demonstrated the continuous formation of protein aggregates large enough to cause light scattering (Yarnpakdee et al., 2009). The differences in turbidity values were most probably due to the variable sizes or rates of protein aggregation, which could be roughly estimated by turbidity or quantified by measuring *z*-average diameter (d_z).

3.6. Effect of $CaCl_2$ on z-average diameter of silver carp myosin

Fig. 4b shows the d_z of the fish myosin during setting at 40 °C. The d_z of myosin aggregates increased from 526.45 to 1279.00 nm when the incubation time was prolonged to 60 min. $CaCl_2$ accelerated the formation of large particles during setting at 40 °C. When incubated with 20, 40, 60, and 100 mM $CaCl_2$ for 60 min, the d_z of the myosin increased from 520.28 to 1626.60, 646.18 to 2464.75, 677.85 to 2696.60, and 599.96 to 3414.4 nm, respectively. The corresponding rates of d_z increment during setting were 12.54, 18.43, 30.31, 33.65 and 46.90 nm/min, respectively, indicating that the myosin incubated with a higher $CaCl_2$ concentration exhibited a higher rate of aggregation and produced bigger aggregates. The increase in the myosin particle sizes was in accordance with the increase in turbidity values (Fig. 4a). Protein aggregation affected, not only the size of the aggregates, but also its surface charge (Fig. 3). These results suggested that Ca^{2+} acted as the bridge among negatively charged residues, particularly COO^-. Furthermore, a significant denaturation of myosin, as well as hydrophobic interactions and disulfide bonds formation during setting, promoted the aggregation of myosin molecules. It is known that denaturation and aggregation behaviors generally influence thermal gelation properties of myosin. Liu et al. (2011) pointed out that the formation of aggregates was presumably a prerequisite for the formation of a good elastic gel.

3.7. Effect of $CaCl_2$ on dynamic rheology of silver carp myosin

Dynamic rheological properties of myosin at 40 °C are illustrated in Fig. 5. The heating procedure could be divided into two phases: phase I (heating from 20 °C to 40 °C at 3 °C/min) and phase II (incubation at 40 °C for 1 h). During Phase I, a drastic increase in G' and decrease in δ were observed at about 34 °C. The rheological parameters of the fish myosin with $CaCl_2$ exhibited a pattern similar to those without $CaCl_2$ in the temperature range 34–40 °C. G' increased and δ decreased with increasing $CaCl_2$ concentration until 60 mM $CaCl_2$, followed by a decrease in G' and an increase in δ. The rates of G' development of the fish myosin at 0, 20, 40, 60, and 100 mM $CaCl_2$ (34–40 °C) were 0.62, 1.52, 2.30, 4.63, and 1.20 Pa/min, respectively. During Phase II, G' (40 °C initially) gradually increased from 1.61 to 9.62 Pa with $CaCl_2$ increasing from 0 to 60 mM, and decreased to 2.81 Pa with the addition of 100 mM $CaCl_2$. G' (40 °C for 60 min) increased from 5.24 to 34.74 Pa, while δ decreased from 11.13° to 5.91° with an increase of $CaCl_2$ concentration from 0 to 60 mM. The G' value of the fish myosin gel with 60 mM $CaCl_2$ showed a 5.62-fold increase over that without $CaCl_2$. However, an excessive amount of $CaCl_2$ (100 mM) could decrease G' (40 °C for 60 min) to 7.34 Pa and increase δ to 9.66°. The rates of G' development of the fish myosin treated with 0,

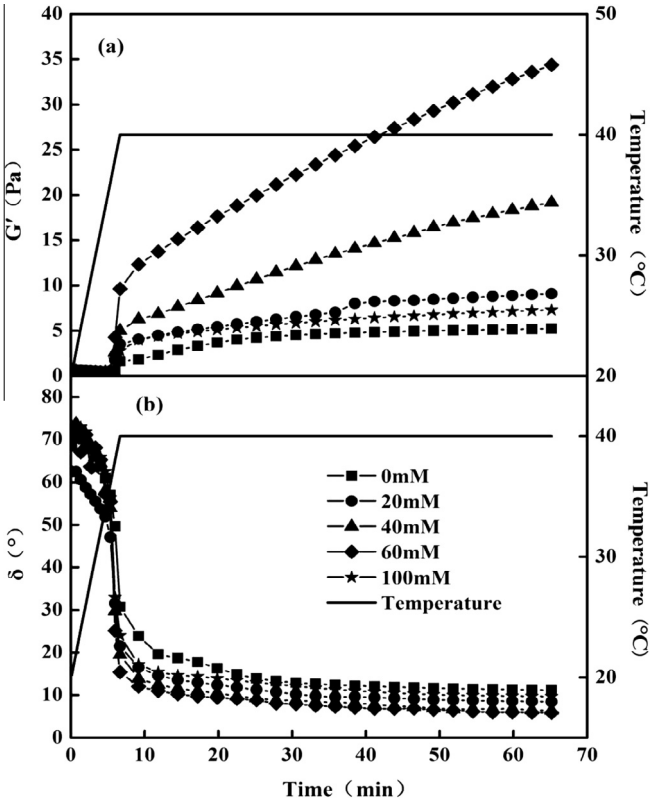


Fig. 5. Storage modulus (a) and phase angle (b) of myosin solution at various $CaCl_2$ concentrations during thermal gelation.

20, 40, 60, and 100 mM $CaCl_2$ were 0.06, 0.09, 0.22, 0.38, and 0.06 Pa/min, respectively. The maximal increasing rate of G' development was obtained at 60 mM $CaCl_2$. The G' values of all the samples in phase I had a similar pattern to those in phase II. From the results of all the samples without heating (incubation for 0 min), it can be deduced that phase I and phase II had the same interactions, which might explain the changes of G' values in phases I and II. Specifically, $CaCl_2$ induced myosin denaturation and then enhanced aggregation via hydrophobic interactions, disulfide bonds and Ca bridges, which probably resulted in further protein aggregation into a gel network. The extent and rate of aggregation of myosin increased with increasing $CaCl_2$ concentration (Fig. 4). 60 mM $CaCl_2$ could be used to improve gelation properties of the fish myosin due to the suitable extent and rate of aggregation. However, the high aggregation rate and the excessive aggregation extent resulting from 100 mM $CaCl_2$ might lead to random aggregation and formation of coarse protein networks, which is associated with poor gelling properties. Ju and Kilara (1998) suggested that the formation of a good gel required an optimal aggregation extent or aggregate size. Based on the aforementioned results, it could be concluded that silver carp myosin treated with 60 mM $CaCl_2$ showed a proper extent and rate of aggregation, which was associated with the highest G' values. Arfat and Benjakul (2012) reported that a greater extent of aggregation occurred with Zn^{2+} (25–100 μM) treatment. They also pointed out that an appropriate amount of Zn^{2+} could promote gel strength of myosin, due to the finer and ordered gel network with smaller voids. However, an excessive amount of Zn^{2+} would result in a looser network with large voids and lower gel strength (Arfat & Benjakul, 2013). Moreover, Hongsprabhas and Barbut (1997) reported that whey protein isolate showed a salting-out effect when treated with 40 mM $CaCl_2$. Thus, it is possible that the myosin treated with 100 mM $CaCl_2$ exhibited a salting-out effect, resulting in the failure of gel formation. This might be another reason for the lower G' value of 100 mM $CaCl_2$ treatment.

4. Conclusions

During setting at 40 °C, both $CaCl_2$ and heating induced conformational changes of silver carp myosin, and $CaCl_2$ induced the unfolding of myosin molecules, as evidenced by the loss of α-helical content. S_0-ANS increased and incubation time (for S_0-ANS to reach the maximum) was reduced with increasing $CaCl_2$ concentration. Total SH content also decreased with increasing $CaCl_2$ concentration, suggesting that $CaCl_2$ promoted the formation of disulfide bonds, myosin denaturation and the subsequent aggregation via hydrophobic interactions and disulfide bonds. Moreover, Ca bridges also formed in myosin aggregates. All of those results were associated with the increase of turbidity and d_z during setting. The extent and rate of aggregation increased with increasing $CaCl_2$ concentration, which could facilitate the network formation of the fish myosin. The highest G' and lowest δ values of the fish myosin were observed during setting at 40 °C with the treatment of 60 mM $CaCl_2$. An excessive amount of $CaCl_2$ (100 mM) would decrease G' values. The overall findings from this research indicate that $CaCl_2$ enhances gelling properties of silver carp myosin by directly inducing conformational changes of myosin and promoting hydrophobic interactions, disulfide bonds and Ca bridges of "set" gel.

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