Available online at www.sciencedirect.com
ScienceDirect
Food Hydrocolloids 21 (2007) 359–367
FOOD HYDROCOLLOIDS
www.elsevier.com/locate/foodhyd

Aggregation and conformational changes of tilapia actomyosin as affected by calcium ion during setting

Jirawat Yongsawatdigul*, Sornchai Sinsuwan

School of Food Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand

Received 28 October 2005; accepted 12 April 2006

Abstract

The effect of $CaCl_2$ on aggregation and conformational changes of tropical tilapia (*Oreochromis niloticus*) actomyosin incubated at 4 and 40 °C was investigated. Aggregation of tilapia actomyosin incubated at 40 °C for 30 min increased with addition of 10–100 mM $CaCl_2$. Formation of higher molecular weight protein (HMP) at 40 °C was enhanced by addition of >10 mM Ca^{2+} ion, but suppressed by 2 mM N-ethylmaleimide (NEM) and 1 mM phenylmethnesulfonyl fluoride (PMSF), suggesting the involvement of endogenous transglutaminase (TGase). Moreover, addition of 10–100 mM $CaCl_2$ destabilized actomyosin as evident by an increase in aniline naphthalenesulfonate surface hydrophobicity (S_0-ANS) and loss of α-helical structure at 40 °C. However, $CaCl_2$ only increased S_0-ANS of actomyosin incubated at 4 °C without disturbing its secondary structure. Both ε-(γ-glutamyl)lysine isopeptide bonds and hydrophobic interactions appeared to be involved in HMP aggregates formed at 40 °C. Breaking force and deformation of actomyosin gels incubated at 40 °C for 30 min increased with added $CaCl_2$ level and reached the maximum at 100 mM $CaCl_2$, corresponding to an increased intensity of HMP observed on 5% SDS-PAGE. Ca^{2+} improved gelation during setting at 40 °C by not only activating endogenous TGase but also promoting hydrophobic interactions among unfolded actomyosin. Setting was also induced to a lesser extent at 4 °C in the presence of >10 mM $CaCl_2$.
© 2006 Elsevier Ltd. All rights reserved.

Keywords: Tilapia; Actomyosin; Calcium ion; Setting; Conformation

1. Introduction

Setting or suwari is referred to a process in which fish protein is mixed with salt and is incubated at either 25 or 40 °C for a period of time before heating to form gel at higher temperature (90 °C) (Niwa, 1992; Lanier, 2000). The resulting gel exhibited higher elasticity. Thus far, endogenous transglutaminase (TGase) is thought to be responsible for inducing the setting effect. TGase (protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) is a transferase that catalyzes the acyl transfer reaction between γ-carboxyamide groups of glutamine and ε-amino groups of lysine, resulting in protein polymers via ε-(γ-glutamyl) lysine cross-linkings (Folk, 1980). Since endogenous TGase is a Ca^{2+}-dependent enzyme, addition of Ca^{2+} to fish protein paste has been reported to activate TGase activity, and thus improve textural properties of fish protein gel (Lee & Park, 1998; Yongsawatdigul, Worratao, & Park, 2002).

Ca^{2+} ion is a destabilizing salt in the Hofmeister series and promotes "salting in" of protein (Baldwin, 1996). Ca^{2+} ion decreases the free energy required to transfer the nonpolar amino acids into water and thus reduces intramolecular hydrophobic interactions, resulting in an increased protein unfolding (von Hippel & Wong, 1965). Protein extractability of ground turkey breast and thigh muscle increased with calcium concentration due to the salting in effect (Nayak, Kenney, & Slider, 1996). Ca^{2+} ion also increased solubilization of C-protein, troponin-T and troponin-I in rabbit *psoas* myofibrils (Taylor & Etherington, 1991). Other destabilizing salts, such as lithium bromide (LiBr), potassium iodide (KI), and potassium thiocyanate (KSCN), have been reported to destroy the α-helical structure of myosin (Nakayama, Niwa, & Hamada, 1983). Based on the aforementioned studies, Ca^{2+} ion might have a direct effect on conformational

*Corresponding author. Tel.: +66 44 224 359; fax: +66 44 224 150.
E-mail address: jirawat@sut.ac.th (J. Yongsawatdigul).

0268-005X/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodhyd.2006.04.006

changes of fish actomyosin in addition to being endogenous TGase activator. However, the role of Ca^{2+} on conformational changes of fish actomyosin molecules during setting has not been systematically investigated.

Knowledge of setting is based mainly on the phenomenon observed from cold water species, particularly Alaska pollock and Pacific whiting (Kamath, Lanier, Foegeding, & Hamann, 1992; Joseph, Lanier, & Hamann, 1994; Park, Yongsawatdigul, & Lin, 1994). Several warm water species, such as bigeye snapper (*Priacanthus tayenus*), threadfin bream (*Nemipterus* spp.), and tropical tilapia (*Oreochromis niloticus*), also exhibited setting (Klesk, Yongsawatdigul, Park, Viratchakul, & Virulhakul, 2000; Yongsawatdigul et al., 2002; Benjakul & Visessanguan, 2003). Higher setting temperature (40 °C) is typically reported in warm water species due to high thermal stability of myosin/actomyosin (Lanier, 2000). However, conformational changes of actomyosin from tropical species during setting, especially as affected by Ca^{2+}, have not been thoroughly investigated. Our objective was to elucidate the effect of Ca^{2+} ion on conformational changes of tropical tilapia (*O. niloticus*) actomyosin during setting.

2. Materials and methods

2.1. Chemical

N-ethylmaleimide (NEM), 8-anilino-1-naphthalene sulfonic acid (ANS), 2-mercaptoethanol (β-ME), pheylmethnesulfonyl fluoride (PMSF), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Imidazole, 5,5′-dinitrobis(2-nitrobenzoic acid) (DTNB) and ethylene glycol-bis (2-aminoetyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were purchased from Fluka (Buchs, Switzerland). All other chemicals used were of analytical grade.

2.2. Actomyosin preparation

Live tilapia (*O. niloticus*) were obtained from the Suranaree University of Technology Farm. Fish were kept in ice and transported to the laboratory within 10 min after catching. All procedures were carried out at 4 °C and actomyosin extraction was conducted according to Ogawa, Nakamura, Horimoto, An, Tsuchiya, & Nakai (1999). Fish mince (100 *g*) was added 500 mL of 50 mM NaCl, 10 mM imidazole, and 0.05 mM PMSF (pH 7.0) and homogenized. The homogenate was centrifuged at 10,000 × *g* for 5 min (RC 28S; Sorvall Co., Newtown, CT., USA). The supernatant containing sarcoplasmic proteins was discarded. The precipitates were washed twice using the same buffer. Subsequently, the pellet was homogenized with 1 L of 0.6 M NaCl in 10 mM imidazole buffer (pH 7.0) and the suspension was centrifuged at 10,000 × *g* for 5 min. The supernatant containing myofibrillar protein was filtered through three-layers of cheesecloth to remove the connective tissue. The filtrate was stirred in 3 L of deionized water to precipitate myofibrillar protein, and then centrifuged at 10,000 × *g* for 15 min. The precipitate was washed in 500 mL of 50 mM NaCl in 10 mM imidazole buffer (pH 7.0). Actomyosin was collected by centrifugation at 10,000 × *g* for 10 min. Water was removed from the pellet by centrifugation at 12,500 × *g* for 15 min. Due to high absorption of imidazole buffer in the circular dichroism (CD) measurement, actomyosin preparation was carried out as described above but using 20 mM Tris-HCl instead.

2.3. Turbidity

Turbidity was measured according to the method of Yongsawatdigul and Park (1999). Actomyosin solutions were diluted to 0.5 mg/mL with 0.4 M NaCl, 10 mM imidazole, containing 10–100 mM $CaCl_2$ (pH 7.0). The negative control was prepared by solubilizing actomyosin with 0.4 M NaCl, 10 mM imidazole, containing 1 mM EGTA (pH 7.0). Diluted actomyosin solutions were placed in a quartz cuvette (light path length of 10 mm). Changes of turbidity were monitored at 320 nm using UV/VIS spectrophotometer (GBC UV/VIS 916; GBC Scientific Equipment PTY, Ltd., Australia) connected with a circulating cooling bath set at 40 °C.

2.4. Determination of soluble aggregates

The extent of actomyosin aggregation at 4 °C for 24 h and 40 °C for 30 min was evaluated using ultracentrifugation. Each sample was ultracentrifugated at 84,200 × *g* for 1 h (XL-100 Ultracetrifuge, Beckman Co., Palo Alto, CA., USA). Protein content was determined by the dye binding method (Bradford, 1976) using BSA as a standard. Remaining protein was calculated as the percentage of soluble protein after ultracentrifugation, taking soluble protein of samples without added Ca^{2+} at each incubating condition as 100%.

2.5. Protein cross-linking studies

Actomyosin solutions were incubated at 40 °C for up to 2 h. Three mL of incubated solutions was mixed in 27 mL of 5% (w/v) SDS solution and heated to 90 °C until complete solubilization. Solubilized proteins were centrifuged at 10,000 × *g* for 20 min. The extent of protein cross-linking was analyzed using 5% SDS-PAGE according to Huff-Lonergan, Parrish, and Robson (1995). Bonding of cross-linked proteins were elucidated by solubilizing the incubated actomyosin solution in various solubilizing buffers, including 3%, 5%, 10% SDS, and 5% SDS mixed with 2% β-ME. The mixtures were heated at 90 °C for 30 min. Protein patterns were analyzed using 5% SDS-PAGE. The effect of TGase inhibitors, namely 2 mM NEM and 1 mM PMSF, on protein patterns was also investigated on SDS-PAGE.

2.6. ANS-surface hydrophobicity

Aniline naphthalene sulfonate surface hydrophobicity (S_0-ANS) was determined by the method of Yongsawatdigul and Park (2003). Actomyosin solutions were diluted to 0.125, 0.25, 0.5, 0.75, and 1 mg/mL in 0.4 M NaCl, 10 mM imidazole, containing various $CaCl_2$ concentrations (10–100 mM) (pH 7.0). The series protein concentration were added 10 µL of 8 mM ANS in 0.1 M imidazole (pH 7.0) and kept under dark for 10 min. Fluorescence intensity was measured using a spectrofluorometer (RF-1501; Shimadzu Co., Kyoto, Japan) at excitation and emission wavelength of 374 and 485 nm, respectively. The relative fluorescence (R) was plotted against percentage of protein concentration. Slope was calculated as S_0-ANS value. The relative fluorescence was defined:

$$R = (F - F_0)/F_0,$$

where F and F_0 are fluorescence intensity of sample containing ANS and ANS solution, respectively.

2.7. Circular dichroism

Changes in secondary structure were monitored using a spectropolarimeter (PS150J; JASCO, Tokyo, Japan) connected with a circulating cooling bath set at 4 and 40 °C. AM solutions were incubated at either 4 °C for 24 h or 40 °C for 30 min, and were diluted to 0.25 mg/mL in 0.4 M NaCl, 20 mM Tris–HCl at various $CaCl_2$ concentrations (10–100 mM) (pH 7.0). The changes of secondary structure were monitored by scanning solutions from 195 to 260 nm in a 0.2 mm quartz cell. Mean molar residue weight of 115 g/mol was used to calculate the molar ellipticities of actomyosin (Price, 1996). α-Helical content was determined by the equation described by Ogawa, Kanamaru, Miyashita, Tamiya, and Tsuchiya (1995).

$$\alpha - \text{Helicity}(\%) = 100 \times \left\{ \frac{[\theta]_{222}}{-40,000} \right\}.$$

When $[\theta]_{222}$ is ellipticity at 222 nm.

2.8. Textural properties

Actomyosin pastes (38.5 mg protein/g) containing final concentrations of 0.4 M NaCl, 10 mM imidazole (pH 7.0) at various $CaCl_2$ concentrations (10–100 mM) were prepared using a mortar and pestle. The pastes were filled into a microplate with a diameter of 5 mm and depth of 10 mm. The filled microplates were placed in a plastic bag and pre-incubated at either 4 °C for 12 h or 40 °C for 30 min. Consequently, samples were heated at 90 °C for 15 min and then were cooled at 4 °C for overnight. The breaking force and deformation were determined using a Texture Analyzer TA-XT2 (Stable Micro System, Surrey, England) with a 2-mm cylindrical probe at a test speed of 1 mm/s. For each treatment, mean values were obtained from at least five measurements.

2.9. Statistical analysis

Two different lots of fish were used. Analysis of variance (ANOVA) was analyzed using the SAS program (SAS Institute Inc, Carry, NC, USA). Differences among mean values were established using the Duncan multiple range test (DMRT) at $p<0.05$. In gel texture studies, a split plot design was applied. The eight levels of Ca^{2+} concentration (0, 10, 20, 30, 40, 50, 70, and 100 mM) were assigned as a main plot factor and the three heating treatments (4 °C/12 h, 40 °C/30 min, 90 °C/15 min) as a split plot factor. Two different lots of actomyosin were applied as a block.

3. Results and discussion

3.1. Aggregation of actomyosin

Turbidity of actomyosin solution in >30 mM $CaCl_2$ sharply increased with incubation time at 40 °C (Fig. 1). Turbidity changed to a greater extent at higher $CaCl_2$ concentration. Gill, Chan, Phonchareon, and Paulson (1992) demonstrated that an increase in turbidity of heated fish myosin solution was the direct result of formation of myosin aggregates. Association of tilapia actomyosin molecules produced aggregates that increased light scattering, resulting in an increased turbidity. Formation of actomyosin aggregates in the absence of Ca^{2+} (EGTA added) and in the presence of endogenous Ca^{2+} (control) at 40 °C was far less than those with added Ca^{2+}. The formation of large aggregates induced by Ca^{2+} at 40 °C was confirmed using ultracentrifugation. Addition of 10 mM Ca^{2+} induced formation of large actomyosin aggregates at 40 °C as evident by approximately 40% decrease in protein content after ultracentrifugation (Fig. 2). An increase in Ca^{2+} concentration from 30 to 100 mM did not further promote actomyosin aggregation.

The effect of Ca^{2+} on actomyosin aggregation at 4 °C was not as pronounced as at 40 °C (Fig. 2). Large aggregates formed after incubation at 4 °C for 24 h were noticed when 50 mM Ca^{2+} was added. It is also noted that aggregation of actomyosin at 4 °C readily occurred at >50 mM $CaCl_2$ without incubation. These results indicated that Ca^{2+} induced aggregation of actomyosin at both 4 and 40 °C. The extent of aggregation at 4 °C was far less than that at 40 °C. Aggregation is typically induced by association of unfolded protein molecules via various interactions, namely disulfide linkages, electrostatic, hydrophobic interactions, and hydrogen bonds (Oakenfull, Pearce & Burley, 1997). Since denaturation temperature of actomyosin from tropical fish is around 35 °C (Yongsawatdigul & Park, 2003), tilapia actomyosin incubated at 40 °C would unfold and aggregate to a greater extent than at 4 °C. Addition of Ca^{2+} appeared to increase aggregate formation, particularly at 40 °C.

3.2. Covalent cross-linking induced by Ca^{2+}

Higher molecular weight proteins (HMP) were found in actomyosin containing Ca^{2+} (>10 mM) and incubated at 40 °C for 30 min (Fig. 3(a)). Lower intensity of HMP was observed in unheated samples. Intensity of HMP also increased with Ca^{2+} concentration (Figs. 3(b) and (c)). More distinct HMP bands were observed at 50–100 mM Ca^{2+} (Fig. 3(c)), corresponding to an increased turbidity (Fig. 1). Since actomyosin aggregates were solubilized using SDS and β-ME at 90 °C, noncovalent bonds were mainly disrupted. Non-disulfide covalent bonds probably participated in HMP formation. When incubation was prolonged in the presence of 70 mM Ca^{2+}, intensity of HMP increased (Fig. 4). Formation of non-disulfide covalent cross-linking in Alaska pollock and Atlantic croaker surimi subjected to the optimal setting condition was also noted (Kamath et al., 1992). Covalent cross-linkings of myosin heavy chain induced by Ca^{2+} has been reported in Alaska pollock (Wan, Kimura, Satake, & Seki, 1994) and threadfin bream surimi (Yongsawatdigul et al., 2002). Endogenous TGase has been reported to be responsible for the formation of these cross-linked polymers. The enzyme catalyzed formation of ε-(γ-glutamyl) lysine bonds, which was not dissociated by SDS, β-ME, and heat.

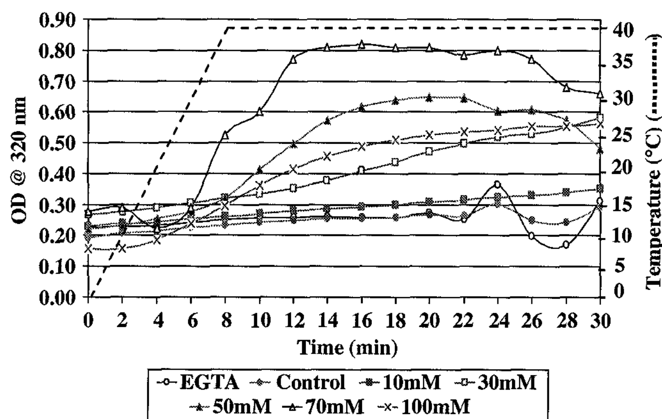

Fig. 1. Turbidity changes of tilapia actomyosin solution (0.5 mg/mL) in 0.4 M NaCl, 10 mM imidazole, containing 0–100 mM $CaCl_2$ (pH 7.0), incubated at 40 °C for 30 min.

To prove if HMP observed in tilapia was a product from the cross-linking reaction catalyzed by endogenous TGase, TGase inhibitors, namely NEM and PMSF (Worratao & Yongsawatdigul, 2005), was added to the reaction mixture. Intensity of HMP was remarkably reduced with addition of TGase inhibitors (Fig. 5), implying that formation of HMP was mediated by endogenous TGase activity. Since the enzyme is Ca^{2+}-dependent, increasing Ca^{2+} concentration activated endogenous TGase, which in turn catalyzed actomyosin cross-linking via ε-(γ-glutamyl) lysine bonds. This also explained the greater extent of actomyosin aggregates formed at 40 °C at higher Ca^{2+} concentration (>10 mM) (Fig. 1). In the absence of added Ca^{2+}, faint HMP bands were noticed after incubation at 40 °C for 2 h (Fig. 6(a)). These results corresponded with low turbidity observed in the sample without added Ca^{2+} (Fig. 1). In addition, HMP bands formed in the absence of Ca^{2+} disappeared when samples were solubilized in 3–10% SDS and 5%SDS + β-ME (Fig. 6(a)). In contrast, HMP formed in the presence of 100 mM Ca^{2+} was still noticeable after solubilizing in these buffers (Fig. 6(b)). These results suggested that different actomyosin cross-links were formed in the absence and presence of Ca^{2+}. In the absence of Ca^{2+}, tilapia actomyosin appeared to aggregate via hydrophobic interactions at 40 °C. Such interactions

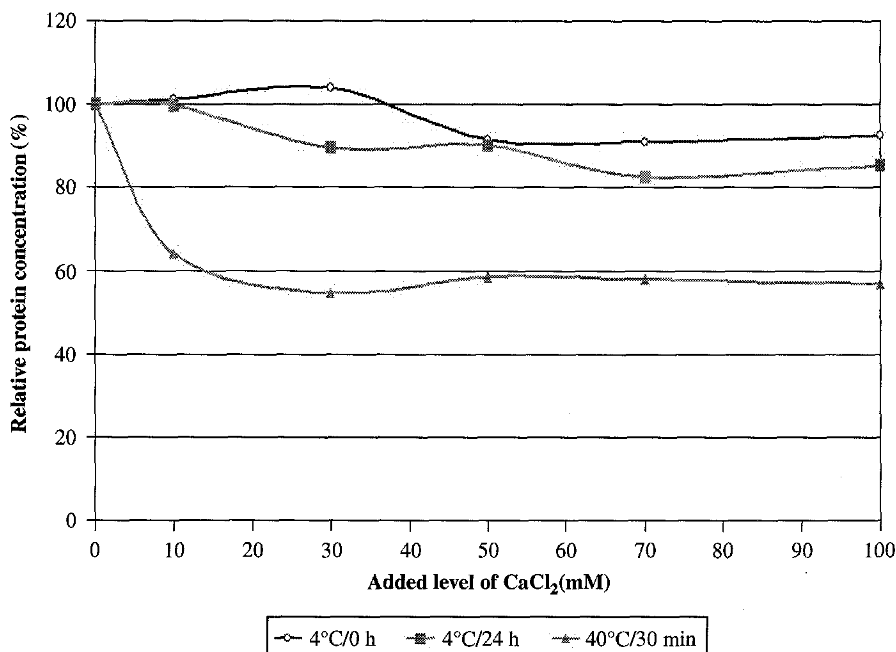

Fig. 2. Relative protein concentration after ultracentrifugation of tilapia actomyosin containing 0–100 mM $CaCl_2$ and incubated at 4 and 40 °C.

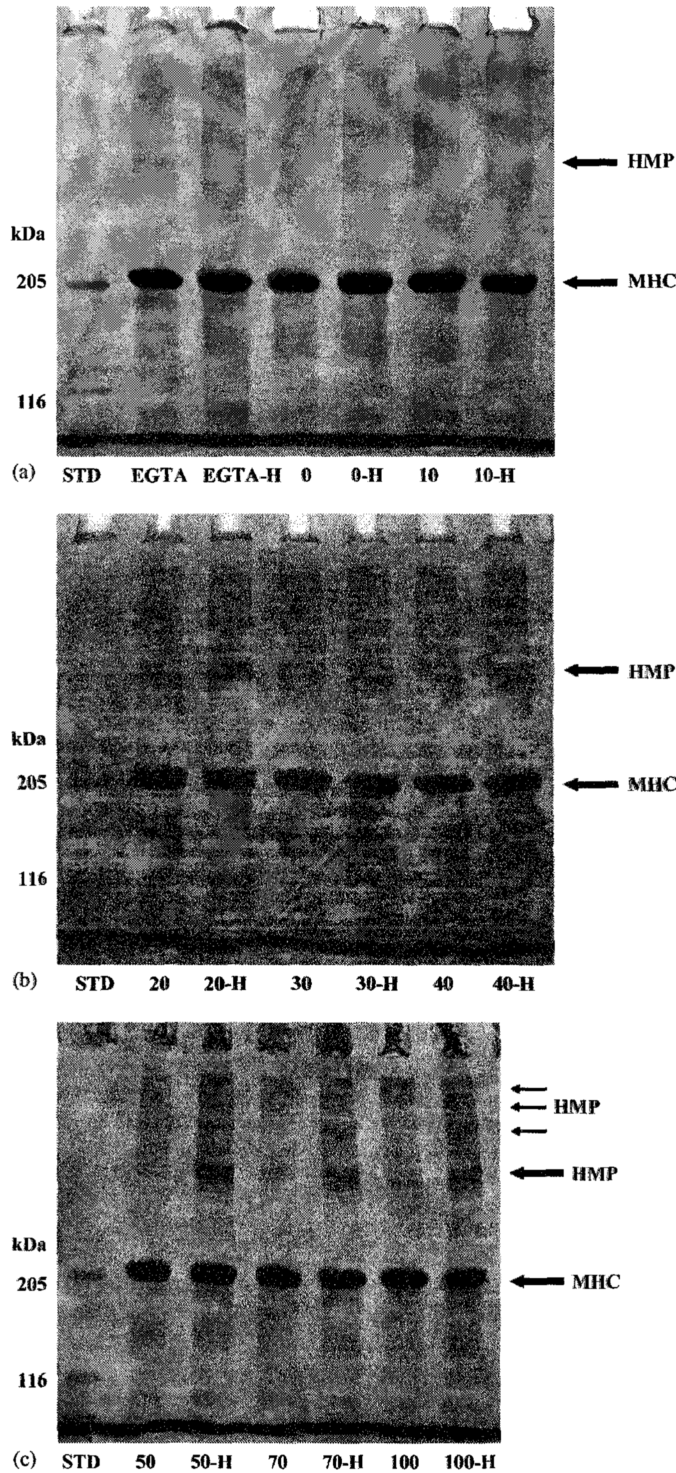

Fig. 3. SDS-PAGE patterns (5% acrylamide) of tilapia actomyosin containing EGTA or 0–10 mM $CaCl_2$ (a), 20–40 mM $CaCl_2$ (b), and 50–100 mM $CaCl_2$ (c). EGTA, 0–100 indicate samples containing EGTA and 0–100 mM $CaCl_2$ concentration at 4 °C, respectively. H indicates samples incubated at 40 °C for 30 min. MHC = myosin heavy chain; HMP = high molecular weight proteins; STD = standard molecular weight.

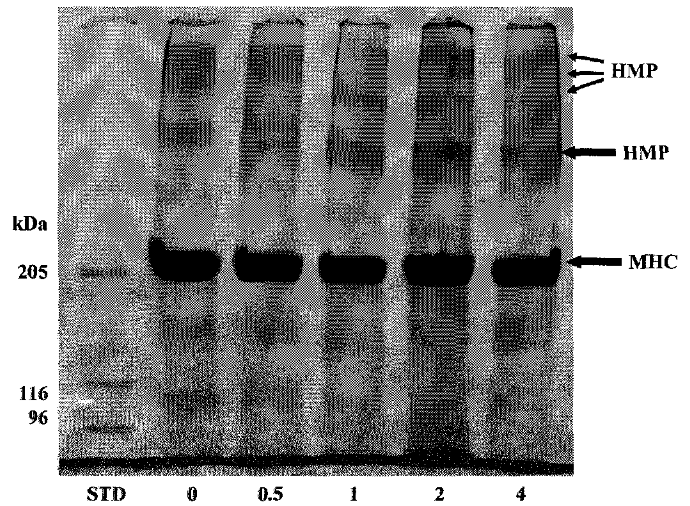

Fig. 4. SDS-PAGE patterns (5% acrylamide) of tilapia actomyosin containing 70 mM $CaCl_2$ and incubated at 40 °C for 0, 0.5, 1, 2, and 4 h. Abbreviations are the same as Fig. 3.

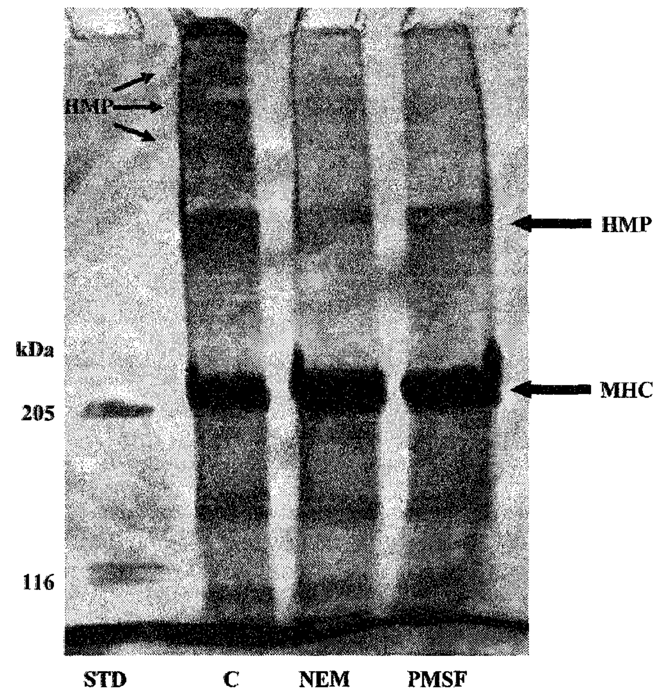

Fig. 5. SDS-PAGE patterns (5% acrylamide) of tilapia actomyosin containing 100 mM $CaCl_2$ with TGase inhibitors and incubated at 40 °C for 2 h. C = control (no inhibitor), NEM, PMSF = inhibitors. Abbreviations are the same as Fig. 3.

were disrupted in SDS solution. When 100 mM Ca^{2+} was added, aggregation of tilapia actomyosin was predominantly formed via non-disulfide covalent bonds, which was presumably catalyzed by endogenous TGase. It should be noted that endogenous TGase activity in this study would be lower than in the surimi system because actomyosin was prepared by extensive washing and subsequent precipitation at low ionic strength. Yongsawatdigul et al. (2002) reported that approximately 40% of TGase activity of threadfin bream surimi was retained after three washing cycles. Due to low residual TGase activity in tilapia actomyosin, formation of HMP was more evident at higher Ca^{2+} concentration (Figs. 3(b) and (c)).

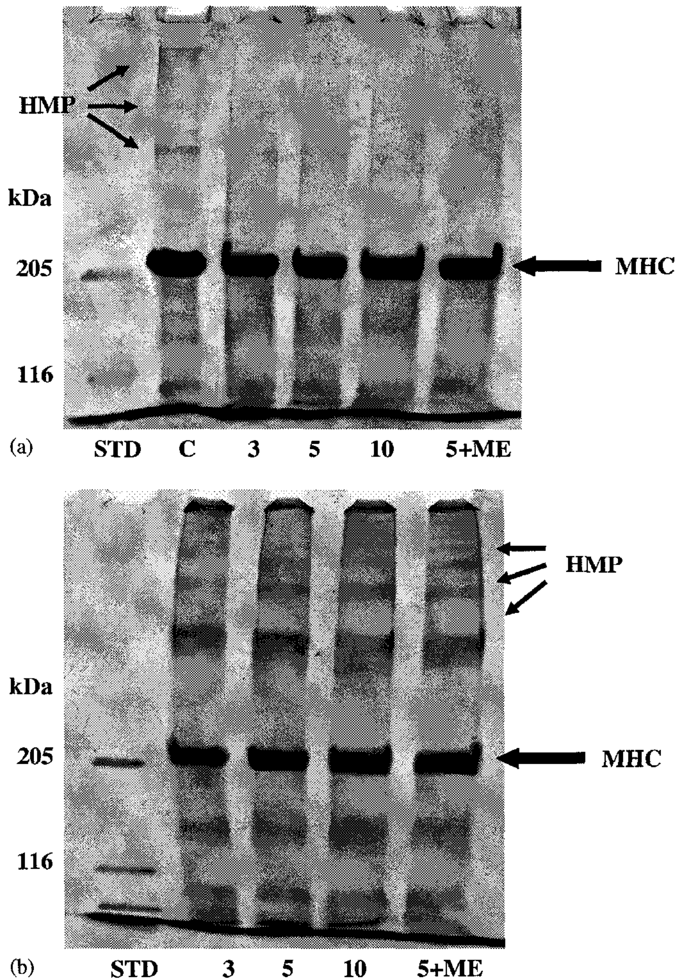

Fig. 6. SDS-PAGE patterns (5% acrylamide) of tilapia actomyosin without added $CaCl_2$ (a) and with 100 mM $CaCl_2$ (b), incubated at 40 °C for 2 h. C = samples without solubilizing buffer; 3, 5, 10 = samples solubilized in 3, 5, 10% SDS, respectively; 5 + ME = samples solubilized in 5%SDS + 2%β-ME. Abbreviations are the same as Fig. 3.

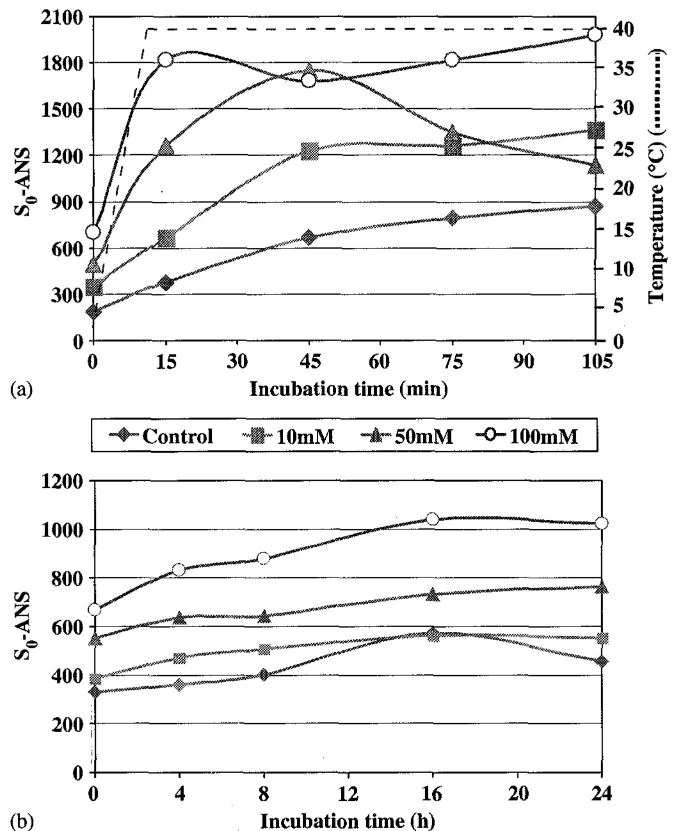

Fig. 7. Effect of $CaCl_2$ concentration on changes of S_0-ANS of tilapia actomyosin incubated at 40 °C (a) and 4 °C (b).

3.3. Surface hydrophobicity

S_0-ANS of tilapia actomyosin without added Ca^{2+} (control) increased with incubation time at 40 °C (Fig. 7(a)). Addition of 10–100 mM Ca^{2+} notably increased S_0-ANS during incubation at 40 °C ($p<0.05$). Actomyosin underwent unfolding when subjected to 40 °C, subsequently exposing hydrophobic amino acids to the aqueous environment. Ca^{2+} further promoted the unraveling of hydrophobic domains on the actomyosin molecule at 40 °C. The exposed hydrophobic groups could undergo intermolecular interactions via hydrophobic interactions. Chan, Gill, and Paulson (1993) also reported that thermal aggregation ability of fish myosin linearly increased with surface hydrophobicity. Thus, Ca^{2+} did not only activate endogenous TGase as typically understood but also promoted the unfolding of actomyosin, which in turn enhanced aggregation at 40 °C. Besides non-disulfide covalent bonds (ε-(γ-glutamyl) lysine cross-links), hydrophobic interactions were also involved in "set" surimi (Niwa, 1992; Lanier, 2000). The effect of Ca^{2+} on surface hydrophobicity was also reported in other proteins. Addition of 1–15 mM $CaCl_2$ induced structural changes in β-lactoglobulin, resulting in an increased hydrophobicity (Jeyarajah & Allen, 1994).

S_0-ANS of actomyosin slightly increased with incubation time at 4 °C ($p<0.05$) (Fig. 7b). S_0-ANS values of actomyosin in the presence of 10–100 mM Ca^{2+} were greater than those of sample without Ca^{2+} ($p<0.05$). These results suggested that Ca^{2+} also induced the unfolding of actomyosin even at low temperature (4 °C), but to a lesser extent than at 40 °C. The exposure of hydrophobic amino acids could also lead to the formation of hydrophobic interactions among actomyosin molecules at 4 °C.

3.4. Circular dichroism

Patterns of CD spectra of tilapia actomyosin showed the predominant α-helical structure at various $CaCl_2$ concentrations (10–100 mM) at 4 °C (Fig. 8(a)). In addition, α-helical content of actomyosin incubated at 4 °C for 24 h was comparable to that without incubation (data not shown). These results suggested that Ca^{2+} had no effect on secondary structure of actomyosin at 4 °C. However, S_0-ANS results revealed that tilapia actomyosin exposed more hydrophobic residues at higher Ca^{2+} concentration when incubation time

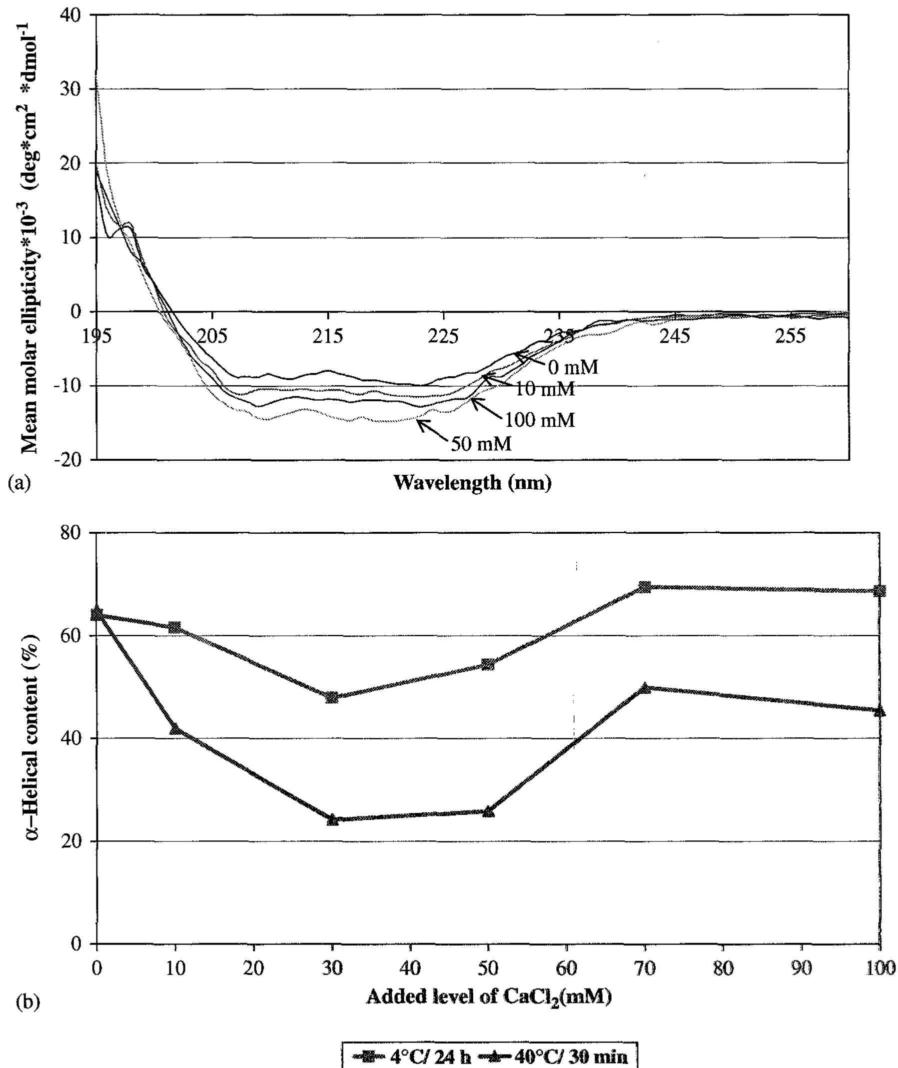

Fig. 8. CD spectra of tilapia actomyosin containing various $CaCl_2$ concentrations at 4 °C (a) and changes of α-helical content of actomyosin at various $CaCl_2$ concentrations (b).

at 4 °C was prolonged (Fig. 7b). It could be speculated that Ca^{2+} only induced conformational changes by exposing hydrophobic amino acids without disturbing the secondary structure of tilapia actomyosin at 4 °C.

When actomyosin was incubated at 40 °C for 30 min without added Ca^{2+}, the α-helical content was comparable to that incubated at 4 °C (Fig. 8(b)), indicating the subtle change in α-helix of tilapia actomyosin at 40 °C in the absence of Ca^{2+}. Ogawa et al. (1999) also reported that the α-helical structure of tilapia actomyosin exhibited high thermal stability at 40 °C. α-Helical content markedly decreased with increased Ca^{2+} concentrations at 40 °C (Fig. 8(b)). Incubation at 40 °C in the presence of Ca^{2+} drastically promoted conformational changes accompanied by the loss of α-helical structure. Both heat and destabilizing effect of Ca^{2+} synergistically induced the unfolding of actomyosin at 40 °C, which was a prerequisite for TGase reactivity and protein aggregation during setting. The greater extent of protein unfolding would allow more glutamine and lysine residues to be exposed to endogenous TGase, resulting in formation of more isopeptide cross-links. In addition, the greater extent of unfolding promoted the exposure of hydrophobic patches and intermolecular hydrophobic interactions. Therefore, it could be postulated that actomyosin of fish exhibiting greater thermal stability would require more Ca^{2+} to initiate the conformational changes for setting phenomenon. This corresponded with the findings of Saeki (1995) who reported that myofibrils of fish with less thermal stability, such as pollock, sardine, and Pacific cod, were more labile to structural changes induced by 50 mM Ca^{2+} at 30 and 38 °C.

3.5. Gel strength

Breaking force and deformation values of actomyosin gels increased with added $CaCl_2$ levels at all studied heating regimes ($p<0.05$) (Figs. 9(a) and (b)). The control (no Ca^{2+} added) heated to 90 °C without pre-incubation did not form a gel, but addition of Ca^{2+} resulted in gelation. Hydrophobic interactions which were enhanced

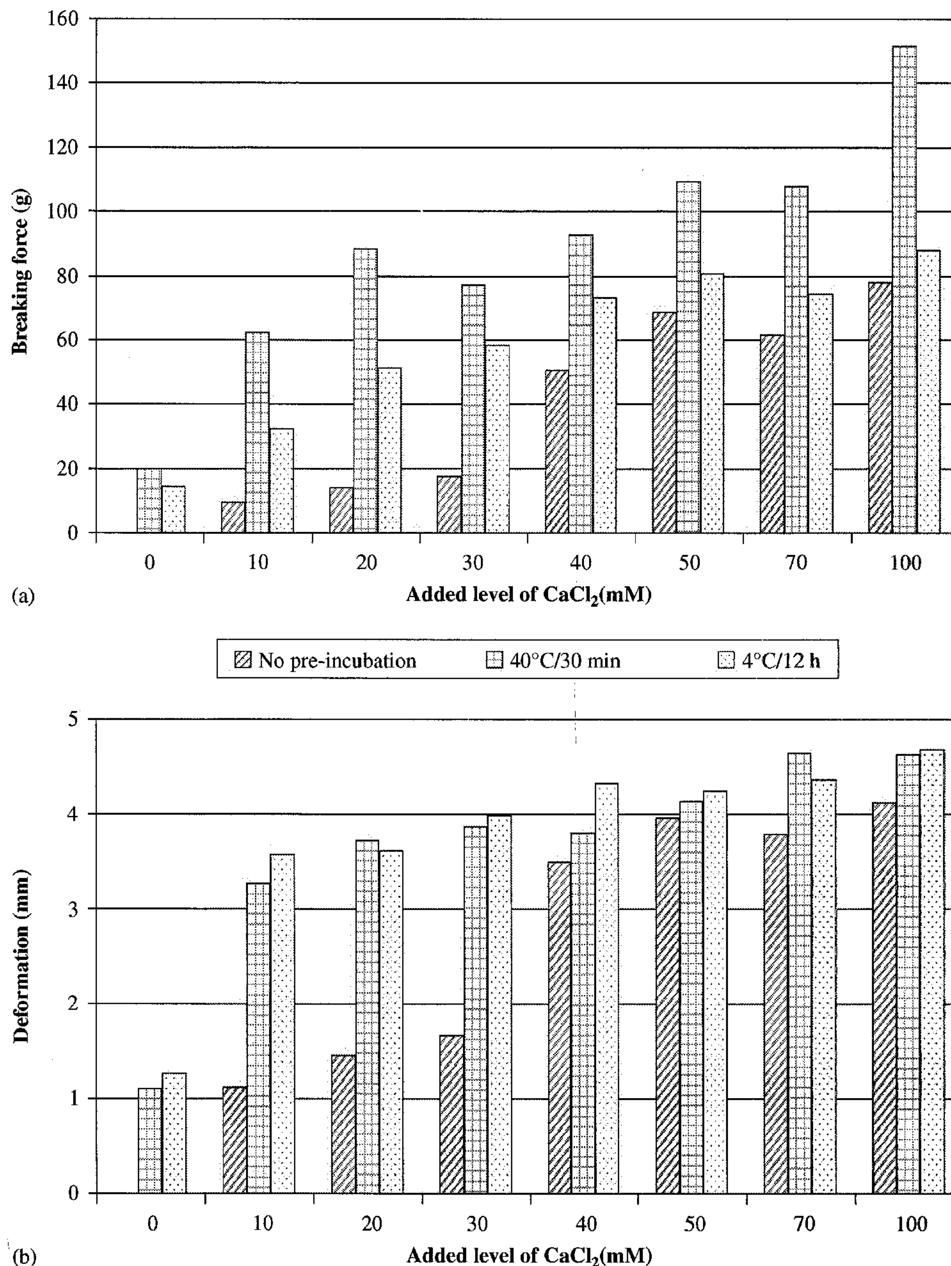

Fig. 9. Effect of $CaCl_2$ concentration and setting condition on breaking force (a) and deformation (b) on actomyosin gels.

by addition of Ca^{2+} could play an important role in stabilizing the gel network of actomyosin heated without pre-incubation. Pre-incubation at 4 °C appeared to enhance both breaking force and deformation of tilapia actomyosin gels, especially at 10–100 mM Ca^{2+} ($p<0.05$). The maximum breaking force was obtained at 40 mM $CaCl_2$ (Fig. 9a). $CaCl_2$ (up to 100 mM) did not increase protein extractability of actomyosin (data not shown). Therefore, an increase in textural properties was not caused by the salting in effect of Ca^{2+} as previously reported in chicken myofibril (Xiong & Brekke, 1991). It has been generally believed that actomyosin from tropical fish species does not "set" at low temperature due to its high thermal stability. Klesk et al. (2000) reported that no setting effect was observed at 4 °C in tilapia surimi. However, our study demonstrated that setting of tilapia actomyosin occurred at 4 °C in the presence of >10 mM Ca^{2+}. The unfolding of actomyosin induced by Ca^{2+} could promote hydrophobic interactions among actomyosin molecules, which perhaps contributed to an increase in textural properties.

As expected, the effect of Ca^{2+} on textural properties of tilapia actomyosin was more pronounced when incubated at 40 °C for 30 min ($p<0.05$) (Figs. 9 (a) and (b)). Ca^{2+} ion activated endogenous TGase activity, enhancing the formation of ε-(γ-glutamyl) lysine cross-links among actomyosin molecules. An increase in textural properties with Ca^{2+} concentration corresponded with formation of HMP. Furthermore, addition of Ca^{2+} in conjunction with pre-incubation at 40 °C induced the unfolding of actomyosin. The greater extent of unfolding promoted a higher

degree of hydrophobic interactions. Both isopeptide cross-links and hydrophobic interactions were enhanced by addition of Ca^{2+}, consequently improved textural properties of actomyosin gel.

4. Conclusions

Ca^{2+} affected gelation of tilapia actomyosin during setting at 40 °C in two different means. Firstly, Ca^{2+} activated endogenous TGase, promoting the formation of ε-(γ-glutamyl) lysine cross-links. Secondly, Ca^{2+} induced the unfolding of actomyosin molecule, resulting in an increased surface hydrophobicity. The unfolded molecules tended to intermolecularly aggregate via hydrophobic interactions. Thus, both ε-(γ-glutamyl) lysine cross-links and hydrophobic interactions played an important role in gelation of tilapia actomyosin set at 40 °C. Ca^{2+} ion also induced the unfolding of actomyosin at 4 °C, leading to enhancement of hydrophobic interactions and improvement in textural properties. Thus, tropical fish with high thermal stability essentially required Ca^{2+} to unfold actomyosin and promote setting.

Acknowledgements

The authors would like to thank Thailand Research Fund for financially supporting this research under Grant RSA/15/2545 and Suranaree University of Technology under Grant SUT3-305-43-24-35. We also thank Dr. Chartchai Kritanai of Institute of Molecular Biology and Genetics, Mahidol University, Salaya, Thailand for technical assistance on circular dichroism measurements and analyses.

References

Baldwin, R. (1996). How Hofmeister ion interactions affect protein stability. *Biophysical Journal, 71*, 2056–2063.

Benjakul, S., & Visessanguan, W. (2003). Transglutaminase-mediated setting in bigeye snapper surimi. *Food Research International, 36*, 253–266.

Bradford, M. (1976). A rapid sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry, 72*, 248–252.

Chan, J. K., Gill, T. A., & Paulson, A. T. (1993). Thermal aggregation of myosin subfragments from cod and herring. *Journal of Food Science, 58*, 1057–1061, 1069.

Folk, J. E. (1980). Transglutaminase. *Annual Review of Biochemistry, 49*, 517–531.

Gill, T. A., Chan, J. K., Phonchareon, K. F., & Paulson, A. T. (1992). Effect of salt concentration and temperature on heat-induced aggregation and gelation of fish myosin. *Food Research International, 25*, 333–341.

Huff-Lonergan, E., Parrish, F. C., Jr., & Robson, R. M. (1995). Effect of postmortem aging time, animal age, and sex on degradation of titin and nebulin in bovine longissimus muscle. *Journal of Animal Science, 73*, 1064–1073.

Jeyarajah, S., & Allen, J. C. (1994). Calcium binding and salt-induced structure changes of native and preheated β-lactoglobulin. *Journal of Agricultural and Food Chemistry, 42*, 80–85.

Joseph, D., Lanier, T. C., & Hamann, D. D. (1994). Temperature and pH effects of tranglutaminase-catalyzed "setting" of crude fish actomyosin. *Journal of Food Science, 59*, 1018–1023.

Kamath, G. G., Lanier, T. C., Foegeding, E. A., & Hamann, D. D. (1992). Nondisulfide covalent cross-linking of myosin heavy chain in "setting" of Alaska pollock and Allantic croaker surimi. *Journal of Food Biochemistry, 16*, 151–172.

Klesk, K., Yongsawatdigul, J., Park, J. W., Viratchakul, S., & Virulhakul, P. (2000). Physical behavior of tilapia (*Oreochromis niloticus*) surimi ges at various thermal treatments as compared with Alaska pollock and Pacific whiting surimi. *Journal of Aquatic Food Product Technology, 9*, 91–104.

Lanier, T. C. (2000). Surimi gelation chemistry. In J. W. Park (Ed.), *Surimi and surimi seafood* (pp. 237–265). New York: Marcel Dekker.

Lee, N., & Park, J. W. (1998). Calcium compounds to improve gel functionality of Pacific whiting and Alaska pollock surimi. *Journal of Food Science, 63*, 969–974.

Nakayama, T., Niwa, E., & Hamada, I. (1983). New type of myosin gel induced by salts. *Agriculture and Biological Chemistry, 47*, 227–233.

Nayak, R., Kenney, P. B., & Slider, S. (1996). Protein extractability of turkey breast and thigh muscle with varying sodium chloride solutions as affected by calcium, magnesium and zinc chloride. *Journal of Food Science, 61*, 1149–1154.

Niwa, E. (1992). The chemistry of surimi gelation. In T. C. Lanier, & C. M. Lee (Eds.), *Surimi technology*, (pp. 389–427). New York: Marcel Dekker.

Oakenfull, D., Pearce, J., & Burley, R. W. (1997). Protein gelation. In S. Damodaran, & A. Paraf (Eds.), *Food Proteins and their applications*, (pp. 111–142). New York: Marcel Dekker.

Ogawa, M., Kanamaru, J., Miyashita, H., Tamiya, T., & Tsuchiya, T. (1995). Alpha-helical structure of fish actomyosin: changes during setting. *Journal of Food Science, 60*, 297–299.

Ogawa, M., Nakamura, S., Horimoto, Y., An, H., Tsuchiya, T., & Nakai, S. (1999). Raman spectroscopic study of changes in fish actomyosin during setting. *Journal of Agricultural and Food Chemistry, 47*, 3309–3318.

Park, J. W., Yongsawatdigul, J., & Lin, T. M. (1994). Rheological behavior and potential cross-liking of Pacific whiting surimi. *Journal of Food Sceince, 59*, 773–776.

Price, N. C. (1996). Circular dichroism in protein analysis. In *Encyclopedia of molecular biology and molecular medicine*. New York: Weinheim.

Saeki, H. (1995). Lowering of thermal stability of fish myofibrillar protein induced by $CaCl_2$. *Fisheries Science, 61*, 535.

Taylor, M. A., & Etherington, D. J. (1991). The solubilization of myofibrillar proteins by calcium ions. *Meat Science, 29*, 211–219.

von Hippel, P. H., & Wong, K-Y. (1965). On the conformational stability of globular proteins. The effects of various electrolytes and nonelectrolytes on the thermal ribonuclease transition. *Journal of Biological Chemistry, 240*, 3909–3923.

Wan, J., Kimura, I., Satake, M., & Seki, N. (1994). Effect of calcium ion concentration on the gelling properties and tranglutaminase activity of walleye Pollack surimi paste. *Fisheries Science, 60*, 107–113.

Worratao, A., & Yongsawatdigul, J. (2005). Purification and characterization of transglutaminase from Tropical tilapia (Oreochromis niloticus). *Food Chemistry, 93*, 651–658.

Xiong, Y. L., & Brekke, C. J. (1991). Gelation properties of chicken myofibrils treated with calcium and magnesium chlorides. *Journal of Muscle Foods, 2*, 21–36.

Yongsawatdigul, J., & Park, J. W. (1999). Thermal aggregation and dynamic rheological properties of Pacific whiting and cod myosins as affected by heating rate. *Journal of Food Science, 64*, 679–683.

Yongsawatdigul, J., & Park, J. W. (2003). Thermal denaturation and aggregation of threadfin bream actomyosin. *Food Chemistry, 83*, 409–416.

Yongsawatdigul, J., Worratao, A., & Park, J. W. (2002). Effect of endogenous transglutaminase on threadfin bream surimi galation. *Journal of Food Science, 67*, 3258–3263.