and others 1990). Limited unfolding of myosin at 25 °C would restrict intermolecular entanglement via any interactions, resulting in formation of soluble aggregates, rather than large aggregates.

At 40 °C, myosin molecules underwent partial unfolding due to thermal denaturation. The partial unfolded molecules exposed the previously buried hydrophobic groups to the aqueous environment, which subsequently re-associated via hydrophobic interactions. As a result, large aggregate formation at 40 °C was evident (Figure 3a). Hydrophobic interactions of unfolded molecules would reduce ANS-binding capacity. This explained why S_o ANS at 40 °C was comparable to that at 25 °C in spite of the greater extent of unfolding occurred at 40 °C. Lanier (2000) suggested that hydrophobic interactions participated in gelation during setting. Thus, Ca^{2+} ion induced the unfolding of myosin, which could in turn enhance hydrophobic interactions among myosin molecules during setting.

S_o ANS of actin also increased with $CaCl_2$ concentration and exhibited higher values than those of myosin at all temperature studied (4, 25 and 40 °C) (Figure 4b). This may be partly due to the inactivation of actin by EGTA used in actin extraction (Turoverov and others 1999). The inactivated actin tended to expose hydrophobic clusters on the surface and showed high affinity to hydrophobic probes (Lehrer 1972). Moreover, existence of large hydrophobic groups on the surface led to self-association of actin monomers (Mazhul and others 2003). Thus, the greater extent of aggregation and exposure of surface hydrophobicity was observed in actin. Transformation of native G-actin to inactivated form resulted in partial unfolded structure, which was more prone to

denaturation. Our study showed that Ca^{2+} induced more open structure of inactivated actin, leading to the aggregate formation via hydrophobic interactions.

Effect of $CaCl_2$ on total SH groups of myosin and actin

Total SH groups of myosin and actin in the absence of $CaCl_2$ at 4 °C were ≈ 6×10^{-5} and 5×10^{-5} mole /g protein, respectively. When myosin was incubated at 40 °C for 2 h, total SH groups decreased to ≈ 4.8×10^{-5} mole /g protein as a result of thermal denaturation. In the presence of Ca^{2+} ion, SH groups of myosin incubated at all studied conditions continuously decreased as $CaCl_2$ concentration increased (Figure 5a). A marked decrease in SH group was observed when incubated at 40 °C for 2 h. The similar trend was also observed in actin (Figure 5b). These results indicated that Ca^{2+} induced the formation of disulfide linkages of both myosin and actin when incubated at 40 °C. The unfolding of myosin and actin induced by $CaCl_2$ resulted in an exposure of free SH groups, which subsequently underwent disulfide interchanges. Similar effect of $CaCl_2$ on the formation of disulfide linkages and hydrophobic interactions were also found in α-crystallin molecules (Valle and others 2002). It should be noted that the extent of disulfide bond formation at 40 °C was greater than that at 25 °C (Figure 5a). It was, therefore, speculated that disulfide bond might be partly responsible for aggregation of myosin set at 40 °C. Addition of $CaCl_2$ to fish protein paste induced hydrophobic interactions and disulfide linkages of myosin and actin at 40 °C to a greater extent than at 25 °C. Besides ε-(γ-glutamyl) lysyl isopeptide bonds catalyzed by Ca^{2+}- dependent endogenous TGase, hydrophobic interactions and disulfide linkages could be involved during setting of fish protein.

When Ca^{2+} ion was not added, setting phenomenon at 25 °C was not observed in surimi made from tropical fish (Kamath and others 1992; Klesk and others 2000). The existing explanation was that tropical fish exhibited higher thermal stability that limited the exposure of reactive groups on myosin molecule for TGase catalytic reaction. However, Yongsawatdigul and others (2002) reported the setting of TB surimi at 25 °C when 0.1% $CaCl_2$ (≈ 10 mM) was added. Our study revealed that addition of Ca^{2+} ion (≥10 mM) increased more exposure of hydrophobic amino groups and more disulfide linkages of myosin and actin, which subsequently contributed to setting phenomenon of TB at 25 °C.

Effect of $CaCl_2$ on Ca-ATPase activity of myosin

Ca-ATPase activity of myosin slightly increased and reached the maximum at 50 mM $CaCl_2$ (Table 1). Further increase of $CaCl_2$ concentration dramatically reduced Ca-ATPase activity. Ca-ATPase activity at 200 mM $CaCl_2$ was about 36 % of that at 50 mM $CaCl_2$. High level of $CaCl_2$ (> 50 mM) induced conformational changes of globular head of myosin, resulting in a decrease of Ca-ATPase activity. The exposure of hydrophobic and changes of total SH groups at $CaCl_2$ < 50 mM was likely to occur at myosin rod, while both globular and rod portions underwent such changes at high $CaCl_2$ concentration (> 50 mM).

Binding of Ca^{2+} to anionic sites on protein structure can induce the unfolding. These binding interactions prevent salt exclusion from protein structure and decrease preferential hydration of salts, resulting in salting-in and destabilization of protein structure (Arakawa and Timasheff 1984). Myosin contained negative charges at pH 7

because pI of myosin is around 4.8-6.2 (Stefansson and Hultin 1994). Thus, the ionic interactions between Ca^{2+} and negatively charged myosin might be responsible for disturbance of native myosin molecules. Ca^{2+} also induced aggregation of β-lactoglobulin (theoretical net charge, Z = -8) by selective binding to carboxylated anions (Simons and others 2002). For myosin, most negative charges are located at myosin rod (Z = - 34 to -52) and followed by myosin light chains (Z = -6 to -27), while globular head myosin has positive charges (Z = 6 to 16) (Bechet and Albis 1989). Therefore, Ca^{2+} was more likely to bind to myosin rod than the globular head. For this reason, the rod portion was more susceptible to conformational changes induced by Ca^{2+} ion.

Effect of $CaCl_2$ on CD spectra of myosin and actin

CD spectra in far UV region of myosin showed predominant α-helix structure (Figure 6a). $CaCl_2$ promoted the loss of secondary structure of both myosin and actin even at 4 °C (Figure 6a,b). The helical content of myosin at 4 °C without $CaCl_2$ was 71.2% and decreased to 51.4% in the presence of 100 mM $CaCl_2$ (Figure 7a). The helical content of myosin incubated at 25 °C for 4 h was slightly decreased with increasing $CaCl_2$ concentration. In contrast, $CaCl_2$ markedly decreased helical content of myosin incubated at 40 °C for 2 h (Figure 7a). Both thermal energy and $CaCl_2$ synergistically contributed to unfolding of myosin at 40 °C, leading to considerable loss of helical structure. Ogawa and others (1995) reported that loss of helical structure of fish actomyosin was a pre-requisite to initiate setting. Therefore, addition of $CaCl_2$ accompanied by incubating at 40 °C enhanced myosin unfolding, which subsequently

resulted in a higher degree of hydrophobic interactions and formation of disulfide linkages.

Low helical content (28.73 %) was observed in actin at 4 °C (Figure 7b). Nagy et al. (1972) reported that actin contained 30% α-helix structure, 10% of β-sheet and the remaining residues did not appear to contribute to the optical activity (1972). α-Helical content of actin decreased when incubated at 25 °C in the presence of 10 mM $CaCl_2$. However, further increase of $CaCl_2$ from 30 to 100 mM did not further decrease helical content of actin. Moreover, α-helical structure of actin incubated at 40 °C for 2 h was completely destroyed at10 mM $CaCl_2$. These results suggested that $CaCl_2$ at ≥10 mM also induced the changes of secondary structure of actin.

Conclusions

Ca^{2+} ion induced conformational changes of TB myosin and actin leading to partial unfolding and exposure of hydrophobic amino acids. The unfolded molecules subsequently aggregated via hydrophobic interactions and disulfide linkages when incubated at either 25 or 40 °C. Such interactions could be important in gel-forming of TB during setting. Thus, $CaCl_2$ did not only enhance gelling properties of TB myosin through activating endogenous TGase but also directly induce conformational changes of myosin and actin, promoting hydrophobic interactions and disulfide linkages of "set" gel.

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Figure legends

Figure 1. SDS-PAGE patterns of TB myosin and actin. S= molecular weight standard, MI= TB mince, MY_1 and MY_2 = myosin from lot 1 and lot 2, respectively. AC_1 and AC_2 = actin from lot 1 and lot 2, respectively. MHC= myosin heavy chain, LC = myosin light chains.

Figure 2. Effect of $CaCl_2$ on turbidity of TB myosin and actin incubated at either 25 or 40 °C in 0.6 M NaCl, 20 mM Tris-maleate, pH 7.0: myosin at 25 °C (a), myosin at 40 °C (b), actin at 25 °C (c), and actin at 40 °C (d).

Figure 3. Remaining protein contents of TB myosin (a) and actin (b) at varied $CaCl_2$ concentration after centrifugation at 78,000 ×g for 1 h.

Figure 4. Effect of $CaCl_2$ on the changes in S_o ANS of TB myosin (a) and actin (b) in 0.6 M NaCl, 20 mM Tris-maleate, pH 7.0.

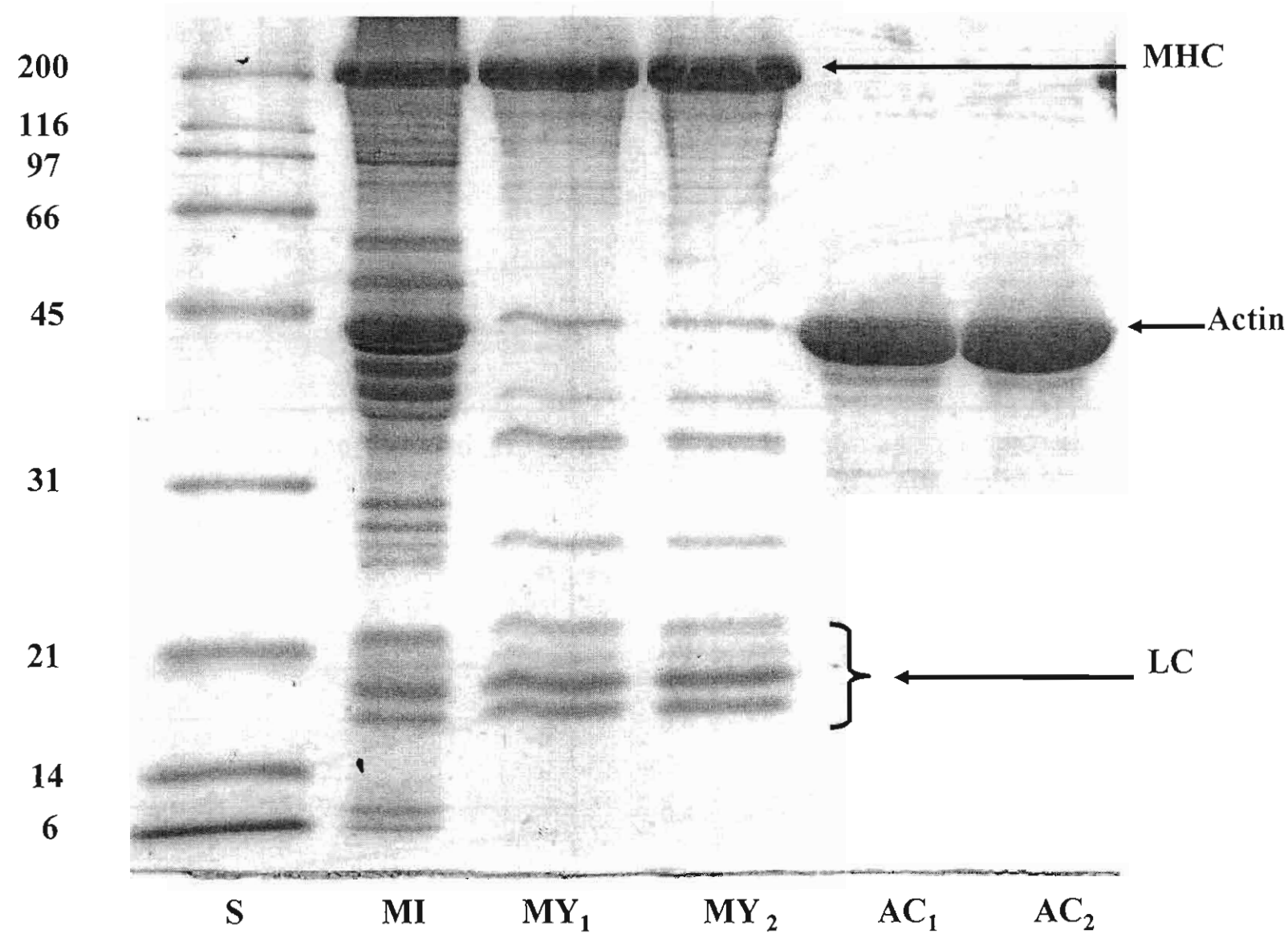
Figure 5 Effect of $CaCl_2$ on the changes in total SH groups of TB myosin (a) and actin (b) in 0.6 M NaCl, 20 mM Tris-maleate, pH 7.0.

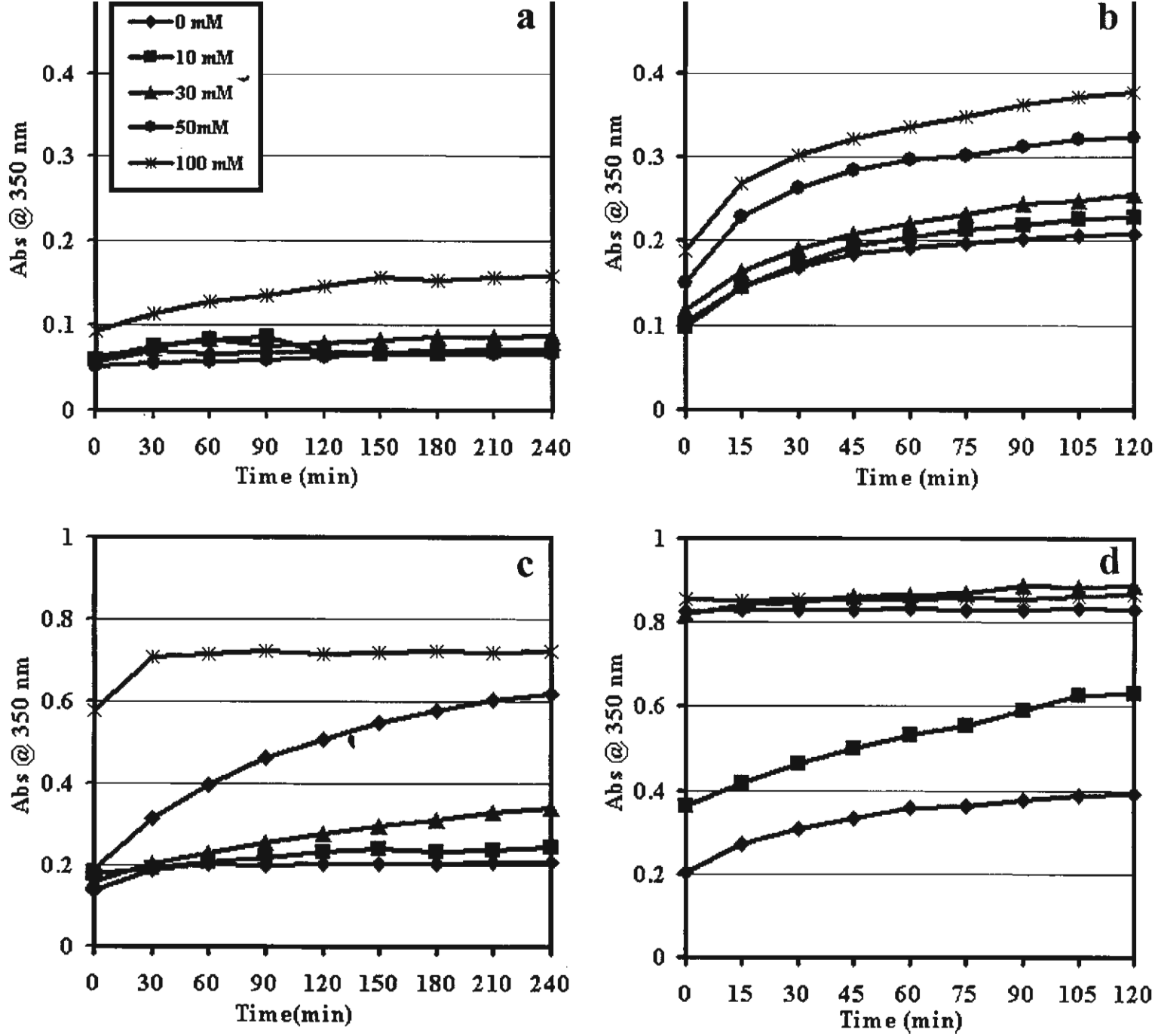
Figure 6 Effect of $CaCl_2$ on CD spectra of TB myosin (a) and actin (b) in 0.6 M NaCl, 20 mM Tris-HCl, pH 7.0 at 4 °C.

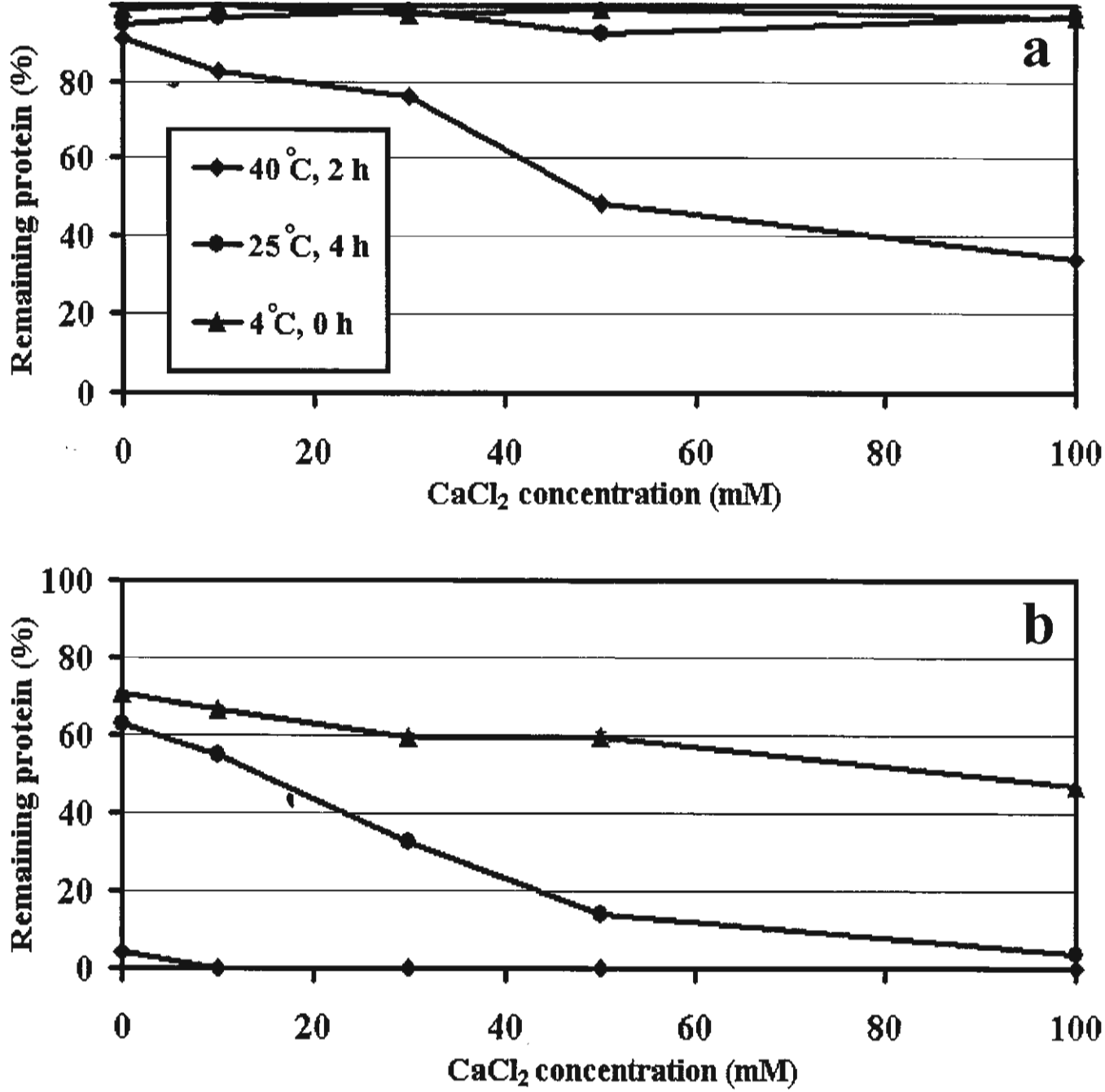
Figure 7 Effect of $CaCl_2$ on the changes in α-helical contents of TB myosin (a), and actin (b).

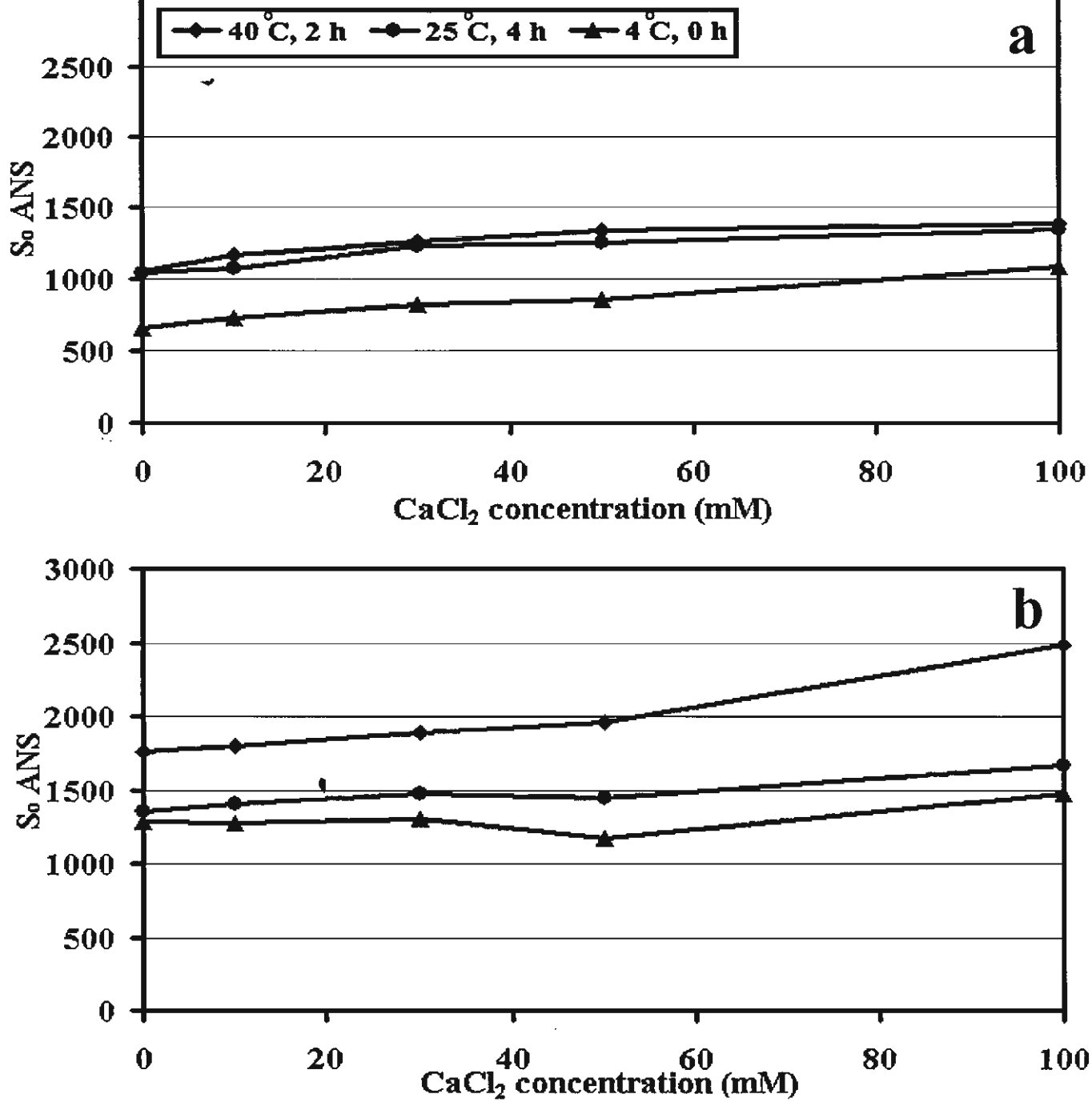
Table 1. Ca -ATPase activity of TB myosin determined at various $CaCl_2$ concentrations at 25 °C.

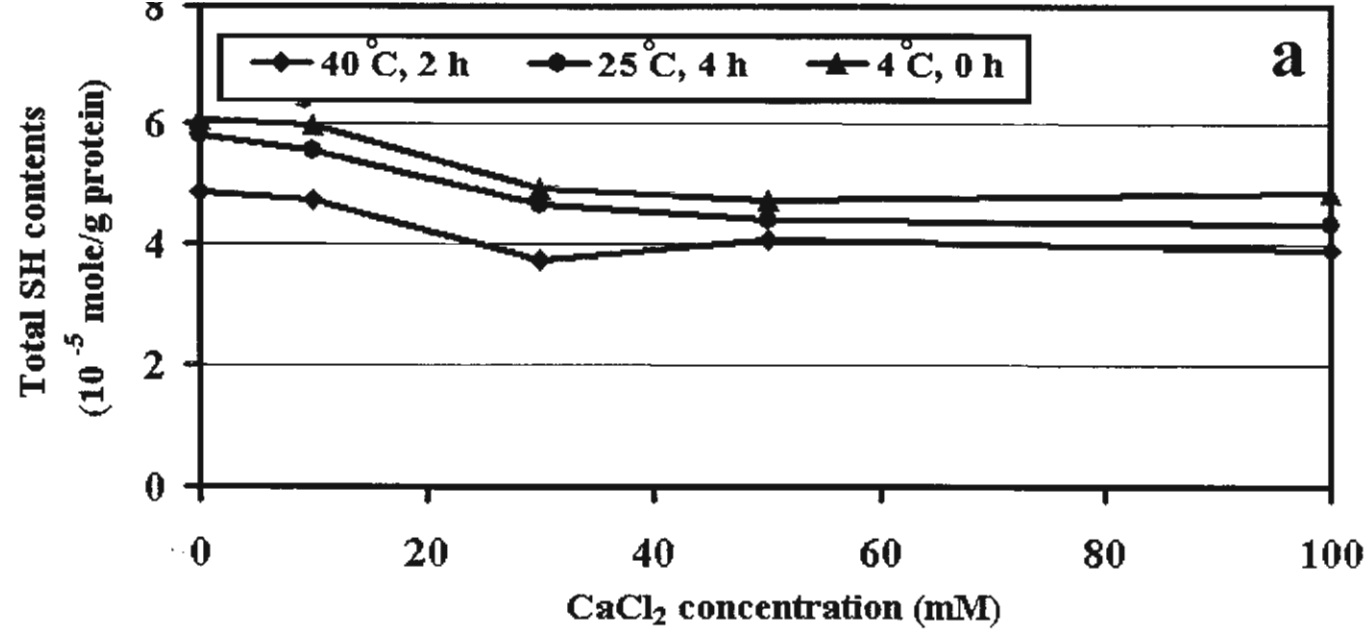
$CaCl_2$ concentration (mM)	Ca-ATPase activity (μ mole Pi/mg protein/min)
0	0
3.3	0.274 ± 0.025
10	0.297 ± 0.013
30	0.313 ± 0.023
50	0.299 ± 0.010
100	0.229 ± 0.011
150	0.185 ± 0.020
200	0.106 ± 0.019

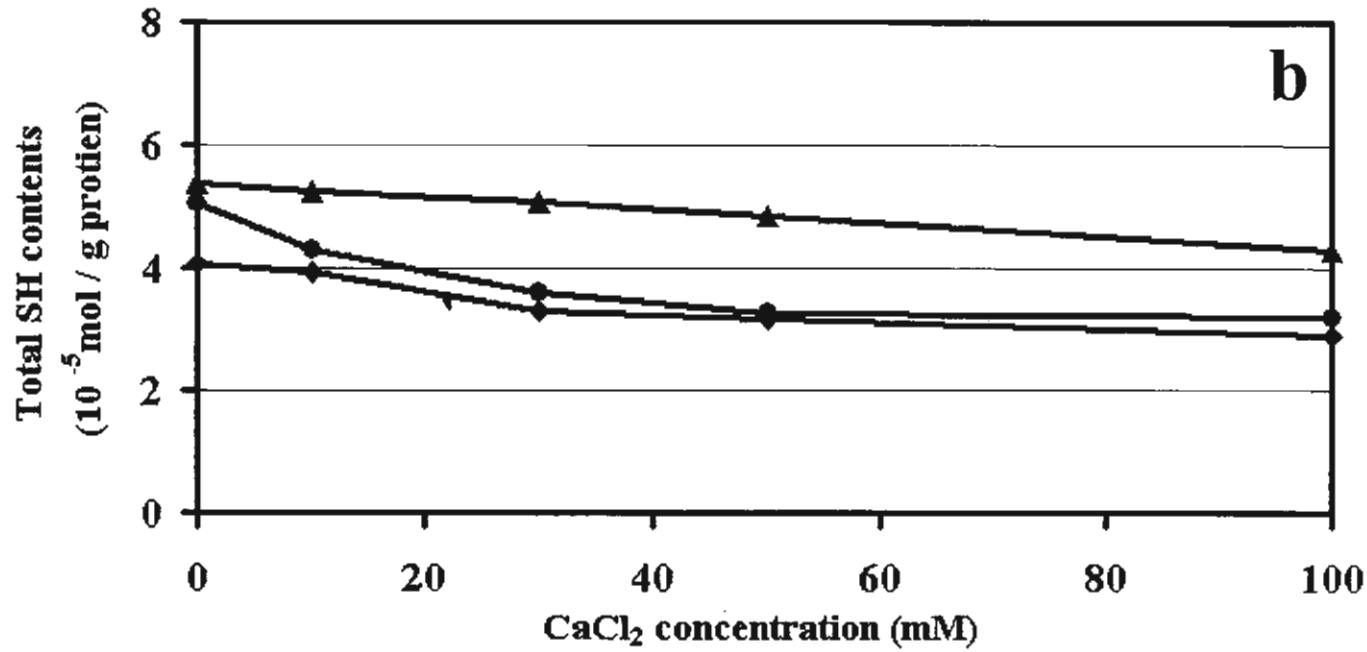

200
116
97
66
45
31
21
14
6
MHC
Actin
LC
S
MI
MY1
MY2
AC1
AC2

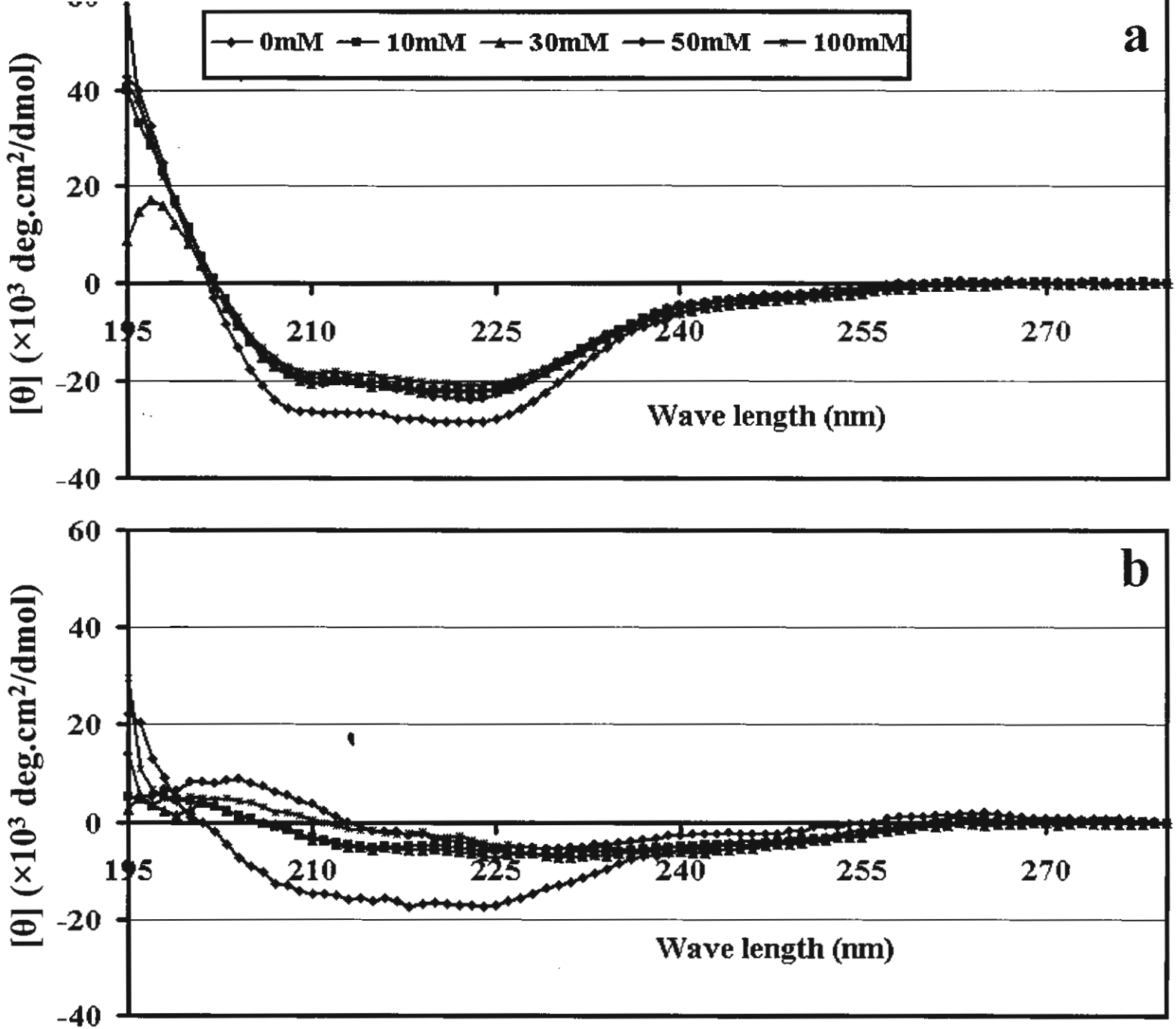

a
0 mM
10 mM
30 mM
50mM
100 mM
Abs @ 350 nm
Time (min)
b
Abs @ 350 nm
Time (min)
c
Abs @ 350 nm
Time(min)
d
Abs @ 350 nm
Time (min)

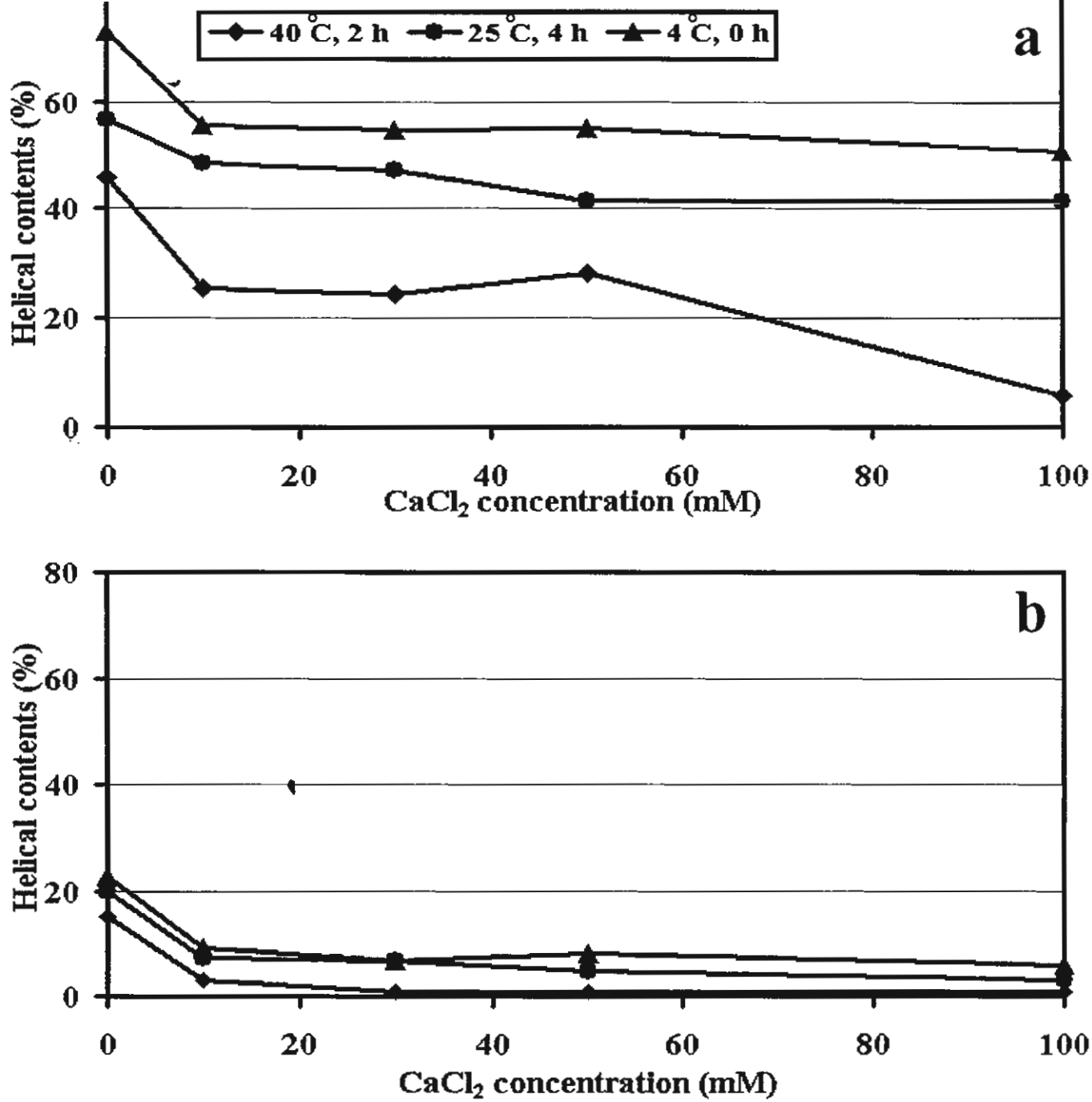

a
Remaining protein (%)
0
20
40
60
80
40 °C, 2 h
25 °C, 4 h
4 °C, 0 h
0
20
40
60
80
100
CaCl2 concentration (mM)
b
Remaining protein (%)
0
20
40
60
80
100
0
20
40
60
80
100
CaCl2 concentration (mM)


40 °C, 2 h
25 °C, 4 h
4 °C, 0 h
a
S₀ ANS
0
500
1000
1500
2000
2500
0
20
40
60
80
100
CaCl₂ concentration (mM)
b
S₀ ANS
0
500
1000
1500
2000
2500
3000
0
20
40
60
80
100
CaCl₂ concentration (mM)


40 °C, 2 h
25 °C, 4 h
4 °C, 0 h
a
Total SH contents
(10 -5 mole/g protein)
0
2
4
6
0
20
40
60
80
100
CaCl2 concentration (mM)


b
Total SH contents
(10 -5 mol / g protien)
0
2
4
6
8
0
20
40
60
80
100
CaCl2 concentration (mM)


0mM
10mM
30mM
50mM
100mM
a
[θ] (×10³ deg.cm²/dmol)
60
40
20
0
-20
-40
195
210
225
240
255
270
Wave length (nm)
b
[θ] (×10³ deg.cm²/dmol)
60
40
20
0
-20
-40
195
210
225
240
255
270
Wave length (nm)


40 °C, 2 h
25 °C, 4 h
4 °C, 0 h
a
Helical contents (%)
0
20
40
60
0
20
40
60
80
100
CaCl2 concentration (mM)
b
Helical contents (%)
0
20
40
60
80
0
20
40
60
80
100
CaCl2 concentration (mM)

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Aggregation and Conformational Changes of Tilapia Actomyosin as Affected by Calcium Ion during Setting

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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 pheylmethnesulfonyl 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 $CaCl_2$.

Key words: 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 °C 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 changes of fish actomyosin in addition to being endogenous TGase activator. However, the role of Ca^{2+} ion pertaining conformation of fish actomyosin molecule 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 & Vissesanguan, 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 (*Oreochromis 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 (*Oreochromis niloticus*) were obtained from the Suranaree University of Technology Farm. Fish were kept in ice and transported to the laboratory within 10 min after catch. All procedure 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 homoginized. The homogenate was centrifuged at 10,000· g for 5 min (RC 28S; Sorvall Co., Newtown, Conn., 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 myofibrilar protein was filtered through three-layers 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 in stead.

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, Calif., 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 the Huff-Lonergan, Parrish, & Robson (1995). Bondings 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 (S_0-ANS)

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 ∝L of 8 mM ANS in 0.1 M imidazole (pH 7.0) and kept under dark for 10 min. Fluorescence intensity was measured by 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 with percentage of protein concentration. Slope was calculated as S_0-ANS value. The relative fluorescence was defined:

$$R = (F-F_o)/F_o$$

Where F and F_o are fluorescence intensity of sample containing ANS and ANS solution, respectively.

2.7. Circular Dichroism (CD)

Changes in secondary structure were monitored using a spectropolarimeter (PS150J; JASCO, Tokyo, Japan) connected with a circulating cooling bath set at 4 °C 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 solution from 260-195 nm in a 0.2 mm quartz cells. 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, Kanamuru, Miyashita, Tamiya, & 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/g) contained final concentration 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, means values were obtained from a least 5 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 Duncan Multiple Range Test (DMRT) at $p<0.05$. In gel texture studies, a split plot design was applied. The 8 levels of Ca^{2+} concentration (0, 10, 20, 30, 40, 50, 70, and 100 mM) were assigned as a main plot factor and the 3 heating treatments (4°C/12h, 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 & 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 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. 3a). Less intensity of HMP was observed in unheated samples. Intensity of HMP also increased with Ca^{2+} concentration (Fig. 3b,c). More distinct HMP bands were observed at 50-100 mM Ca^{2+} (Fig. 3c), 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). Cross-linked myosin heavy chain of pollock surimi increased with added Ca^{2+} concentration (Wan, Kimura, Satake, & Seki, 1994). Cross-linking of myosin heavy chain of threadfin bream surimi was observed when incubated at 25 and 40 °C in the presence of 0.1-0.3% Ca^{2+} (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.

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. 6a). These results corresponded with low turbidity observed in the sample without added Ca^{2+} (Fig. 1). HMP bands disappeared when samples were solubilized in 3-10% SDS and 5%SDS+β-ME (Fig. 6a). In contrast, HMP formed in the presence of 100 mM Ca^{2+} remained after solubilizing in these buffers (Fig. 6b). 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 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 3 washing cycles. Due to low residual TGase activity in tilapia actomyosin, formation of HMP was more evident at higher Ca^{2+} concentration (Fig. 3b,c).

3.3. Surface hydrophobicity (S_0-ANS)

S_0-ANS of tilapia actomyosin without added Ca^{2+} (control) increased with incubation time at 40 °C (Fig 7a). Addition of 10 to 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 & Paulsen (1993) also reported that thermal aggregation ability of fish myosin linearly increased with surface hydrophobicity. Thus, Ca^{2+} did not only activated 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 dichroisms (CD)

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. 8a). In addition, α-helical content of actomyosin incubated at 4 °C for 24 h was comparable to that without incubation (4 °C/0 h) at all studied $CaCl_2$ concentrations (Fig. 8b). 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 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 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 4 °C (Fig. 8b), indicating the subtle change in α-helix of tilapia actomyosin at 40 °C. Ogawa et al. (1999) also reported that α-helical structure of tilapia actomyosin exhibited high thermal stability at 40 °C. α-Helical content markedly decreased with increased Ca^{2+} concentrations (Fig. 8b). 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 ($p<0.05$) (Fig. 9a,b). The control (no added Ca^{2+}) heated to 90 °C without pre-incubation did not form gel, but addition of Ca^{2+} resulted in gelation. 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 ($p<0.05$) (Fig. 9a,b). Ca^{2+} ion activated endogenous TGase activity, enhancing the formation of ε-(γ-glutamyl) lysine crosslinks 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 crosslinks 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 crosslinks. 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 crosslinks 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.

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Figure captions

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.

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% acylamide) 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% acylamide) 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% acylamide) 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.

Fig. 6 SDS-PAGE patterns (5% acylamide) 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).

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).

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

Figure No: Fig. 1

Legend: Turbidity changes of tilapia actomyosin solution

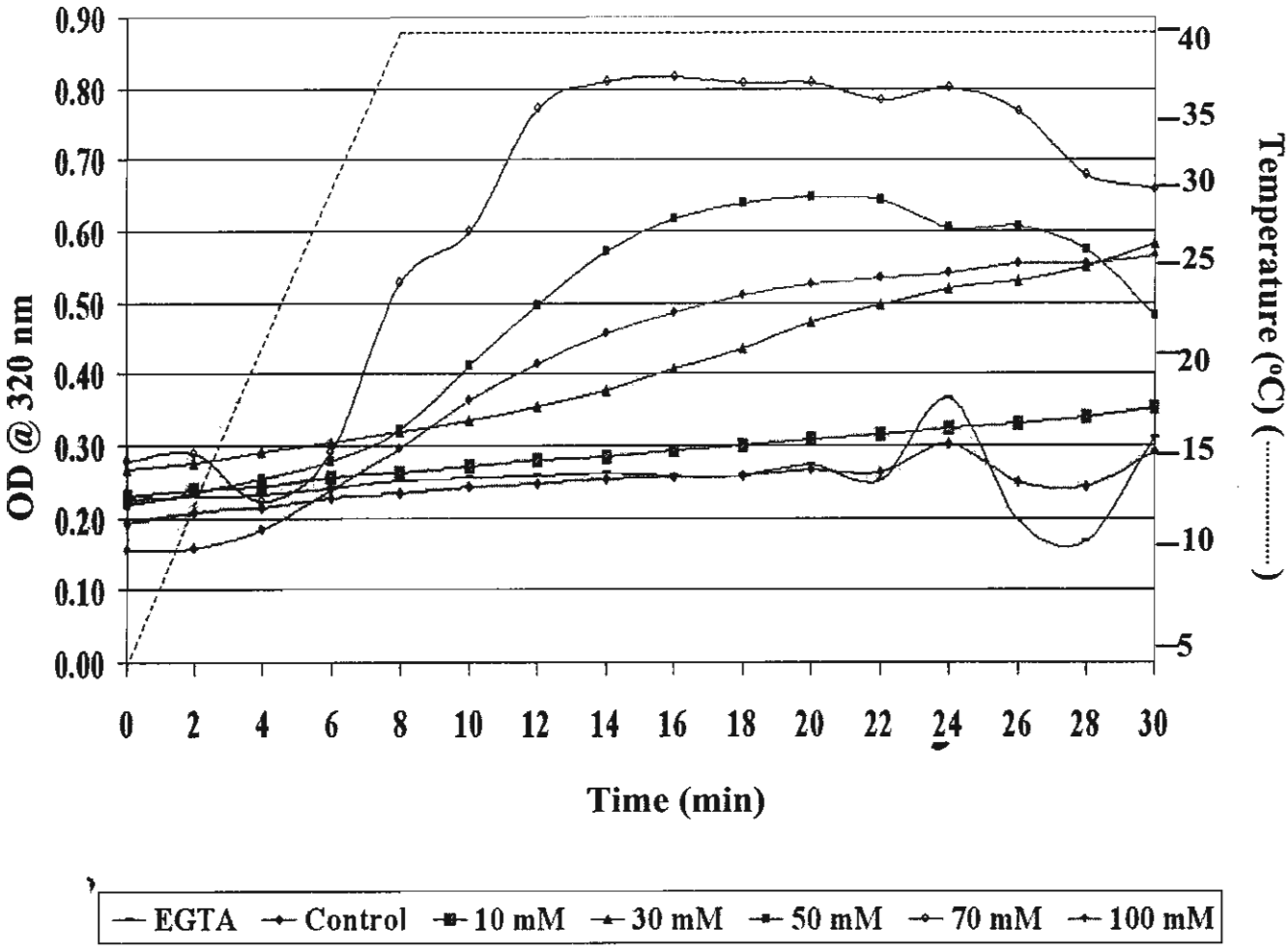


Figure No: Fig 2

myosin ...

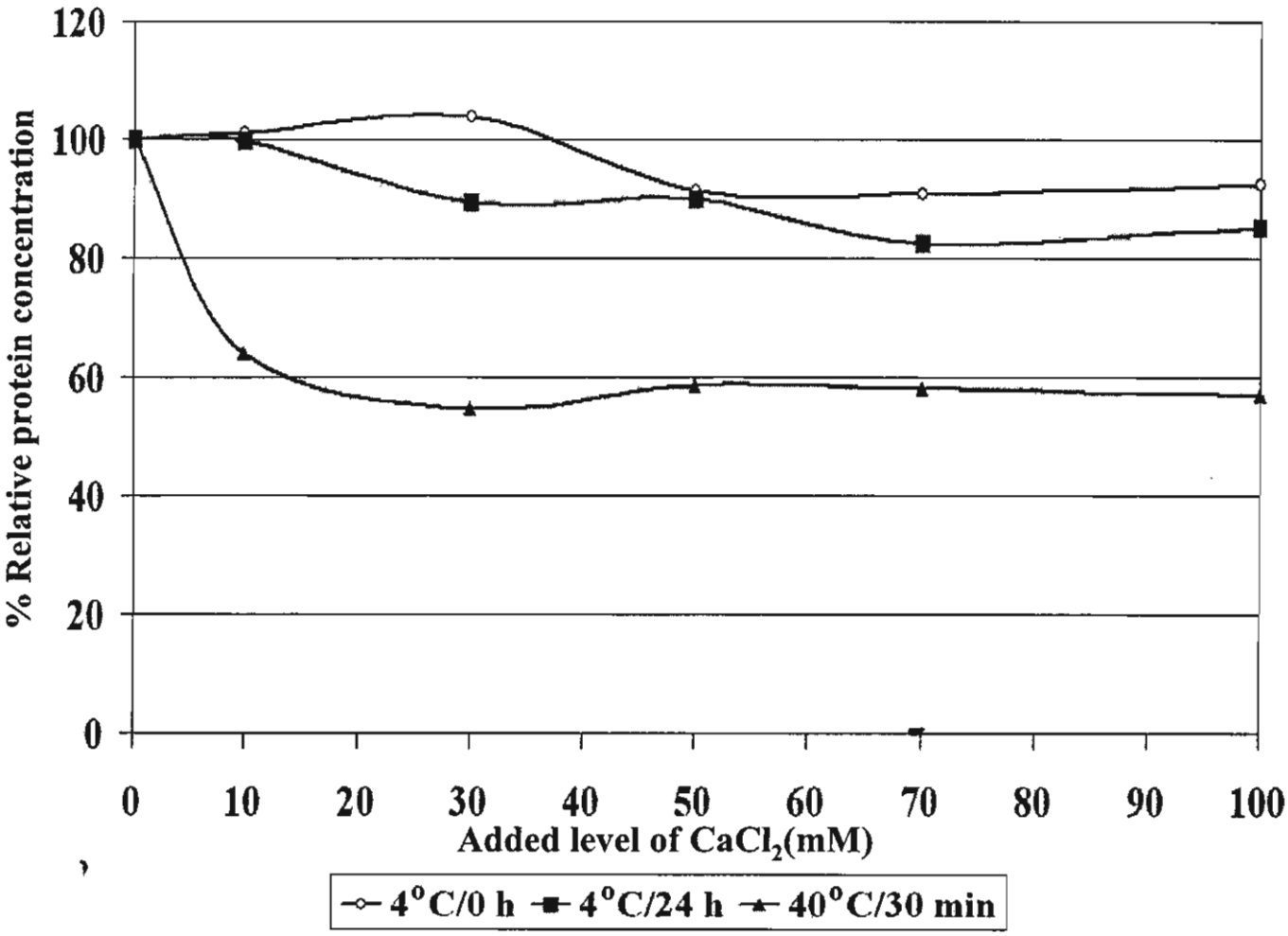


Figure No: Fig 8

at 4ýaC (a) and changes of ýý-helical content of actomyosin at various CaCl2 concentrations (b)....

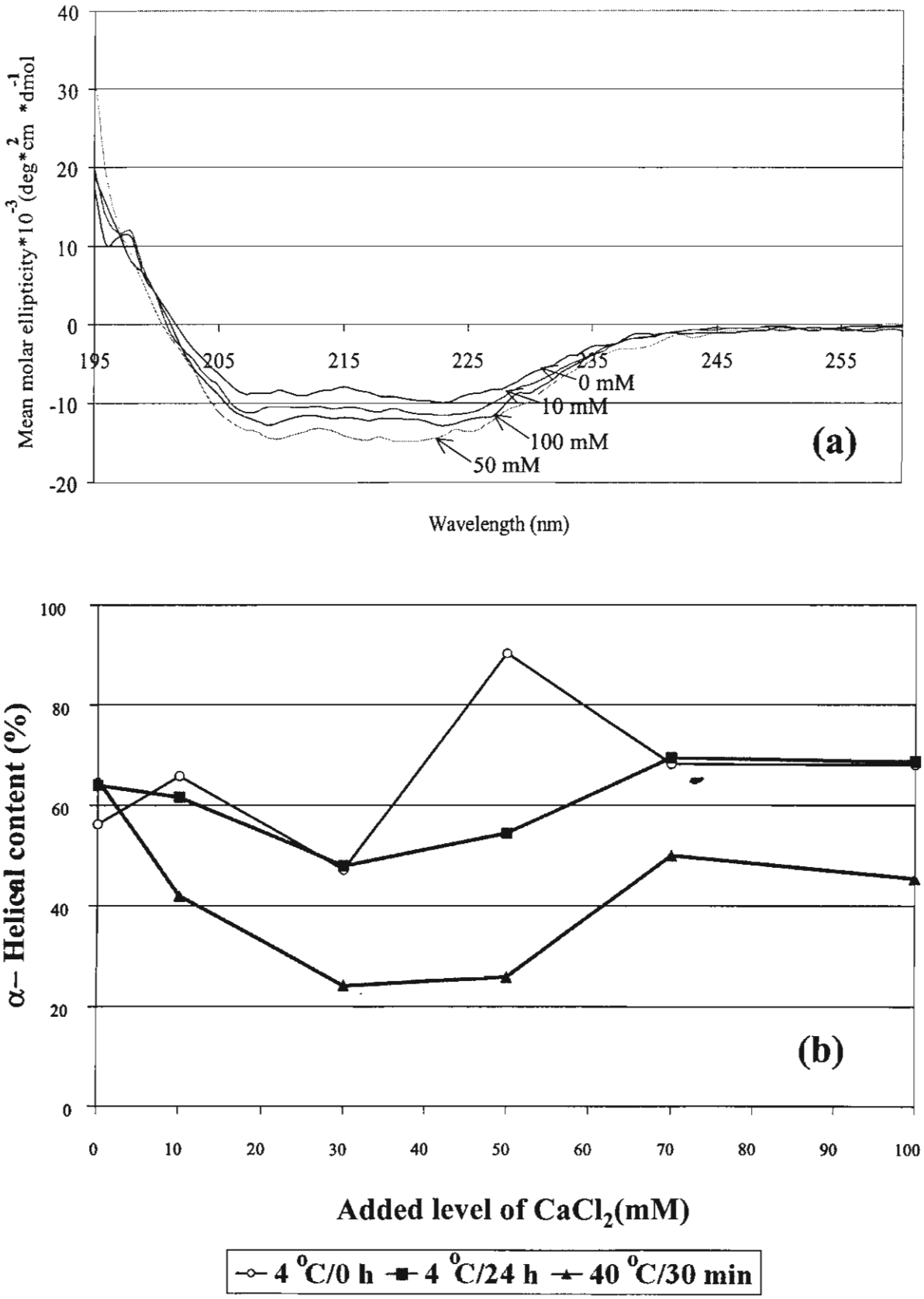
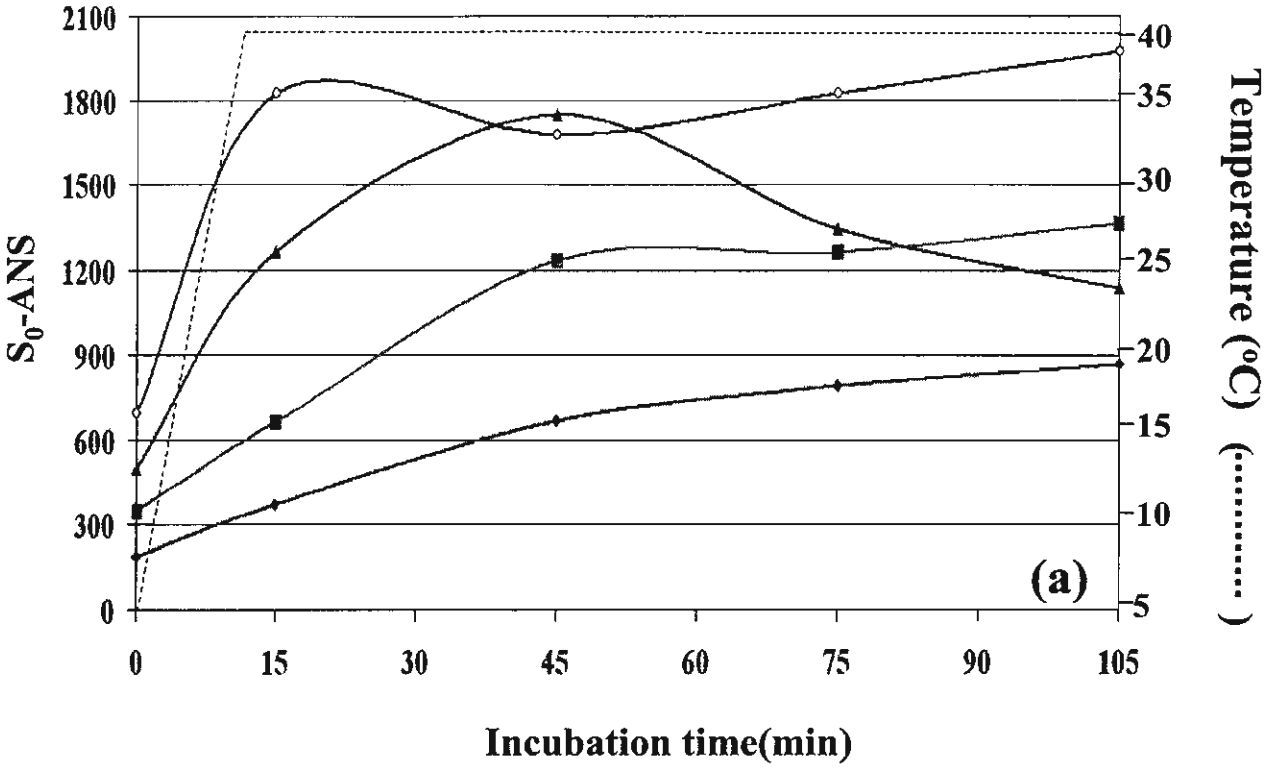
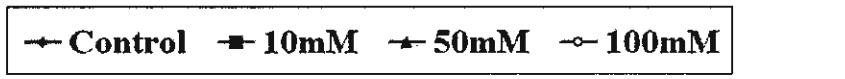


Figure No: Fig. 7

incubated at 40ýaC (a) and 4ýaC (b)....





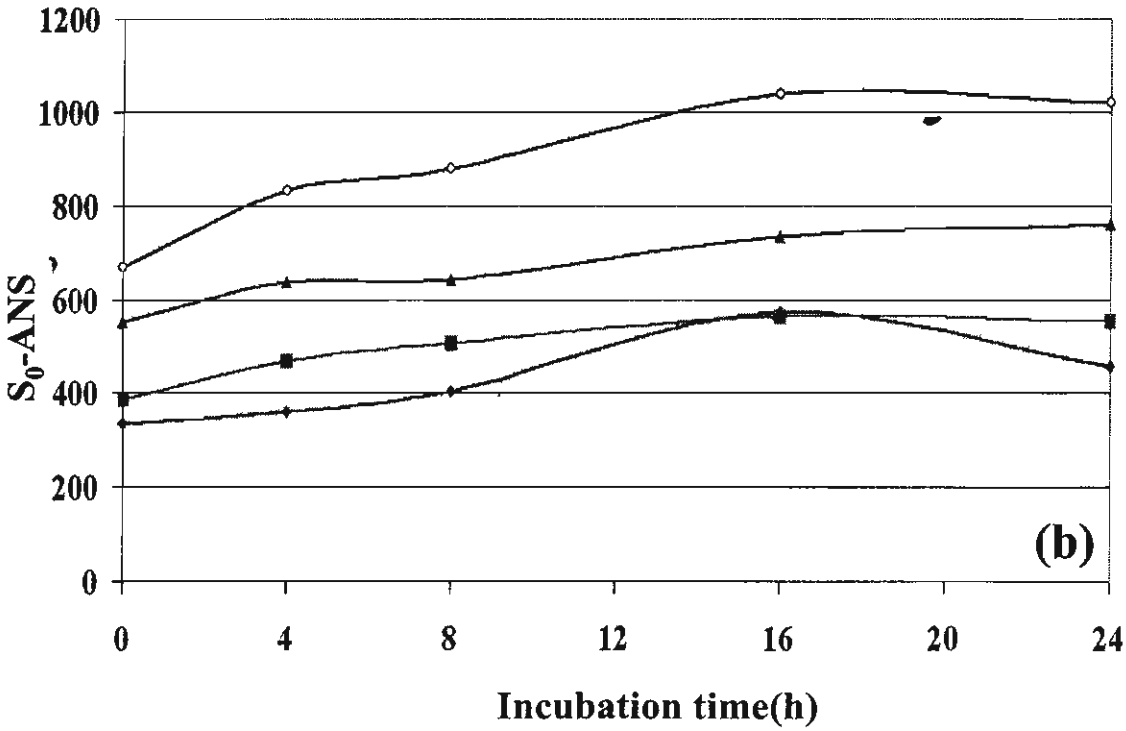
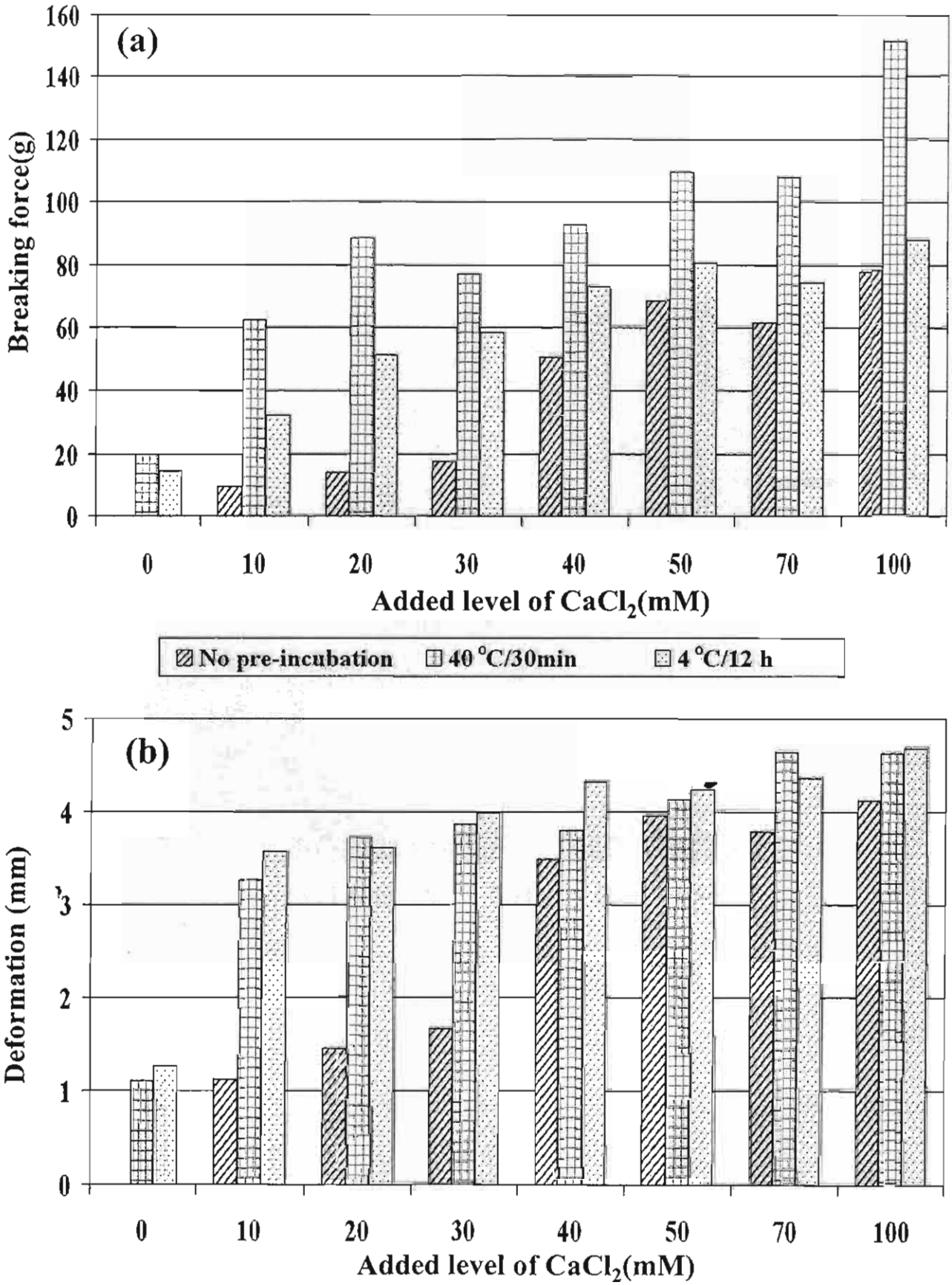
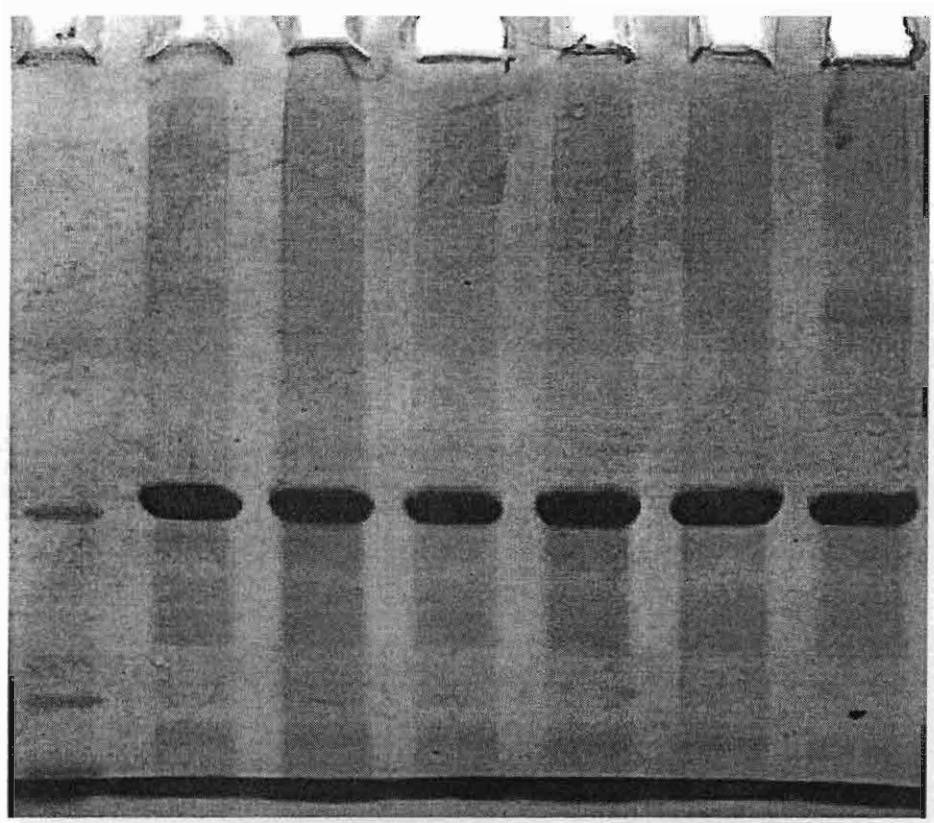


Figure No: Fig. 9

) and deformation (b) on actomyosin gels. ...



(a)



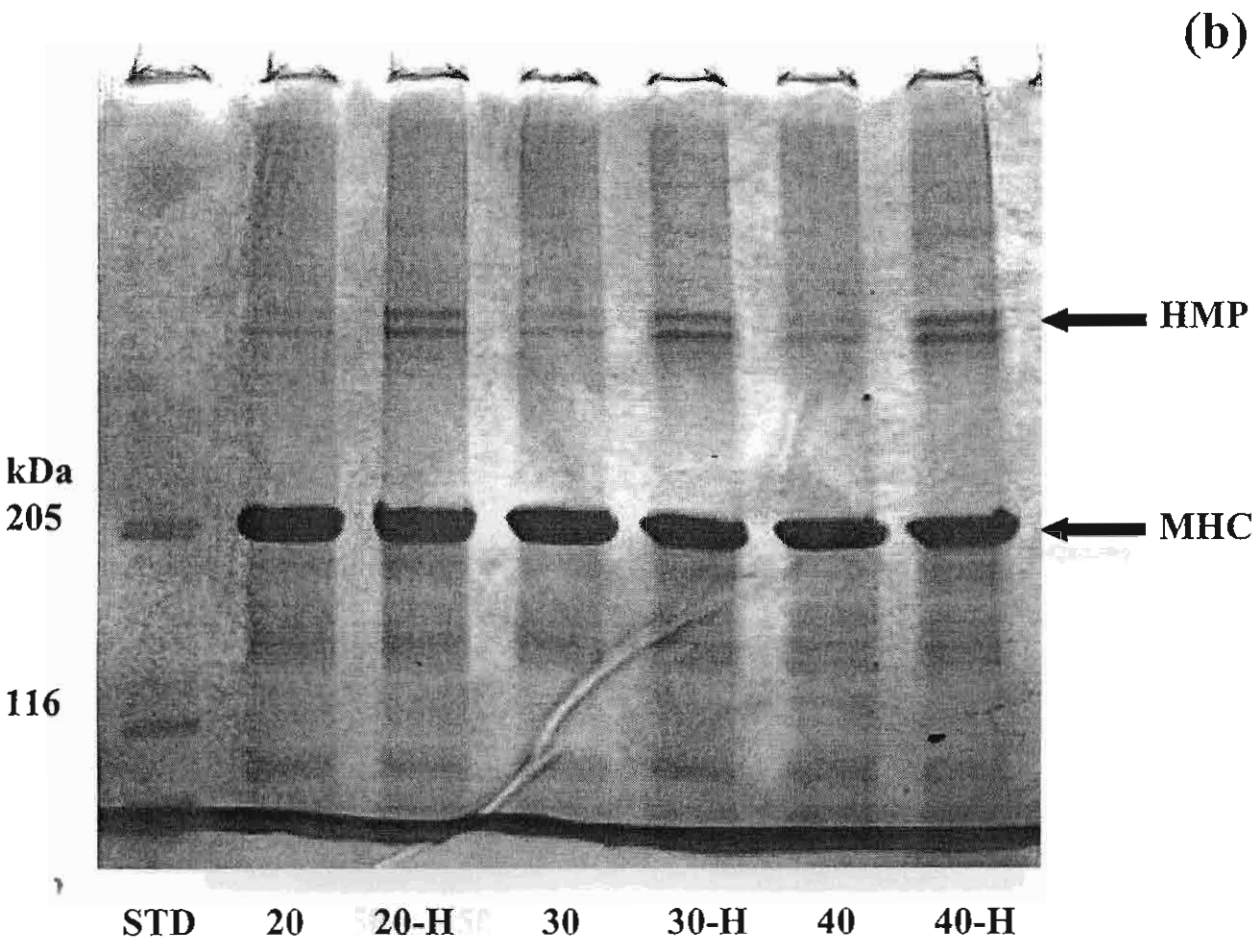

(b)
HMP
MHC
kDa
205
116
STD
20
20-H
30
30-H
40
40-H

Figure No: Fig. 3

Legend: SDS-PAGE patterns (5% acylamide) of tilapia actomyosin

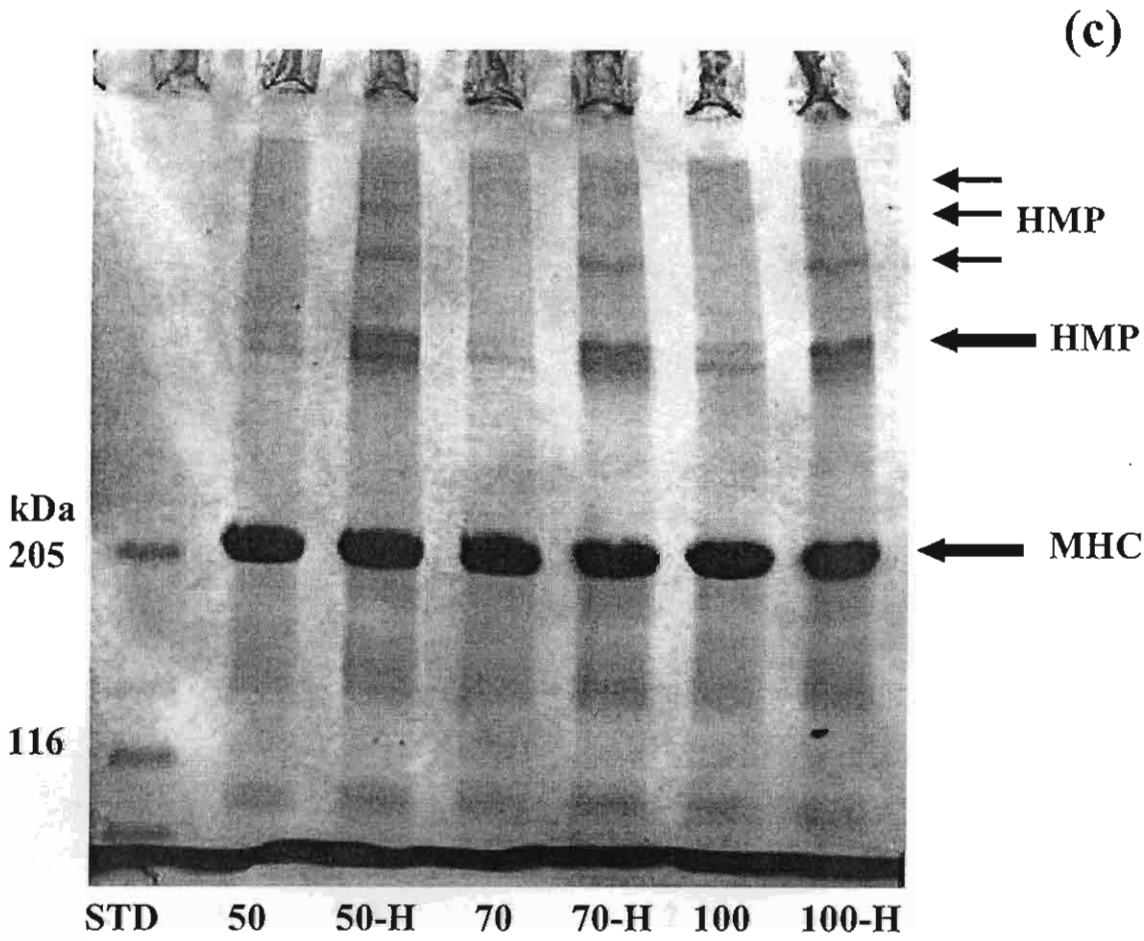
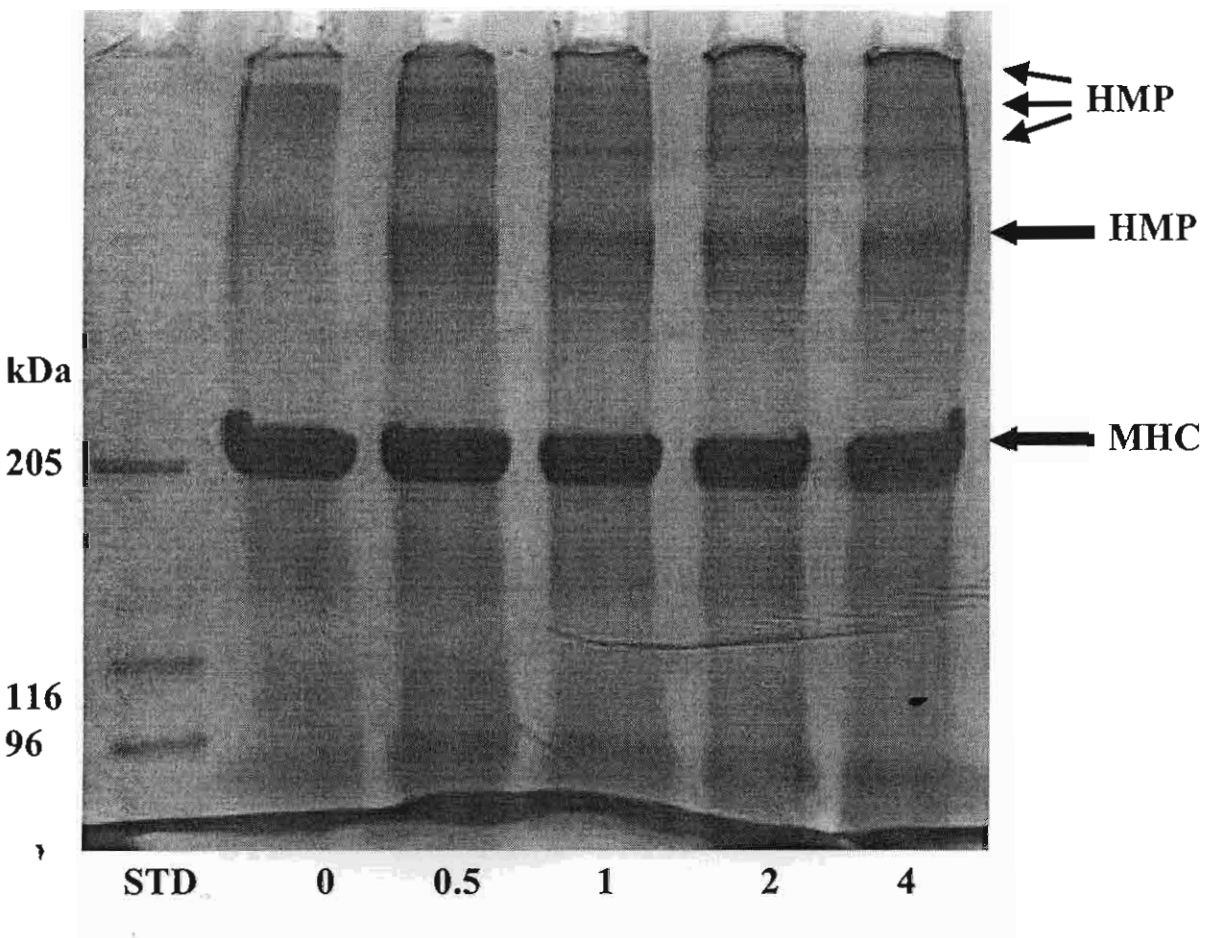


Figure No: Fig. 4

CaCl2 ...



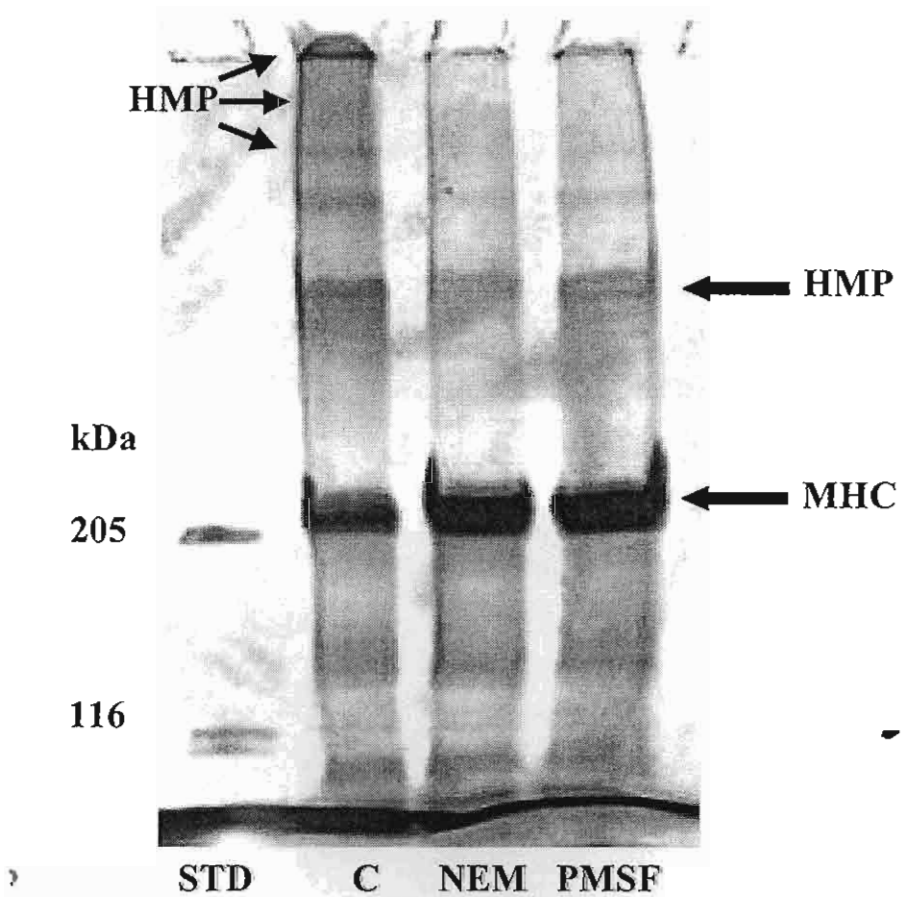

HMP
HMP
MHC
kDa
205
116
STD
C
NEM
PMSF

Figure No: Fig. 6

I2 (a) and with 100 mM CaCl2 (b), ...