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Thermal stability of fish natural actomyosin affects reactivity to cross-linking by microbial and fish transglutaminases

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ABSTRACT

Natural actomyosin (NAM) from Pacific whiting (PW) showed thermal transition temperatures by circular dichroism at 31.8 and 43.1 °C, which were lower than those of threadfin bream (TB) NAM, 35.0 and 49.3 °C. Endothermic transitions of PW-NAM by differential scanning calorimetry were at 31.8, 42.1 and 75.3 °C, compared to 36.1, 50.9 and 78.4 °C for TB-NAM. Based on surface hydrophobicity, α-helical content, and solubility, PW-NAM unfolded to a greater extent than did TB-NAM when incubated at 25 °C for 4 h and 40 °C for 2 h, suggesting its lower thermal stability. Transglutaminase generally catalyzed more extensive cross-linking of PW-myosin heavy chain (MHC) than TB-MHC, and the MHC cross-linking mediated by microbial transglutaminase (MTG) was greater than by fish transglutaminase (FTG). Textural properties of PW-NAM gels increased approximately 3.6–6.1-fold and 1.3–1.5-fold in the presence of MTG and FTG, respectively.

1. Introduction

Natural actomyosin (NAM) is a major component responsible for gelation of muscle proteins. Pre-incubation of fish NAM in the presence of salt under the appropriate conditions prior to heating results in formation of a stronger gel. This process is typically known as "setting". One of the important mechanisms involved in the setting phenomenon is the formation of inter- and intramolecular isopeptide bonds of NAM catalyzed by transglutaminases (TGases) (Lee, Lanier, Hamann, & Knopp, 1997). Thermal unfolding of NAM has been reported to be a pre-requisite for setting (Ogawa, Kanamaru, Miyashita, Tamiya, & Tsuchiya, 1995). Kishi, Nozawa, and Seki (1991) observed that the reactivity of TGases toward NAM depends on the amount of reactive residues, which in turn depends on conformational changes of NAM.

The stability of NAM has been reported to be closely related to habitat temperatures. Muscle proteins extracted from fish living in tropical habitats, including tilapia, threadfin bream, and bigeye snapper, unfold at relatively high temperature and they can be set at about 40 °C (Esturk, Park, & Thawornchinsombut, 2004; Howell, Matthews, & Donnelly,1991). In contrast, low setting temperatures of 4–25 °C are usually applied for muscle proteins from fish living in cold and temperate water habitats and exhibiting lower thermal stability, such as Pacific whiting, Alaska pollock, and Atlantic croaker (Kamath, Lanier, Foegeding, & Hamann, 1992). Thermal denaturation of NAM from fish living at different habitats could thus affect the reactivity of TGases.

TGases catalyze an acyl-transfer reaction in which γ-carboxamide groups of peptide-bound glutaminyl residues serve as acyl donors, while lysine residues of proteins may act as acyl acceptors, resulting in ε-(γ-glutamyl) lysine cross-links. TGase can be classified into Ca^{2+}-dependent and Ca^{2+}-independent types. Ca^{2+}-dependent TGases have been found in several tissues, including blood, plasma, and liver (Folk & Finlayson, 1977). Endogenous TGase from fish muscle proteins (Yongsawatdigul, Worratao, & Park, 2002) as well as liver TGase from red sea bream (Yasueda, Kumazawa, & Motoki, 1994), walleye pollock (Kumazawa, Nakanishi, Yasueda, & Motoki, 1996), and threadfin bream (TB) (Hemung & Yongsawatdigul, 2008) have been reported to catalyze cross-linking and polymerization of fish myosin heavy chain (MHC), which is important for setting of surimi. On the other hand, Ca^{2+}-independent TGases, isolated from *Streptoverticillium mobaraense* (Ando et al., 1989), are known as microbial TGase (MTG) and have also been applied to increase the textural properties of surimi from some fish species (Jiang, Hsieh, Ho, & Chung, 2000). However, MTG exhibits different characteristics from Ca^{2+}-dependent TGases, including molecular weight, crystal structure, active site arrangement, catalytic mechanism, and substrate requirement (Ohtsuka, Ota, Nio, & Motoki, 2000). Cross-linking reactivity of MTG during setting of NAM may also be different from that of Ca^{2+}-dependent TGase and

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should be elucidated. However, the catalytic reactivity of microbial and fish TGases on substrates with different thermal stability has not been systematically compared. Understanding reactivity of TGases towards NAM would lead to a means for improving textural properties of fish proteins.

Therefore, the objectives of this research were to compare the thermal stability of NAM extracted from TB and Pacific whiting (PW), and to investigate the potential differences in the cross-linking of NAM from these two species catalyzed by fish (threadfin bream liver) TGase (FTG) and MTG. TB and PW were selected as representatives of tropical and temperate fish species, respectively, which are important for surimi production in Southeast Asia and North America, respectively. The results of this study could lead to better understanding of the reactivity of different TGases on setting phenomena and provide critical information for setting of fish proteins from different habitats.

2. Materials and methods

2.1. Materials

PW *(Merluccius productus)* were caught on July 15th and 25th, 2006 off the coast of Vancouver Island, British Columbia, Canada (near 48.5 °N and 124.7–125.5 °W). Fish were packed with ice in polystyrene foam boxes and transported to the Food, Nutrition and Health Laboratory at the University of British Columbia, Vancouver. Tissue from the nape of the fish was removed to evaluate the level of infection by *Kudoa paniformis* and *K. thyrsites* (Samanarayaka, Ho, & Li-Chan, 2006). The fish with undetectable infection were vacuum-packed and kept at −20 °C until analyzed. TB (*Nemipterus* sp.) were caught in the South China Sea and kept frozen at −20 °C until use.

Monodansylcadaverine (MDC), sodium dodecyl sulfate (SDS), *N*,*N*′-dimethylated casein (DMC), and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma Chemicals (St. Louis, MO, USA). Dithiothreitol (DTT) was obtained from ICN Biomedicals (Aurora, Ohio, USA). Bicinchoninic acid (BCA) protein assay kit and bovine serum albumin (BSA) were purchased from Pierce (Rockford, IL, USA). The fluorescence probe, 6-propionyl-2-dimethylaminonaphthalene (PRODAN), was obtained from Molecular Probes (Eugene, OR, USA). Other chemicals and reagents used were of analytical grade.

2.2. NAM preparation

NAM from both TB and PW was prepared according to Yongsawatdigul and Park (2003) with slight modifications. The extraction was carried out at 0–4 °C to minimize protease activity. Fish muscle (10 g) was homogenized using an IKA homogenizer (Ultra Turrax T25 Basic Labortechnik, IKA Works, Inc., Wilmington, NC, USA) for 2 min with 10 volumes of low ionic strength buffer (20 mM Tris–Cl, pH 7.5 containing 50 mM NaCl and 0.05 mM PMSF). The homogenate was centrifuged at 5000*g* (Sorvall RC-5 C Plus, Dupont, Wilmington, Del., USA) for 10 min before collecting the pellet. The process was repeated one more time. Subsequently, the pellet was homogenized with 100 ml of high ionic strength buffer (20 mM Tris–Cl, pH 7.5 containing 0.6 M NaCl) before centrifugation at 10,000*g* for 10 min. The supernatant was collected and diluted with three volumes of cold de-ionized (DI) water to precipitate NAM. The precipitates obtained by centrifugation at 10,000*g* for 10 min were dissolved in 20 mM Tris–Cl, pH 7.5 containing 0.6 M NaCl and the resulting preparation, referred to as NAM, was used within a week. Protein concentration of NAM was determined by the BCA protein assay with BSA as a standard.

2.3. Thermal transitions of NAM

2.3.1. Circular dichroism (CD)

NAM was dissolved in 0.6 M NaCl, 20 mM Tris–Cl, pH 7.5 and diluted to obtain final concentration of 0.05 mg/ml. CD spectra were measured using a J-810 spectropolarimeter (JASCO, Tokyo, Japan) equipped with a JASCO temperature control device (PFD-425S, JASCO, Tokyo, Japan). A quartz cell of 2 mm path length was used to measure the ellipticity at 222 nm $[\theta]_{222}$, with N_2 gas purging throughout the measurement. The NAM sample was heated from 10 to 80 °C at the heating rate of 1 °C/min. Molar mean ellipticities ($[\theta]$) were calculated using a mean residue weight of 115 g/mol as described by Price (1996). α-Helical content (%) was estimated from $[\theta]_{222}$ according to the equation developed by Ogawa, Ehare, Tamiya, and Tsuchiya (1993).

2.3.2. Differential scanning calorimetry (DSC)

DSC experiment was performed with a multiple cell differential scanning calorimeter model 4100 (Calorimetry Sciences Co., Lindon, UT, USA). Approximately 0.5 g of NAM (15 mg/ml in 0.6 M NaCl, 20 mM sodium phosphate buffer at pH 7.5) was weighed into a DSC ampoule, which was then sealed tightly with a lid to prevent evaporation during heating. The reference ampoule was filled with buffer at the same weight. All ampoules were heated from 1–90 °C with a scan rate of 1 °C/min. The instrumental sensitivity was 10 μcal/°C. The DSC data were analyzed using the software package, Origin, developed by MicroCal (MicroCal, Northampton, MA, USA) according to Nakaya, Kakinuma, and Watabe (1997).

2.4. Physicochemical changes of NAM after incubation

2.4.1. General

NAM solution (1 mg/ml in 20 mM Tris–Cl, pH 7.5 containing 0.6 M NaCl) were incubated at 25 and 40 °C for 4 and 2 h, respectively. These incubation temperatures are typically used as setting conditions (Park, Yongsawatdigul, & Lin, 1994; Yongsawatdigul et al., 2002). All incubated NAM solutions were diluted to desired concentration before analyzing as described below.

2.4.2. Changes in secondary structure analyzed by CD

The incubated NAM solutions were diluted to 0.05 mg/ml (20 mM Tris–Cl, pH 7.5 containing 0.6 M NaCl) and CD spectra were measured at each incubated temperature by scanning from 195–300 nm at 100 nm/min, using response of 2 s and the bandwidth of 1.5 nm. CD spectra of control (NAM without incubation) were measured at 10 °C. The spectra were expressed as the averaged spectra from triplicate measurements.

2.4.3. Changes in surface hydrophobicity

Surface hydrophobicity (So PRODAN) of NAM was determined according to Alizadeh-Pasdar and Li-Chan (2000) with slight modifications. PRODAN stock solution was prepared at 0.32 mg/ml in methanol and kept at −20 °C until use. The incubated samples and control (NAM without incubation) were diluted with buffer (20 mM Tris–Cl, pH 7.5 containing 0.6 M NaCl) to obtain the serial dilutions of 0, 0.0625, 0.125, 0.25, 0.5, and 1 mg/ml. To 2 ml of each diluted protein solution, 10 μl of stock PRODAN was added, followed by incubation in the dark for 15 min. The relative fluorescence intensity (RFI) of all samples was measured with a Shimadzu RF-540 spectrofluorometer (Shimadzu Co., Kyoto, Japan) at excitation/emission wavelength of 365/445 nm with slits of 5 nm. RFI of protein (without PRODAN) was also measured at each concentration, and subtracted from the RFI of sample with PRODAN at each concentration to obtain the net RFI. Surface hydrophobicity (S_0 PRODAN) was obtained from the regression slope of the net RFI versus protein concentration (%). S_0 PRODAN values of incu-

bated NAM were compared to the control (without incubation) and expressed as the fold increase of S_0 PRODAN.

2.4.4. Changes in protein solubility

The incubated NAM solutions were centrifuged at 10,000*g* for 15 min at 4 °C before collecting the supernatant. The soluble protein content in the supernatants was determined by the BCA protein assay and expressed as percentage relative to the soluble protein content of the control NAM.

2.5. TGase preparation and activity assay

2.5.1. TGase preparation

Partial purification of FTG was performed as described in details by Hemung and Yongsawatdigul (2008). In brief, the enzyme was purified using DEAE-Sephacel, hydroxyapatite, Sephacryl-200, and hi-trap heparin chromatographies. The partially purified TGase preparation was mixed with 20% sucrose at a ratio of 1:1 before lyophilization. The lyophilized FTG was kept at −20 °C throughout the study. Lyophilized FTG was reconstituted with cold DI water and was exchanged with buffer (20 mM Tris–Cl, pH 7.5 containing 2 mM DTT) using a 10 kDa molecular weight cut-off membrane (Nanosep®, Pall Life Science, Ann Arbor, MI, USA).

MTG from *S. mobaraense* was supplied by Ajinomoto Co. Inc., (Tokyo, Japan). The enzyme was dissolved in DI water to obtain final protein concentration of 5 mg/ml and was exchanged with the same buffer used for FTG.

2.5.2. TGase activity assay

TGase activity based on the incorporation of MDC into DMC was measured according to the method described by Takagi, Saito, Kikuchi, and Inada (1986) with slight modifications. The reaction mixture contained 1 mg/ml DMC, 15 μM MDC, 70 mM Tris–Cl (pH 7.5), 5 mM $CaCl_2$, 3 mM DTT and 100 μl of enzyme. $CaCl_2$ was omitted in the reaction for MTG. After incubation at 37 °C for 10 min, ammonium sulfate was added to obtain final concentration of 42 mM in order to stop the reaction. The fluorescence intensity of sample (FI_S) was measured at excitation and emission wavelength of 350 and 480 nm, respectively, using a Shimadzu RF-540 spectrofluorometer (Shimadzu Co., Kyoto, Japan). Blanks (FI_b) were prepared in a similar manner except that the enzyme was substituted with DI-water and FI was measured without incubation. TGase activity (U) was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of MDC into DMC within 1 min at 37 °C using a enhancing factor of 3.2 and 1.5 for FTG and MTG, respectively.

2.6. Characterization of NAM after incubation with TGases

2.6.1. General

NAM solutions were incubated with TGases in the reaction mixture containing 3 mg/ml NAM in 0.6 M NaCl, 5 mM DTT, 5 mM $CaCl_2$, 20 mM Tris–Cl, pH 7.5, and 3 U TGase/ml. $CaCl_2$ was omitted in the reaction catalyzed by MTG. For the control, the enzyme was also replaced by DI water. Samples were incubated at either 25 or 40 °C for 4 or 2 h, respectively. Cross-linked NAM solutions were analyzed as described below.

2.6.2. Ca^{2+}-ATPase activity

Two hundred microlitres of NAM reaction mixture containing TGase was taken to determine residual Ca^{2+}-ATPase activity. NAM solutions were pre-incubated at 25 °C for 5 min in the presence of 17 mM Tris–Cl, pH 7.5 and 3.3 mM $CaCl_2$. ATP was subsequently added to the final concentration of 0.67 mM before further incubation for 10 min. Chilled trichloroacetic acid (TCA) was added at a final concentration of 5% to stop the reaction. After centrifugation at 3000*g* for 10 min, the supernatant was collected to determine inorganic phosphate (Pi) according to Black and Jones (1983) using KH_2PO_4 as a standard. Ca^{2+}-ATPase activity was expressed as nmol of released Pi/mg protein/min at 25 °C.

2.6.3. NAM cross-linking

Cross-linked NAM was determined by sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS–PAGE). Sample was mixed with treatment buffer to contain final concentration of 2% SDS, 0.02% BME, and 0.01% bromophenol blue before boiling for 20 min. The boiled samples were centrifuged at 5000*g* for 5 min. The soluble proteins in supernatant were separated on a PhastGel 10–15% gradient gel under reducing conditions using a PhastSystem™ (GE Healthcare, Upsala, Sweden).

2.7. Textural properties of NAM gels

NAM pastes containing final concentration of 123.1 ± 9.6 and 119.2 ± 2.4 mg/g for TB and PW, respectively, in 0.6 M NaCl, 20 mM Tris–Cl (pH 7.5) were prepared using mortar and pestle, before dividing into three portions. In the first portion, $CaCl_2$ was added to obtain final concentration of 5 mM for the sample containing FTG. $CaCl_2$ solution was substituted with the equal volume of DI-water for the control and samples with added MTG. MTG and FTG were added to NAM pastes at 1 U/mg protein. The pastes were filled into a microplate with a diameter of 8 mm and 10 mm depth. The filled plates of PW-NAM were wrapped with a plastic bag and incubated at 25 °C for 4 h, whereas those of TB-NAM were carried out at 40 °C for 2 h. These temperatures are the optimal setting temperatures of each fish species (Park et al., 1994; Yongsawatdigul et al., 2002). Samples were then heated at 90 °C for 15 min before cooling at 4 °C overnight. The breaking force and deformation were determined using a Texture Analyzer TA-XT2 (Stable Micro System, Surrey, England) equipped with a 3 mm diameter cylindrical probe at a test speed of 1 mm/s. The average breaking force and deformation values were obtained from seven measurements.

3. Results and discussion

3.1. Conformational changes of NAM

3.1.1. CD measurement

The content of α-helical structure of PW-NAM started to decrease at around 25 °C, and showed two thermal transitions with denaturation temperatures (T_d) at 31.8 and 43.1 °C (Fig. 1). TB-NAM exhibited higher thermal stability than PW-NAM as evident by higher T_d at 35.0 and 49.3 °C. The changes in α-helical content are mainly attributed to the unfolding of myosin rods (Margossian & Lowey, 1982). Ogawa et al. (1995) indicated that the most unstable portion of fish myosin was in the light meromyosin (LMM). Rogers, Karr, Biedermann, Ueno, and Harrington (1987) reported that thermal stability of myosin rods shifted along with the working temperature of their respective muscles. These studies have led to speculations that myosin unfolding is initiated in the LMM region upon heating. The results from our study indicate that the myosin rod of temperate water species is more thermally labile than that of tropical species.

3.1.2. DSC measurement

Transition temperature (T_m) values of PW-NAM were found at 31.8, 42.1, and 75.3 °C, while those of TB-NAM were at 36.1, 50.9, and 78.4 °C, respectively. The multiple transitions of NAM observed in this study are in agreement with changes reported previously in fish myofibrils (Howell, Matthews, & Donnelly, 1991), and in myosin from snapper and cod (Davies, Bardsley, & Ledward,

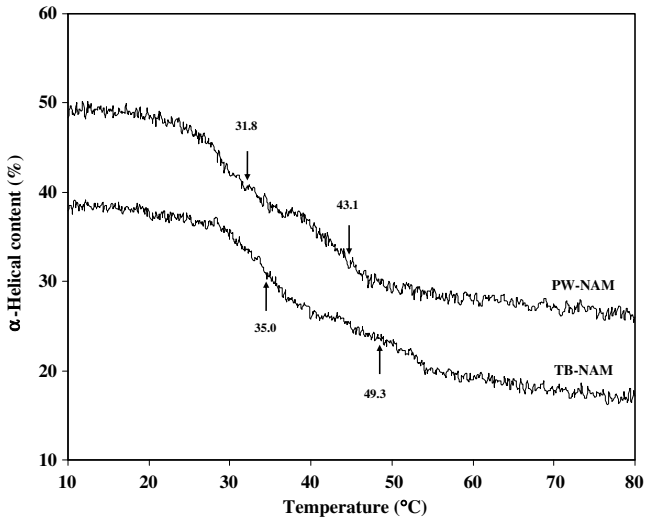

Fig. 1. Changes in α-helical content of PW- and TB-NAM in 0.6 M NaCl and 20 mM Tris–Cl, pH 7.5 at heating rate of 1 °C/min. Denaturation temperatures (T_d values) are indicated by the arrows.

1988). T_m values of carp myosin acclimatized to 10 °C were found at 33.9 and 47.4 °C and these values corresponded to denaturation of the LMM rod as described by Nakaya et al. (1997). The T_d values of each NAM observed by CD representing the melting of helical structure (Fig. 1) were similar to the first two T_m values of respective NAM observed by DSC. Thus, the two lower T_m values could represent denaturation temperature of the myosin rod, while the highest T_m value could be attributed to denaturation of actin, as reported by Davies et al. (1988).

The multiple transitions of both species in the present study suggested the independent structural changes of myosin subfragments and actin. T_m values of TB-NAM were higher than those of PW, confirming the higher thermal stability. Similar endothermic peaks of TB-NAM were also observed previously (Yongsawatdigul & Park, 2003). Togashi, Kanimuma, Nakaya, Ool, and Watabe (2002) suggested that thermal stability of fish myosin is closely related to habitat temperature. Based on these results, NAM from fish living in temperate habitat showed lower thermal stability than that of tropical fish.

3.2. Physicochemical changes of NAM after incubation

3.2.1. Changes in secondary structure

It has been reported that the α-helical structure is the dominant pattern of secondary structure in the myosin rod (Margossian & Lowey, 1982). After incubation at 25 °C, the α-helical contents of NAM from TB and PW decreased by 33.1% and 69.7%, respectively (Table 1). Lower reduction in α-helical content of TB-NAM may reflect its higher stability when compared to PW-NAM. The α-helical content reduction of TB-NAM and PW-NAM after incubation at 40 °C was observed to be 78.0% and 89.7%, respectively. Ogawa et al. (1995) indicated that setting was initiated by unfolding of α -helical structure. Optimum setting temperature of PW-NAM has been reported to be at 25 °C (Park et al., 1994), which coincided with the high degree of unfolding of PW-NAM observed in this study. Yongsawatdigul et al. (2002) also suggested the optimum setting temperature of TB-NAM at 40 °C. Our study demonstrated that the extent of TB-NAM unfolding at 40 °C for 2 h was comparable to that of PW-NAM at 25 °C for 4 h. In comparison, TB-NAM only unfolded slightly when incubated at 25 °C for 4 h. This could explain why TB surimi hardly set at this condition (Yongsawatdigul et al., 2002). These results suggested that the changes in secondary

Table 1
Reduction in α-helical content of TB-NAM and PW-NAM after incubation at various conditions

NAM	Incubation condition	α-Helical reduction[a] (%)
TB-NAM	25 °C/4 h	33.1 ± 1.35
	40 °C/2 h	78.0 ± 0.43
PW-NAM	25 °C/4 h	69.7 ± 1.48
	40 °C/2 h	89.7 ± 1.09

The mean values of reduction in α-helical content are from triplicate measurements by circular dichroism.

[a] α-Helical reduction was calculated as the % reduction relative to the α-helical content of each NAM at 10 °C.

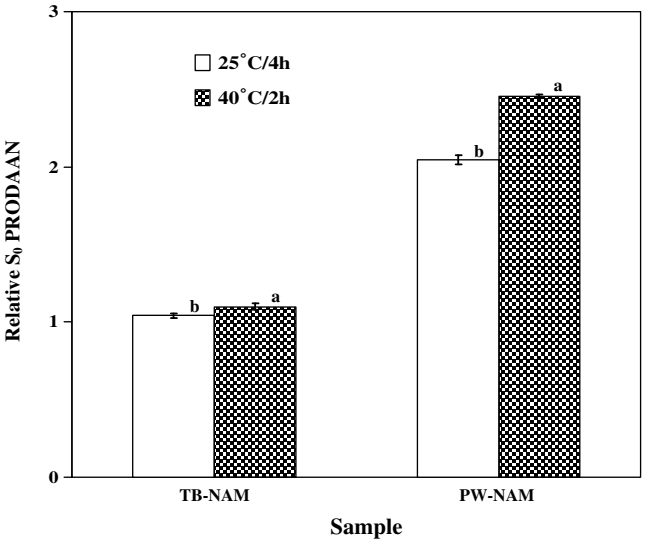

Fig. 2. Relative S_0 PRODAN of incubated NAM expressed as the ratio of S_0 PRODAN of incubated NAM to that of control (without incubation). 25 °C/4 h and 40°C/2 h = incubation at 25 °C for 4 h and 40 °C for 2 h, respectively. Different letters indicate significant differences within species ($P < 0.05$).

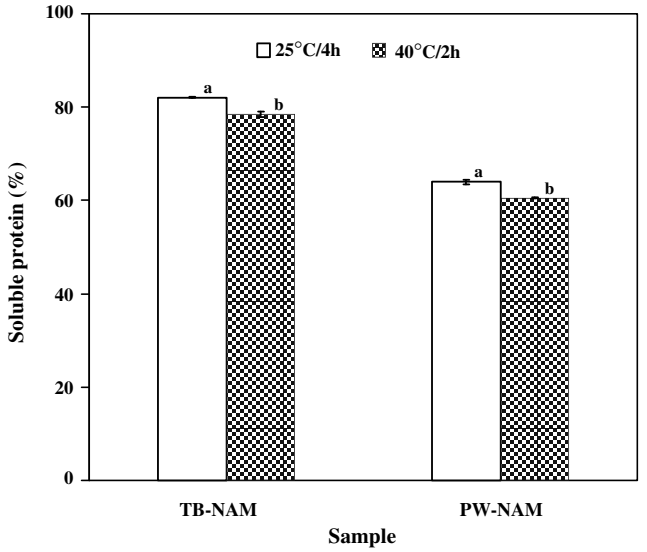

Fig. 3. Soluble proteins of incubated NAM expressed as percentage compared to control (without incubation). 25 °C/4 h and 40 °C/2 h = incubation at 25 °C for 4 h and 40 °C for 2 h, respectively. Different letters indicate significant differences within species ($P < 0.05$).

structure appeared to be the prime factor governing setting phenomenon of NAM.

3.2.2. Changes in surface hydrophobicity

PRODAN has been used as fluorescence probe for the hydrophobic residues accounting for surface hydrophobicity (S_0 PRODAN) and could also be used to monitor thermal denaturation of proteins by probing the hydrophobic patches that may become exposed from the hydrophobic core by protein unfolding. After incubation at 25 °C, S_0 PRODAN of PW-NAM increased more than 2-fold compared to the control NAM, while that of TB increased only slightly (Fig. 2). The increase in S_0 PRODAN of TB-NAM incubated at 40 °C was higher than that at 25 °C. These increases in S_0 PRODAN correlated with the extent of secondary structural changes (Table 1), implying that the exposure of hydrophobic residues may have resulted from unfolding of myosin rod, coiled coil domain. The amino acid sequences that adopt a coiled coil structure display a characteristic seven-residue repeat pattern (haptad repeat), designated *a, b, c, d, e, f,* and *g* (McLachlan & Karn, 1982). The hydrophobic residues are concentrated in the interior of the chain at the *a* and *d* positions to stabilize the coiled coil structure via hydrophobic interactions. However, the presence of α-helix breakers (Pro and Gly) and α-helix unrelated residues (Asp and Thr) results in less stability of α-helical proteins. The large gaps containing helix breakers have been observed in LMM of the least stable myosin, walleye pollock myosin (Togashi et al., 2002). It may be speculated that PW-NAM could possibly contain helix breakers at the myosin rod, which may lead to unfolding of PW-NAM to a greater extent than TB-NAM after incubation under similar conditions.

3.2.3. Protein solubility

Solubility of PW-NAM after incubation at either 25 or 40 °C was lower than that of TB-NAM (Fig. 3). These results indicate that PW-NAM underwent intra- and inter-molecular interactions to form

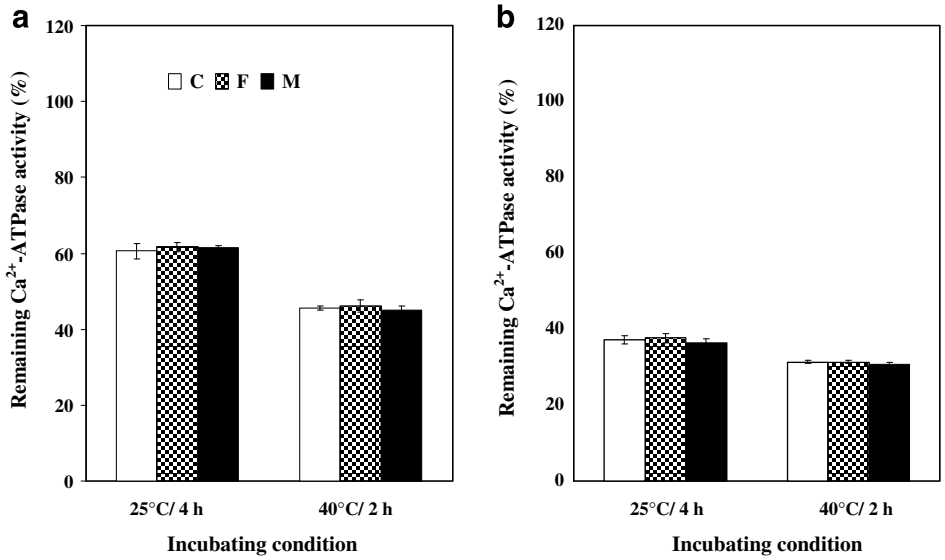

Fig. 4. Ca^{2+}-ATPase activity of TB-NAM (a) and PW-NAM (b) remaining after incubation at various conditions as follows: C: control (without enzyme); M: MTG; F: FTG; 25 °C/4 h and 40 °C/2 h = 25 °C for 4 h and 40 °C for 2 h, respectively; Ca^{2+}-ATPase activity of each species (TB- or PW-NAM) without incubation was considered to be 100%.

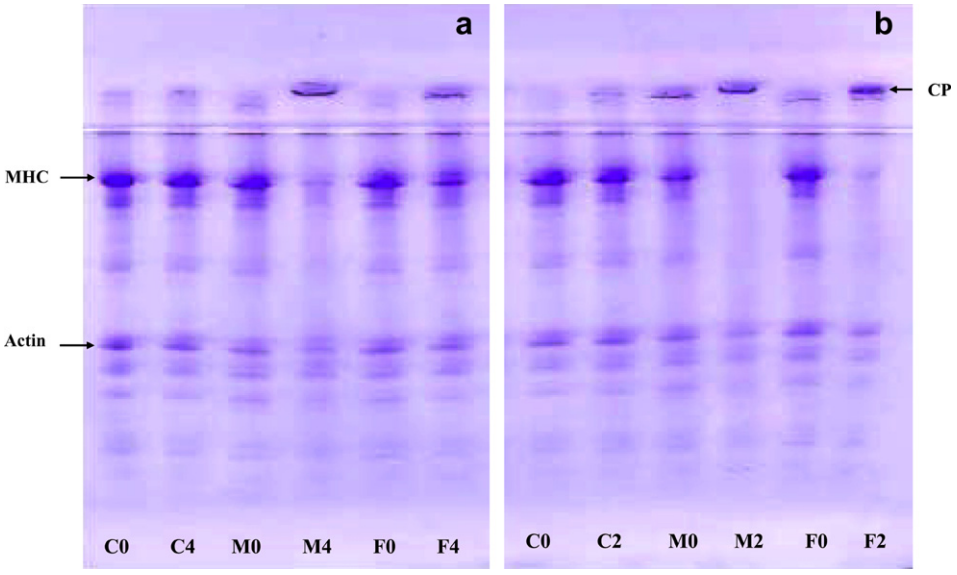

Fig. 5. SDS–PAGE patterns of TB-NAM incubated at 25 (a) and 40 °C (b) in the absence or presence of TGases, as follows: C: control (without enzyme); M: MTG; F: FTG; for the designated time in h (0, 2 or 4 h). CP: cross-linked polymers; MHC: myosin heavy chain.

aggregates to a greater extent than did TB. Hydrophobic interactions of cod myosin rod were initiated by thermal aggregation (Chan, Gill, & Paulson, 1993). It is likely that hydrophobic interactions were responsible for aggregate formation of NAM, and it may be postulated that hydrophobic interactions were also important for setting of fish muscle proteins. It should be noted that TB-NAM after incubation exhibited higher solubility concomitant with lesser exposure of hydrophobic amino acids. This implies that hydrophobic interactions formed during setting of tropical fish would be less than that of temperate species.

3.3. Cross-linking of NAM by TGases

3.3.1. Changes in Ca^{2+}-ATPase activity

After incubation at 25 and 40 °C, the Ca^{2+}-ATPase activity of TB-NAM remained at ~60% and 45%, respectively (Fig. 4a), while that of PW-NAM dramatically decreased to remain at <40% and 30%, respectively, (Fig. 4b). It can be noted that Ca^{2+}-ATPase activity of TB-NAM remained at higher level than that of PW at all studied conditions ($P < 0.05$), confirming higher thermal stability of NAM from tropical habitat. Johnston, Frearson, and Goldspink (1973) also found that the inactivation half-life of myofibrillar ATPase correlated well with habitat temperature of fish.

Addition of TGases had no effect on Ca^{2+}-ATPase activity of either PW- or TB-NAM. Similarly, Huang, Seguro, Motoki, & Tawada, (1992) reported that the ATPase activity of rabbit myosin was not significantly affected by the presence of MTG, and also demonstrated that the isolated globular head of myosin (S1) was not cross-linked, while the myosin rod portion was quickly cross-linked. Our results implied that the cross-linking sites might not be located at the myosin head. Seki, Nakahara, Takeda, Maruyama, and Nozawa (1998) also suggested that glutamine residues at S2 region of carp myosin were the preferential sites for endogenous TGase.

3.3.2. Protein patterns by SDS–PAGE

Cross-linking of TB-NAM was observed by incubation at 25 °C for 4 h with FTG and MHC appeared to be the favored substrate, as evident by a reduction of MHC in conjunction with the formation of cross-linked polymers (CP) on SDS–PAGE (Fig. 5a). These results implied that setting of TB surimi at this condition could be possible when endogenous TGase is activated. Yongsawatdigul et al. (2002) also found cross-linking of TB-MHC at 25 °C in the presence of 0.1% $CaCl_2$. It should be noted that at 25 °C, MTG catalyzed cross-linking of TB-MHC to a greater extent than did FTG, despite the low degree of unfolding (33.1% reduction in α-helix, Table 1). This suggests that although the conformation of NAM would affect the reactivity of its residues to the action of TGase and subsequent formation of cross-links, it played a less vital role in the catalytic action of MTG than FTG. The smaller molecular size of MTG may have allowed the enzyme to more easily access the reactive groups within NAM molecule. In addition, it is possible that MTG and FTG are catalyzing different reactive groups on NAM; in other words, the reactive group for MTG may be located at the surface of NAM molecule, while those for FTG might be in a buried position. In this case, conformational changes of the NAM substrate would, thus, be far more vital in governing catalytic reactions of FTG than MTG.

Cross-linking of TB-MHC catalyzed by both enzymes was greater at 40 °C than at 25 °C (Fig. 5b). TB-MHC completely disappeared by the action of MTG, while the monomeric TB-MHC was still observed in the presence of FTG. The extent of TB-MHC cross-linking by MTG correlated well with degree of α-helical reduction (Fig. 5a; Table 1). Degree of substrate unfolding directly governed the exposure of reactive groups, namely glutamine and lysine, which were critical for TGase reactivity.

Due to the extensive unfolding of PW-NAM at both 25 and 40 °C (Table 1), the cross-linking of PW-MHC catalyzed by MTG markedly occurred at both conditions as evident by the complete disappearance of MHC (Fig. 6a and b). Unfolding of NAM rendered more reactive groups for TGase. Kishi et al. (1991) also reported that conformational changes of carp-NAM allowed the certain glutamine residues to become reactive. Reactivity of NAM as a substrate for TGase mainly depended on its conformation induced by thermal unfolding.

It should be mentioned that PW-MHC did not completely disappear by the action of FTG (Fig. 6a and b). These results clearly illustrated that FTG catalyzed MHC cross-linking to the lesser extent than did MTG. Equivalent units of activity of the two enzymes were added to NAM, based on TGase activity determined using incorporation of MDC into DMC. However, the enzymes might exhibit different specificity between DMC and MHC. Incorporation of a small

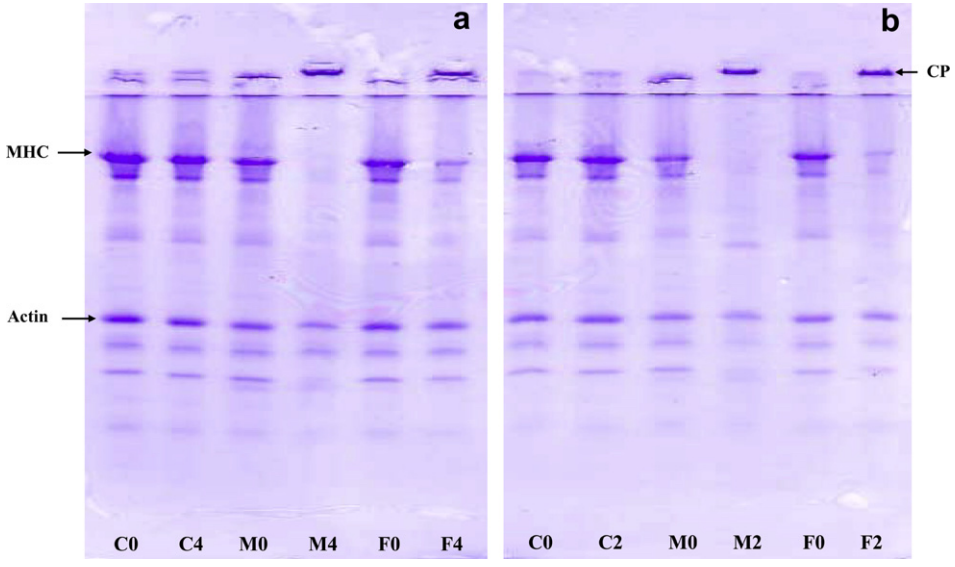

Fig. 6. SDS–PAGE patterns of PW-NAM incubated at 25 (a) and 40 °C (b) in the absence or presence of TGases. Abbreviations are the same as described for Fig. 5.

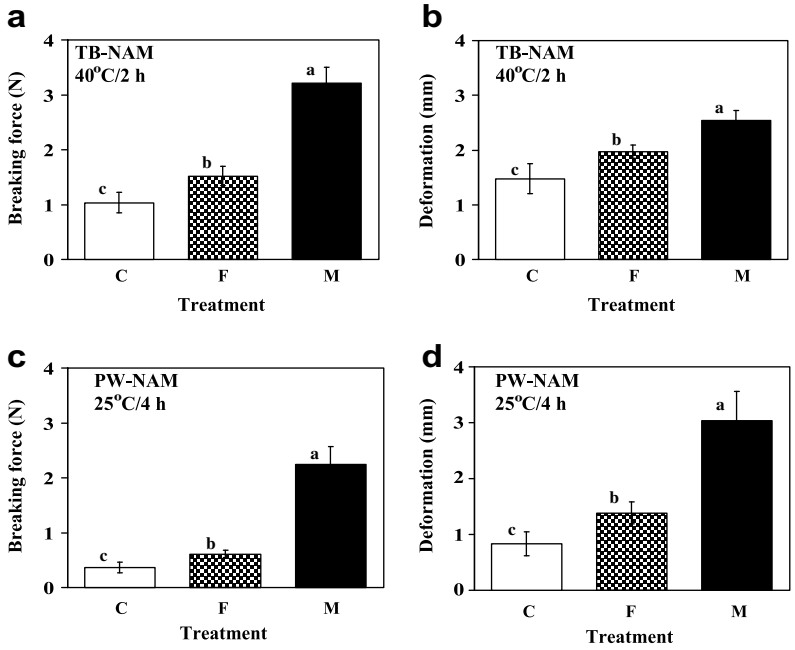

Fig. 7. Breaking force and deformation values of TB-NAM gels (a and b) and PW-NAM gels (c and d) prepared by setting at 40 °C for 2 h and at 25 °C for 4 h, respectively. C: control; F: FTG; M: MTG; different letters within each figure indicate significant difference ($P < 0.05$).

amine such as MDC into casein would be different from cross-linking of lysine residues to glutamine residues along NAM molecule, particularly with regard to steric hindrance and accessibility of reactive group.

3.4. Textural properties of NAM gels

Textural properties of TB-NAM and PW-NAM gels were measured after setting at 40 °C for 2 h and 25 °C for 4 h, respectively, which are the conditions typically applied for these species. FTG improved breaking forces and deformation values of TB-NAM gels by 1.3- and 1.5-fold, respectively (Fig. 7a and b). Approximately 1.7-fold increase in those values was also observed for PW-NAM gels (Fig. 7c and d). Since FTG is a Ca^{2+}-dependent TGase and showed comparable characteristic as fish muscle TGase (Hemung and Yongsawatdigul, 2008), its catalytic reaction would represent the action of endogenous TGase in setting.

Textural properties of TB-NAM were much more improved by MTG with increases in breaking force and deformation values for 3.1- and 1.7-fold, respectively (Fig. 7a and b). Jiang et al. (2000) also reported that MTG enhanced breaking force of golden threadfin bream by almost 3-fold after setting at 40 °C. Breaking force and deformation values of PW-NAM gels added MTG were also improved by 6.1- and 3.6-fold, respectively (Fig. 7a and b). It can be noticed that textural improvement of NAM gels corresponded well with the degree of MHC cross-linking observed on SDS–PAGE (Figs. 5 and 6). Seguro, Kumazawa, Ohtsuka, Toiguchi, and Motoki (1995) also reported that gel strength of kamaboko made from Alaska pollock steadily increased as MHC cross-linking increased. The higher degree of MHC cross-linking catalyzed by MTG resulted in greater textural improvement than that catalyzed by FTG. Textural improvement of NAM gels depends on the extent of MHC cross-linking, which ultimately depended on the types of TGase and the thermal stability of NAM itself.

Setting of PW-NAM was pronounced even at low temperature (25 °C), which is far from the optimum temperature of FTG (50 °C) and MTG (55 °C) (Ando et al., 1989; Hemung & Yongsawatdigul, 2008). These results indicated the importance of NAM conformation on catalytic reaction of TGase.

4. Conclusions

NAM from fish living in temperate habitat underwent greater conformational changes than tropical fish under setting conditions at either 25 °C for 4 h or 40 °C for 2 h and such changes directly governed the extent of MHC cross-linking catalyzed by TGases. NAM conformation and types of TGase were important for MHC cross-linking reactions. Unfolding of NAM played a much more critical role for FTG than MTG in catalyzing protein cross-linking. TB-MHC could be cross-linked even at 25 °C by both TGases. Based on Ca^{2+}-ATPase activity results, cross-linking sites by both MTG and FTG might not be located at the myosin head. Textural properties increased with the extent of MHC cross-linking. MTG catalyzed MHC cross-linking to the greater extent than FTG.

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