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Thermal denaturation and aggregation of threadfin bream actomyosin

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Abstract

Threadfin bream (*Nemipterus bleekeri*) actomyosin formed insoluble aggregates at >40 °C. Conformational changes, as measured by surface hydrophobicity, began at >30 °C and continued to increase with heating temperature. Reactive sulfhydryl groups increased as heating progressed and decreased at 50 °C, indicating the formation of disulfide linkages of threadfin bream actomyosin at >50 °C. Two distinct a-helical transition temperatures of actomyosin were found at 36.1 and 47.9 °C, while major endothemic transitions were at 38.4, 51.0, and 80.7 °C. Storage modulus (G') started to increase at 34.5 °C, implying the simultaneous occurrence of denaturation and aggregation. Gel network formation began to develop at >41 °C.

Keywords: Actomyosin; Threadfin bream; Thermal denaturation; Aggregation

1. Introduction

Threadfin bream (*Nemipterus* spp.) is a major resource for surimi production in Thailand. Gel forming ability of surimi is mainly contributed by actomyosin (Yasui, Ishioroshi, & Samejima 1981). Gelation of food proteins is generally governed by denaturation and aggregation. Denaturation is a process in which proteins undergo conformational changes, primarily unfolding, without alteration of the amino acid sequence (Hermansson, 1979). The denatured proteins aggregate in an ordered fashion to form a continuous network structure (Beveridge, Arntfield, & Murray, 1985). Thermal denaturation and aggregation of fish actomyosin and myosin have been widely studied among cold water species (Bea, Wagner, Crupkin, & Anon, 1990; Chan, Gill, & Paulson, 1992; Sano, Ohno, Otsuka-Fuchino, Matsumoto, & Tsuchiya, 1994; Visessanguan, Ogawa, Nakai, & An, 2000). Ogawa, Ehara, Tamiya, and Tsuchiya (1993) reported that myosin of fish living in colder temperatures is more labile than of those living in warmer temperatures. Therefore, denaturation and aggregation patterns of fish from different temperature habitats would be varied.

Knowledge of protein denaturation and aggregation is a critical basis on which to manipulate the gel-forming ability of fish actomyosin. It was suggested that myosin tail mainly contributed to the development of gel networks (Sano, Noguchi, Matsumoto, & Tsuchiya, 1990). Chan et al. (1992) reported that herring, cod, and silver hake aggregated in different fashions, accounting for differences in gel elasticity between the three species. In addition, gel elasticity of cod myosin was improved by slow heating that promoted the aggregation of denatured myosin (Yongsawatdigul & Park, 1999). However, infbrmation related to denaturation and aggregation patterns of tropical fish actomyosin is very limited. Fundamental knowledge underlying the gelation mechanism is primarily based on cold water species. Such knowledge might impair the optimum utilization of surimi from tropical fish actomyosin. Therefore, our objective was to investigate the thermal denaturation and aggregation patterns of threadfin bream actomyosin.

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2. Materials and methods

2.1. Preparation of fish actomyosin

Threadfin bream (*Nemipterus bleekeri*) was caught off the Gulf of Thailand and immediately transported in ice, packed in polystyrene foam boxes, to a laboratory at Suranaree University of Technology. Fish weight was about 50–100 g. Actomysosin was prepared from the dorsal muscle according to the method of Ogawa et al. (1999) with slight modification as follows: fish muscle (50 g) was mixed in five volumes of cold phosphate buffer (pH 7.0) containing 50 mM KC1, 20 mM potassium, and 0.05 mM phenylmethanesulfonyl fluoride (PMSF). Homogenization was conducted in a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA) for 2 min. The homogenate was centrifuged at $5000\times g$ for 10 min at 4 °C. The precipitate was homogenized with the same buffer and centrifuged once more. The resultant residue was homogenized in 500 ml (0.6 M KCI and 20 mM potassium phosphate, pH 7.0) and then centrifuged at $10{,}000\times g$ for 5 min at 4 °C. The supernatant was collected and diluted with three volumes of cold distilled water (4 °C). The precipitate was collected by centrifugation at $10{,}000\times g$ for 10 min at 4 °C and used as actomyosin throughout the study.

2.2. Heat treatment

One mililitre of actomyosin solution (2.5 mg/ml) in buffer (0.6 M KC1 and 20 mM potassium phosphate, pH 7.0) was heated from 5 to 90 °C in a temperature-controlled water bath (NesLab, Portsmouth, NH, USA) at a heating rate of 1 °C/min. The sample was covered with parafilm and aluminium foil during heating to avoid evaporation. Upon reaching each studied temperature, the actomyosin solution was immediately cooled in ice water and centrifuged at $5000\times g$ for 15 min. The supernatant was collected and analysed for protein content, surface hydrophobicity, and total and reactive sulfhydryls.

2.3. Soluble protein

Protein concentration of the supernatant was determined by the dye binding method (Bradford, 1976) using bovine serum albumin as a standard.

2.4. Surface hydrophobicity

Surface hydrophobicity (S_0) of actomyosin was determined using a hydrophobic fluorescence probe, 1-anilino-8-napthalenesulfonate (ANS), according to Hayakawa and Nakai (1985). Actomyosin solution (1 mg/ml) was diluted with 0.6 M KCl, 20 mM potassium phosphate buffer (pH 7.0) to obtain a series of protein concentrations, from 0 to 1 mg/ml. To 2 ml of each protein solution, 10 μl of 8 mM ANS in 0.1 M potassium phosphate buffer (pH 7.0) was added and mixed well. Samples were kept under dark conditions for 10 min. Fluorescence intensity of the mixture was measured using a luminescence spectrophotometer (LS 50B, Perkin-Elmer, Beaconsfield, UK) at excitation and emission wavelengths of 374 and 485 nm, respectively, and a 5 nm width for both the excitation and emission slits. S_0 of each sample was calculated from the slope of the relative fluorescence (R) vs. percentage (w/v) protein concentration. The relative fluorescence was defined according to Monahan, German, and Kinsella (1995) as follows:

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

where F is the fluorescence of the protein–ANS conjugate and F_o is the reading of the ANS solution without actomyosin.

2.5. Total and reactive sulfhydryls (SHs)

Total SHs were determined according to Jiang, Hwang, and Chen (1988). To 1 ml of actomyosin (4 mg/ml), 9 ml of buffer, containing 50 mM potassium phosphate buffer, 10 mM ethylenediaminetetraacetic acid (10 mM), 0.6 M KCI, 8 M urea (pH 7.0) were added. To 4 ml of the resultant mixture 0.4 ml of 0.1% 5, 5′-dinitrobis(2-nitrobenzoic acid) was added. The mixture was incubated at 40 °C for 25 min. The absorbance was measured at 412 nm to calculate the total SH groups using the extinction coefficient of 13,600 $M^{-1}cm^{-1}$ (Ellman, 1959). Reactive SH groups were conducted by incubating the reaction mixtures in the absence of urea at 4 °C for 1 h.

2.6. Circular dichroism (CD)

The purified actomyosin was diluted to a protein concentration of 0.19 mg/ml with 0.6 M KCI, 20 mM phosphate buffer (pH 7.0). CD spectra were taken with a J-720 spectropolarimeter (JASCO, Tokyo, Japan) operating with nitrogen gas purging at 222 nm and a bandpass of 2 nm. The instrument was equipped with a JASCO thermal control device (PTC-348W, JASCO, Tokyo, Japan). A 1 mm path length quartz cell was used. Actomyosin solutions were heated from 10 to 80 °C at a heating rate of 1 °C/min. The instrument was calibrated for intensity accuracy at 290.5 nm and 192 nm, respectively, using (1*S*)-(+)-10-camphorsulfonic acid (Sigma-Aldrich, St. Louis, MO, USA). Molar ellipticities of actomyosin were determined using a mean residue weight of 115 g/mol, as described by Price (1996). α-Helicity (%) was estimated from ellipticities at

222 nm, $[\theta]_{222}$, by the following equation (Ogawa, Kanamura, Miyashita, Tamiya, & Tsuchiya, 1995):

$$\%\ \alpha\text{-Helicity} = 100 \times \{[\theta]_{222}/-40,000\}$$

2.7. Differential scanning calorimetry (DSC)

DSC studies were performed in a DuPont 910 differential scanning calorimeter (DuPont Co., Wilmington, DE). The temperature calibrations were performed using indium. Actomyosin solutions (75 mg/ml) in 0.6 M KCI, 20 mM phosphate buffer (pH 7.0) were weighed to 18–20 mg, wet basis, in DSC hermetic pans, ensuring good contact between the sample and the capsule bottom. An empty pan was used as a reference. Samples were scanned at 10 °C/mm over the range 5–95 °C. Helium gas was purged through the purging port at 40 ml/min during heating. Quadruplicate samples with reproducible thermograms were analysed.

2.8. Oscillatory dynamic—measurement

Development of an actomyosin network was measured as a function of temperature using a CS-50 rheometer (Bohlin Instruments, Inc., Cranbury, NJ, USA). Actomyosin samples (75 mg/ml in 0.6M KCI, 20 mM phosphate buffer, pH 7.0) at 4 °C were placed between parallel plates (20 mm) with a gap of 1 mm. To avoid sample drying during heating, a plastic cover (trapper) with a moistened sponge inside was used. The sample was heated from 10 to 80 °C at a heating rate of 1 °C/min. Maximum input strain for dynamic analysis was 0.02 at a frequency of 0.1 Hz, a value found to be in the linear viscoelastic region for actomyosin in this study.

2.9. SDS-PAGE

Degradation of actomyosin during heating was studied on SDS–PAGE. Actomyosin solutions heated at 1 °C/min to various temperatures were removed from the water bath and an equal volume of 10% SDS (w/v) (90 °C) was immediately added. The mixture was centrifuged at 5000×*g* for 10 mm. The supematant was used for gel electrophoresis as described by Laemmli (1971). Stacking gel and separating gel were made of 4% (w/v) and 10% (w/v) polyacrylamide, respectively. The amount of protein loaded onto the polyacrylarnide gel was 40 μg. The separated proteins were stained with 0.125% Coomassie brilliant blue R-250 (Bio-Rad, Hercules, CA), and destained in a solution containing 25% ethanol and 10% acetic acid. A wide range molecular weight standards (Sigma-Aldrich, St. Louis, MO, USA) included rabbit myosin (205 kDa), β-galactosidase (116 kDa), rabbit phosphorylase *b* (97 kDa), fructose-6-phasphate kinase (84 Wa), bovine serum albumin (66 kDa), bovine glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), α-lactalbumin (14.2 kDa), and aprotinin (6.5 kDa)

3. Results and discussion

3.1. Protein solubility

Solubility of actomyosin remained constant between 10 and 30 °C and started to decrease at temperatures above 40 °C (Fig. 1). Solubility gradually decreased

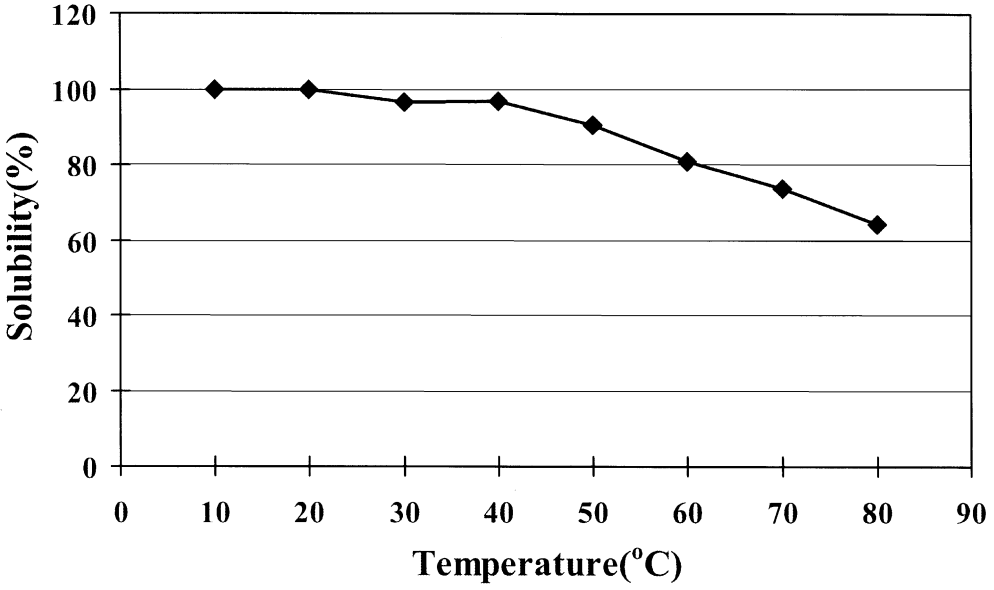

Fig. 1. Changes in solubility of threadfin bream actomyosin heated at 1 °C/min.

afterwards and reached a minimum of 60% at 80 °C. This is similar to that of Sano et al. (1994) who reported a marked decrease in the solubility of carp actomyosin between 40 and 80 °C. Yongsawatdigul and Park (1999) found that aggregation of cod and Pacific whiting myosin, as followed by absorbance at 320 nm, began to increase from 20 °C. Our results implied that threadtin bream actomyosin aggregated to form insoluble actomyosin at higher temperatures than cold water species, such as cod and Pacific whiting.

3.2. Surface hydrophobicity (S_0)

S_0 of actomyosin continually increased at temperatures above 30 °C and reached a maximum at 70 °C (Fig. 2). S_0 indicates an exposure of nonpolar amino acids that unfold in a polar environment. Hence, the conformation of threadfin bream actomyosin began to unfold and expose the buried nonpolar amino acids at temperatures above 30 °C.

The extent of conformational changes increased with temperature. A slight decrease in S_0 above 70 °C suggested the involvement of hydrophobic residues in the actomyosin aggregation. A significant increase in ANS fluorescence was also reported in carp actomyosin from 30 to 50 °C (Sano et al., 1994), while that of Pacific whiting (Yongsawatdigul & Park, 1999) and arrowtooth flounder (Visessanguan et al., 2000) began to increase at 20 °C. The changes of hydrophobicity of rabbit and chicken myosin exhibited a single transition temperature of 44 °C (Wicker, Lanier, Hamann, & Akahane, 1986). The rabbit light meromyosin (LMM) were unfolded and non-polar amino acids exposed at 35–40 °C (Morita & Yasui, 1991).

Changes in S_0, of threadfin bream actomyosin could mainly be contributed by its myosin. It should be noted that actomyosin from threadfin bream, a tropical fish, underwent conformational changes at temperatures similar to carp and rabbit, but at higher temperatures than cold water species such as, cod, Pacific whiting (Yongsawatdigul & Park, 1999) and arrowtooth flounder (Visessanguan et al., 2000).

3.3. Total and reactive sulfhydryls (SHs)

Reactive SH groups started to increase at 30 °C, attained a maximum at 50 °C, and decreased at temperatures higher than 50 °C (Fig. 3). An increased reactive SH indicates unfolding of actomyosin. Total SH groups remained unchanged up to 30 °C and gradually decreased from 40 to 80 °C (Fig. 3), suggesting the formation of disulfide linkages upon heating. Decreased reactive SH groups implies the formation of inter and/or intra-molecular disulfide bonds at >50 °C. It appears that the formation of disulfide linkages occurred simultaneously with protein unfolding. These results were in agreement with that of Sano et al. (1994), who found formation of disultide linkages of carp actomyosin at 30–50 °C. Ishioroshi, Samejima, and Yasui (1981) indicated that oxidation of SH groups took place only in heavy meromyosin (HMM) and not in light meromyosin (LMM). This could lead us to hypothesize that oxidation of SH groups at the globular head of myosin is initiated at 30 °C, resulting in disulfide linkages.

3.4. CD

α-Helix of threadfin bream actomyosin decreased gradually as heating temperature extended above 30 °C, suggesting that the α-helical portion of actomyosin unfolded with increased temperature (Fig. 4). α-Helix

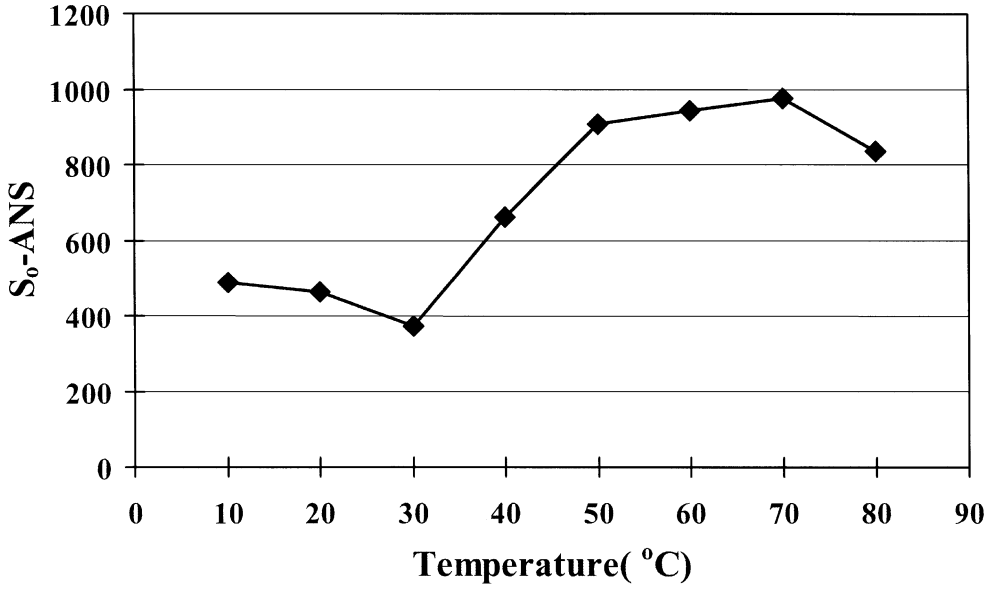

Fig. 2. Changes in surface hydrophobicity (S_0) of threadfin bream actomyosin heated at 1 °C/min.

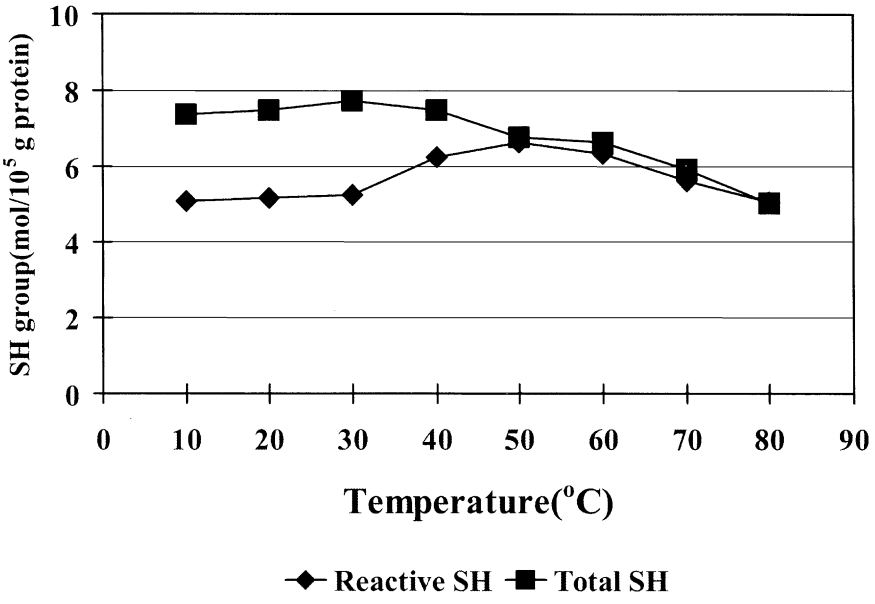

Fig. 3. Changes in reactive and total suithydryl (SH) groups of threadfin bream heated at 1 °C/min.

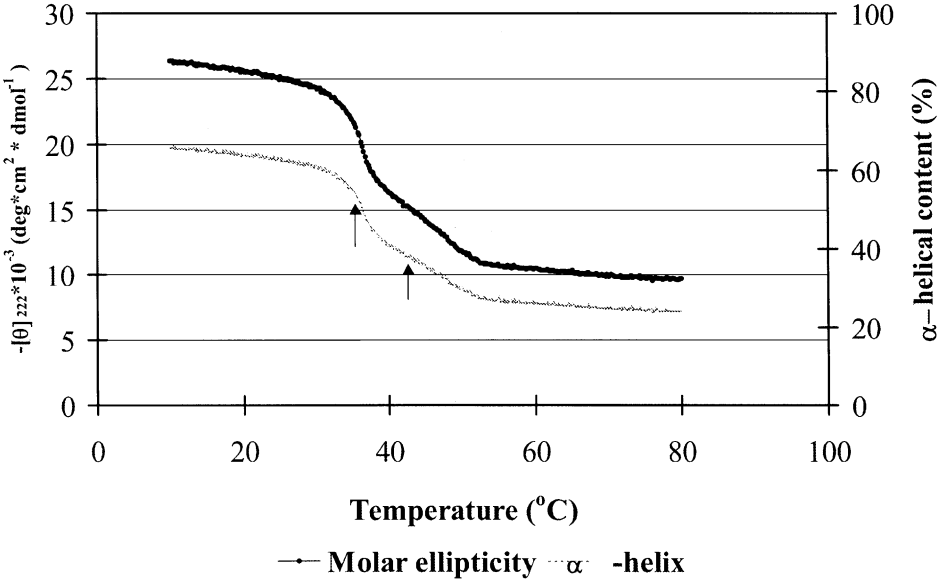

Fig. 4. Changes in molar ellipticity at 222 nm and α-helical content of actomyosin heated at 1 °C/min.

content before heating was 66%, while it decreased to 24.2% upon heating to 80 °C, indicating that approximately 63% of the α-helical portions of the actomyosin molecules unfolded by heating to 80 °C. Two distinct cooperative transitions of threadfin bream actomyosin, exhibited by different slopes, were found at 36.1 and 47.9 °C, respectively (Fig. 4).

The transition of actomyosin helix–coil occurs in myosin because α-helicity of actomyosin belongs to the myosin tail (Ogawa et al., 1995). Burke, Hirnmelfarb, and Harrington (1973) reported that the melting profile of rabbit myosin rod was also biphasic, with transition temperatures at 44 and 55 °C. Multiphasic transitions of myosin rod, which indicated the existence of a quasi-independent melting domain within the structure, were reported by Smyth, Smith, Vega-Warner, and O'Neill (1996). In addition, two sharp decreases in the helicity of pollock myosin, occurring between 28.2 and 41.3 °C, were reported by Togashi, Kakinima, Nakaya, Ooi, and Watanabe (2002). Alaska pollock *(Theragra chalcogramma)* lives at low environmental temperatures in the range 2–5 °C (Togashi et al., in press) and its myosin exhibited lower α-helical transition than actomyosin of threadfin bream. It is therefore postulated that the two transitions observed in our study were contributed by unfolding of the α-helical portion of the myosin rod.

3.5. Differential scanning calorimetry (DSC)

Threadfin bream actomyosin exhibited three major transitions at 38.4, 51.0, and 80.7 °C with onset temperatures of 36.5, 47.0. and 76.2 °C, respectively. Enthalpies of denaturation of each major transition were 0.152, 0.169, and 0.251 J/g respectively. Wright, Leach, and Wilding (1977) reported that actomyosin of rabbit exhibited three transitions, with myosin transitions at 51.5, 60 °C and actin transition at 73 °C. Three endothermic transitions have also been reported in DSC thermograms of rabbit (Wright et al., 1977; Wright & Wilding, 1984) and fish myosin (Lo et al. 1991; Togashi et al., 2002). Multiple transitions of myosin imply structural changes in discrete regions of the myosin molecule, namely the hinge, head, and rod regions (Wright & Wilding, 1984). Lo et al. (1991) reported that the S-1 sub-fragment of black marlin showed one transition at 41 °C, but the rod sub-fragment gave two peaks at 41 and 62 °C, respectively.

In our study, the first two transitions from DSC were similar to the transition temperatures obtained from the α-helical content (Fig. 4). Therefore, the transitions at 36.5 and 47.0 °C could result from structural changes of threadtin bream myosin, while transition of actin was at 76.2 °C. Lower transition temperatures of threadfin bream actomyosin indicated its lower thermal stability as compared with rabbit. Thermal stability of myosin increases as the species adapts to higher environmental temperatures (Davies, Bardsley, Ledward, & Poulter, 1988; Hasting, Rodger, Park, Matthews, & Anderson, 1985).

3.6. Oscillatory dynamic rheology

Storage moduli (G') of threadfin bream actomyosin started to increase at 34.5 °C, reached the first peak at 38.8 °C, and gradually decreased before steadily increasing again at 46.3 °C (Fig. 5). An initial increase of G' indicated the cross-linking of myosin filament, resulting in the transformation from a viscous sol to an elastic network (Egelandsdal, Fretheim, & Samejima, 1986). The onset of gel network development, based on G' (34.5 °C), was slightly lower than the melting temperature obtained from CD spectra (36.1 °C) and the onset of denaturation temperature obtained from DSC (36.5 °C). These results indicated that aggregation of actomyosin and denaturation might have occurred simultaneously.

Hydrophobic interactions and disulfide linkages also appeared to be involved in aggregation of actomyosin (Figs. 2 and 3). As actomyosin unfolded its helical structure, hydrophobic interactions and disulfide linkages prevailed. Formation of such interactions was greater at relatively high temperature (>50 °C) when the extent of unfolding was increased (Fig. 4), corresponding to a stronger gel network at >46.3 °C.

The decline of G' after reaching the first peak has also been reported in myosin (Egelandsdal et al., 1986; Wu, Hamann & Foegeding, 1991; Yongsawatdigul & Park 1999) and myofibrillar proteins (Xiong & Blanchard, 1994a, 1994b). There is no evidence of myosin heavy chain (MHC) or actin degradation in the studied temperature range (Fig. 6). Therefore, a decreased G' was unlikely to be caused by proteolysis. Helix-to-coil

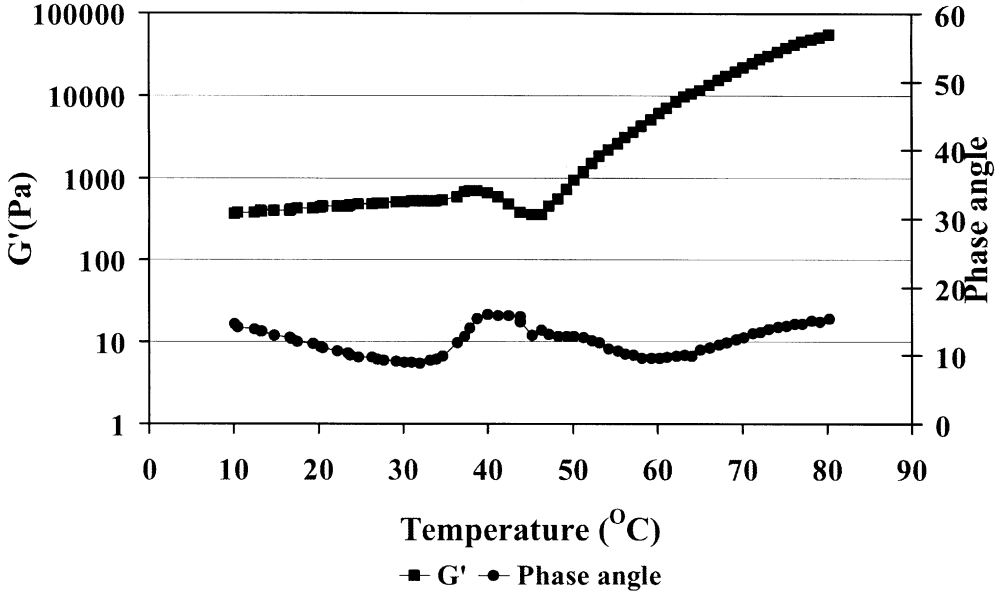

Fig. 5. Changes in storage modulus (G') and phase angle of threadfin bream actomyosin.

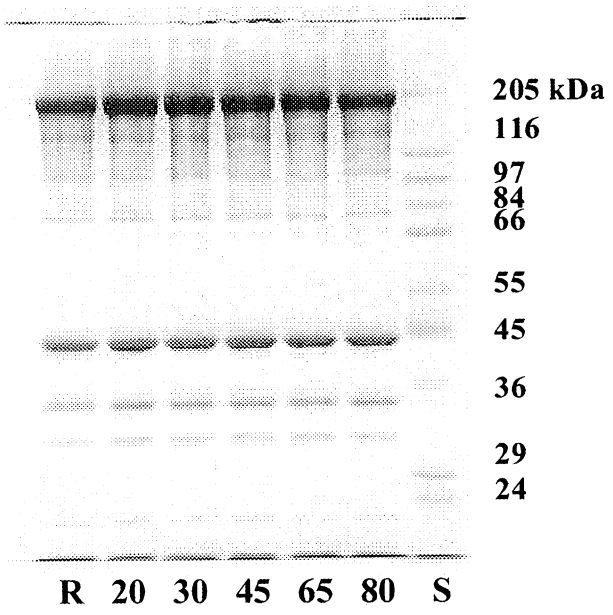

Fig. 6. SDS-PAGE patterns of threadfin bream actomyosin heated to various temperatures at 1 °C/min. R, unheated actomyosin; S. broad-range molecular weight standard. Numbers indicate final heating temperature.

transformation of myosin leads to a large increase in the fluidity of semi-gels and may disrupt some protein networks that have already been formed, resulting in a declined storage modulus (Xiong & Blanchard, 1994a). Subsequently, formation of new bonds produced a more permanent protein network structure, observed at 46.3 °C.

The phase angle, a ratio of G''/G', gradually decreased from 14 to 9 °C when heated from 10 to 32 °C (Fig. 5). It then increased to a maximum at 41 °C and decreased thereafter, corresponding to an increase of G'. It appeared that threadfin bream actomyosin underwent conformational changes and unfolded its helical structure at 36 °C, based on DSC thermogram and CD spectra. Denatured proteins, especially myosin, aggregated to form gel networks. This supports the idea that denaturation and aggregation processes occurred simultaneously.

As the temperature increased to >41 °C, unfolding of myosin progressively increased, allowing the formation of intermolecular bonds, namely hydrophobic interactions and disulfide linkages. As a result, an increase of G' and a decrease of phase angle were observed at >41 °C. Therefore, development of a strong gel network of threadfin bream actomyosin required a relatively high temperature (>41 °C). Visessanguan et al. (2000) reported a similar pattern of the phase angle of myosin extracted from arrowtooth flounder, except that a decrease of phase angle was at 35 °C, instead of 41°C, as found in our study, probably due to a difference in the thermal stability of each species. In addition, a decrease of phase angle of Pacific whiting surimi began at 30 °C (Esturk, Park, & Kim, 2003). This could imply that, not only denaturation, but also aggregation, of threadfin bream actomyosin took place at relatively higher temperatures than those of cold water species.

Yongsawatdigul, Park, and Worratao (2002) reported that threadfin bream surimi exhibited an increased breaking force when pre-incubated at 40 °C up to 2 h, followed by heating at 90 °C. This is known as the "setting" phenomenon. High temperature setting was also reported in tropical tilapia (*Oreochromis niloticus*) surimi (Klesk, Yongsawatdigul, Park, Viruihakul, & Viratchakul, 2000). In contrast, coldwater fish, such as Pacific whiting and Alaska Pollock, typically exhibited low temperature setting at 25 and 5 °C, respectively (Klesk et al., 2000; Park, Yongsawatdigul, & Lin, 1994). Denaturation and aggregation patterns of threadfin bream found in our study can explain the setting mechanism of threadfin bream surimi, which requires a relatively higher temperature. Pre-incubation of actomyosin (surimi) sol at 40 °C allows myosin to unfold and aggregate to form a three-dimensional gel network, resulting in an enhancement of gel strength.

4. Conclusion

Actomyosin of threadfm bream underwent thermal denaturation and aggregation at relatively high temperatures compared to coldwater species, as shown by increased surface hydrophobicity, reactive sulfhydryl group, loss of α-helix structure of myosin, and changes in G' as well as phase angle. Disulfide linkages and hydrophobic interactions appeared to be critical for the gel network development of threadfin bream actomyosin.

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