Effects of Proteolysis and Mechanism of Gel Weakening in Heat-Induced Gelation of Fish Myosin

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Addition of papain decreased the onset temperature and the rate at which G' developed during heat-induced gelation of arrowtooth flounder myosin. Frequency sweep results revealed that G' markedly decreased in proportion to the amount of papain added. However, use of E-64, a cysteine proteinase inhibitor, reversed the effects of papain and protected myosin heavy chain from degradation. DSC thermograms indicated papain significantly decreased the enthalpy required to induce myosin denaturation without significant changes in onset and maximum transition temperatures. Thermal denaturation kinetics indicated decreases in both the activation energy and the rate of myosin denaturation. CD studies revealed a rapid decrease in α-helical content, indicating the initial degradation of myosin molecules mostly occurred in the tail region. These results suggested that proteolysis affected thermal properties and reactivity of myosin during heating. Although myosin gel could be formed, structural disruption resulted in lower gelling ability and rigidity of the formed gel.

Keywords: *Proteolysis; myosin; gelation; Arrowtooth flounder*

INTRODUCTION

Functional properties of a protein play an important role in determining the textural qualities and physical behavior of a food during preparation, processing, and storage. Ability to form a gel upon heating is an important functional property of myosin, which is considered to be the primary component responsible for surimi gel formation (Sano et al., 1988). Myosin is a multidomain protein with two large heavy chains and four light chains arranged into an asymmetrical molecule with two globular heads attached to a long α-helical rodlike tail (Privalov, 1982; Lopez-Lacomba et al. 1989). Both head and tail portions of myosin have been shown to participate in gel formation (Taguchi et al., 1987; Chan et al., 1993). Structural changes that occur in the head and tail portions of myosin, as a result of thermal denaturation, enable myosin to form gels through protein−protein interactions. However, the native conformation of myosin is of primary importance for proper gelation. To form highly viscoelastic gels, sequential unfolding of different protein structural domains and ordered formation of protein−protein interactions are essential (Sharp and Offer, 1992).

The gelling property of myosin is highly related to species and the conditions of myosin preparation. Species differences in the gel-forming ability of fish muscle are attributed to differences in the cross-linking of myosin heavy chain (MHC) associated with the surface hydrophobicity displayed by the unfolded domains of the MHC and the temperature at which such domains unfold (Wicker et al., 1986; Chan et al., 1992). It is postulated that the cross-linking ability of the MHC may be localized at one or more discrete portions of the molecule, and any structural/functional differences in these loci among different fish myosins might explain species differences in gel-forming ability (Chan et al., 1993). In addition to the species, the ability to form myosin gel is also related to structure and has been shown to be highly dependent on ionic environment, pH, and heating (Xiong, 1997).

Proteolytic degradation of myofibrillar proteins, particularly myosin, is a major concern in the manufacture of surimi, especially for those produced from soft-textured fish species which contain high levels of endogenous proteinases, that is, cathepsins and heat stable alkaline proteinase (An et al., 1996). Proteolysis can take place during post-mortem storage and processing and results in undesirable flavor and texture alterations (Asghar and Bhatti, 1987). The gel-softening phenomenon or "modori" observed at 50−70 °C was attributed to myosin hydrolysis by some heat-activated proteases (Lin and Lanier, 1980; Deng, 1981). Quality variations within a species during different seasons has been reported to be associated with proteolysis (Beas et al., 1991). During and after spawning, or before the feeding season, fish flesh loses its gel-forming ability as a result of increased proteolytic activity, which leads to the proteolytic breakdown of muscle tissue. Such proteolysis is accompanied by a decreased level of myofibrillar protein and an increased moisture retention in the tissue. Proteolysis has been shown to be detrimental to surimi quality by substantially lowering the gel strength (Morrissey et al., 1995). However, the mechanism by which proteolysis interferes with surimi gelation has not been clearly elucidated. Thus, the

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objective of this study was to investigate how proteolysis affects heat-induced gelation in the model system prepared with fish myosin and papain. The action of papain closely resembles that of fish cysteine proteinases, which play an important role in tissue softening of fish (Yamashita and Konagaya, 1990; Rawlings and Barrett, 1994; Seymour et al., 1994). Arrowtooth flounder (ATF) was used in this study because it is an abundant fish species caught off the Pacific region of North America and its texture suffers from high levels of autolytic activity in the muscle.

MATERIALS AND METHODS

Reagents. Potassium chloride (KCl), sodium azide (NaN_3), Tris base, 1-(L-*trans*-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), 2-mercaptoethanol (βME), ethylene glycol bis(β-aminoethyl) ether *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), magnesium acetate, Tris maleate, adenosine triphosphate (ATP), sodium bicarbonate, magnesium chloride, sodium phosphate, potasium phosphate, glycerol, and 2× crystallized papain (EC 3.4.22.4) were purchased from Sigma Chemical Co. (St Louis, MO). Phenylmethanesulfonyl fluoride (PMSF) was purchased from Calbiochem Co. (La Jolla, CA). Sodium dodecyl sulfate (SDS) and urea were purchased from Bio-Rad Laboratories (Hercules, CA).

Fish Samples. Arrowtooth flounder were provided by the National Marine Fisheries Service, Utilization Research Laboratory, Kodiak, AK. Trawl-caught arrowtooth flounder were obtained fresh and hand-filleted. The fillets were vacuum-packed in polyethelene bags, frozen immediately at −20 °C, and transported in dry ice overnight to the OSU Seafood Laboratory. Frozen fillets were kept frozen at −50 °C until used.

Myosin Preparation. Myosin was extracted according to the method of Martone et al. (1986) with slight modifications. All steps were performed at 0−4 °C to minimize proteolysis and protein denaturation. Arrowtooth flounder fillets were finely chopped and added with 10 volumes of buffer A (0.10 M KCl, 1 mM PMSF, 10 μM E-64, 0.02% NaN_3, and 20 mM Tris-HCl, pH 7.5). After incubation on ice for 10 min with occasional stirring, washed muscle was recovered by centrifugation at 1000*g* for 10 min. The pellet was suspended in 5 volumes of buffer B [0.45 M KCl, 5 mM βME, 0.2 M $Mg(CH_3COO)_2$, 1 mM EGTA, and 20 mM Tris-maleate, pH 6.8], and ATP was added to a final concentration of 10 mM. The mixture was kept on ice for 1 h with occasional stirring and centrifuged at 10000*g* for 15 min. Supernatant was collected and added slowly with 25 volumes of 1 mM $NaHCO_3$ followed by incubation for 15 min on ice. Precipitated myosin was collected by centrifugation at 12000*g*, resuspended gently with 5 volumes of buffer C (0.50 M KCl, 5 mM βME, and 20 mM Tris-HCl, pH 7.5), and added with 3 volumes of 1 mM $NaHCO_3$ and $MgCl_2$ to a final concentration of 10 mM. The mixture was kept overnight on ice prior to centrifugation at 22000*g* for 15 min. Myosin was recovered as pellet and used immediately or stored at −20 °C in 50% glycerol.

Electrophoretic Analysis of ATF Myosin. Extracted myosin was analyzed on sodium dodecyl sulfate−polyacrylamide gel electrophroresis (SDS−PAGE) according to the method of Laemmli (1970) to determine its purity and the extent of autolysis, if any, due to the possible presence of endogeneous proteases associated with myosin. About 0.5 g of myosin was incubated in a 55 °C water bath for different time intervals. After incubation, 2.5 mL of prewarmed (80 °C) 5% (w/v) SDS was immediately added and the solution was then held in an 80 °C water bath until the proteins were solubilized. Solubilized samples were mixed at a 1:1 (v/v) ratio with the SDS−PAGE sample buffer containing 1.5 M βME and boiled for 3 min. The samples, 10 μg, were loaded on the gel made of 4% stacking and 10% separating gels and subjected to electrophoresis at a constant voltage of 75 V using a Mini Protean II apparatus (Bio-Rad Laboratories Inc., Richmond, CA). After electrophoresis, the gels were stained with 0.125% Coomassie brilliant blue R-250 in 25% ethanol and 10% acetic acid and destained with 25% ethanol and 10% acetic acid. Molecular weights of the proteins were estimated using high and low molecular weight standards (Sigma Chemical Co. High molecular weight standards included rabbit muscle myosin (M_r = 200000), β-galactosidase (M_r = 116000), phosphorylase *b* (M_r = 97000), fructose-6-phosphate kinase (M_r = 84000), bovine serum albumin (M_r = 66000), glutamic dehydrogenase (M_r = 55000), ovalbumin (M_r = 45000), and glyceraldehyde-3-phosphate dehydrogenase (M_r = 36000). Low molecular weight standards included bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase (M_r = 29000), trypsinogen (M_r = 24000), trypsin inhibitor (M_r = 20000), α-lactalbumin (M_r = 14200), and aprotinin (M_r = 6500).

The intensity of protein bands was quantitated by scanning gels with an HP Deskscan II (Hewlett-Packard Co., Minneapolis, MN) and analyzing the image using a software package (National Institutes of Health Image 1.54, Washington, DC).

Papain Activity Assay. Papain activity was determined by using BANA as a substrate according to the modified method of Abe et al. (1994). Papain stock solution was prepared in 50 mM sodium acetate, pH 4.5, at a concentration of 3.2 mg/mL. To obtain the optimal absorbance range, papain solution was diluted 20 times in the same buffer, and 50 μL of the diluted solution was preincubated at 40 °C for 10 min with 450 μL of the assay buffer (25 mM sodium phosphate, pH 7.0, containing 20 mM βME). The reaction was started by adding 200 μL of 6.5 mM BANA, and the mixture was incubated at 40 °C for 10 min. The reaction was stopped by adding 1 mL of 2% (v/v) HCl in ethanol, and the color was developed by adding 1 mL of 0.06% (w/v) *p*-dimethylaminocinnamaldehyde in ethanol. Reaction products were measured spectrophotometerically at 540 nm. A blank was run in the same manner except that enzyme was added after the addition of the stopping reagent. One activity unit was defined as changes in absorbance of 1 at 540 nm per minute.

Determination of Degree of Proteolysis of ATF Mince. The extent of proteolysis of ATF mince by papain was monitored according to the modified method of Greene and Babbitt (1990). Three grams of chopped muscle was heated linearly from 25 to 80 °C at the rate of 1.3 °C/min with or without papain added at 2.5 and 5.0 mU (millunits)/mg of protein. The reaction was stopped by adding 27 mL of ice-cold 5% (w/v) trichloroacetic acid (TCA). The mixture was homogenized for 1 min using a Polytron (Brinkmann Instruments, Westbury, NY) and kept on ice for 1 h followed by centrifugation at 5000*g* for 10 min to collect the supernatant. Oligopeptide content in the supernatant was measured according to the method of Lowry et al. (1951). Proteolytic activity was expressed as micromoles of tyrosine released per minute.

Autolysis Pattern and Effect of Papain on Fish Mince. Three grams of chopped muscle was heated linearly from 25 to 80 °C at the rate of 1.3 °C/min with or without papain added at 2.5 and 5.0 mU/mg of protein. The reaction was stopped by adding 27 mL of 5% (w/v) SDS preheated to 95 °C. The mixture was homogenized for 1 min using a Polytron (Brinkmann Instruments) and incubated at 85 °C for 30 min. Insoluble debris was removed by centrifugation at 7800*g* for 10 min, and supernatant was used for electrophoretic analysis as previously described under Electrophoretic Analysis of ATF Myosin.

Dynamic Rheological Measurements. Rheological changes of myosin during thermal gelation were analyzed according to the method of Visessanguan et al. (2000) using a Bohlin CS-50 rheometer (Bohlin Instruments, Inc., Cranbury, NJ), operated in the small-amplitude oscillatory mode. Samples containing papain and/or E-64 were freshly prepared at 0 °C before each run. The amount of enzyme added was equivalent to 2.5 and 5 mU/mg of protein, respectively, as the activity was determined using BANA as a substrate. Samples were sheared at a fixed frequency of 0.1 Hz with a constant strain amplitude of 0.015 during heating over the range of 25−80 °C at 1 °C/min. Data were collected every 60 s during shearing

measurements. Network development was studied in real time by measuring changes in the storage modulus (G'), loss modulus (G''), and loss tangent (tan δ) as a function of increasing temperature. At the end of the heating cycle, samples were cooled and held at 25 °C for 2 min. The resulting myosin gels were characterized by measuring dynamic properties as a function of oscillatory frequency using the same strain amplitude used in thermal scan.

Differential Scanning Calorimetry (DSC). DSC studies were performed in a DuPont 910 differential scanning calorimeter (Du Pont Co., Wilmington, DE) according to the method of Visessanguan et al. (2000). Samples containing papain at 2.5 mU/mg of protein were prepared at 0−4 °C fresh before each run. Samples with or without added papain were sealed in hermatic DSC pans and accurately weighed to 14−20 mg of wet weight on a semimicro balance (Mettler Instrument Corp., Hightstown, NJ). All samples were scanned at 10 °C/min over the range of 2−100 °C using a DuPont mechanical cooling accessory. An empty hermatic pan was used as a reference. At least three determinations were made, and the most representative thermogram with the average onset and transition temperatures was reported.

Thermal Denaturation Kinetic Studies. Thermal denaturation kinetic constants were calculated according to a dynamic method (Wagner and Añon, 1985). Samples in the presence and absence of papain (2.5 mU/mg of protein) were scanned from 2 to 100 °C at heating rates of 2, 5, 7, 10, 13, 16, and 20 °C/min at least twice according to an ANSI-ASTM E698/79 (1979) method. On the basis of a plot of the logarithm of the program rate versus the peak temperature, corresponding activation energy (E_a), pre-exponential factor of the Arrhenius equation (Z), and rate constant (K_d) of myosin denaturation were calculated using eq 1 (Ozawa, 1970)

$$-\ln(\beta/T^2_{max}) = \ln(ZR/E_a) - E_a/RT_{max} \quad (1)$$

where β represents the heating rate (K min^{-1}), T_{max} the peak temperature (K), and R the gas constant (cal/mol/K).

α-Helical Content. α-Helical content of myosin was analyzed using a JASCO A-500 spectrophotometer (Japan Spectroscopic Co., Ltd., Tokyo) equipped with a temperature control unit (Visessanguan et al., 2000). Myosin pellet was dissolved in cold 0.6 M KCl/20 mM potassium phosphate, pH 7.0, and centrifuged at 7800*g* for 10 min. The supernatant was used for circular dichroism (CD) analysis after dilution to 0.4 mg/mL in the same buffer. Papain was added to the supernatant at the ratio of 2.5 mU/mg of protein at 0 °C before each run. α-Helical content was estimated using eq 2 on the basis of $[\theta]_{222}$ of poly(L-glutamic acid), which possesses the complete helical structure (Holzwarth and Doty, 1965):

$$\alpha\text{-helical content} = (100 \times 115 \times [\theta]_{obs})/(10 \times C'' \times -40000) \quad (2)$$

$[\theta]_{obs}$ and C'' represent the observed ellipticities at 222 nm and protein concentration, respectively.

Protein Determination. Protein concentration was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard. Myosin was first solubilized for 5 min in 5% (w/v) SDS preheated to 95 °C and further incubated at 85 °C for 30 min. Insoluble debris was removed by centrifugation at 7800*g* for 10 min, and supernatant was used for protein analysis.

Statistical Analyses. Obtained data were analyzed by analysis of variance (ANOVA). Mean difference was determined using the least significant difference (LSD) multiple-range test (Statgraphics version 6.0, Manugistics Inc., Rockville, MD). Significance of difference was established at $p \leq 0.05$. The least-squares linear regression was applied and slopes were compared by using Student's t test (Ramsey and Schafer, 1996).

RESULTS

Purity and Autolytic Activity Associated with Extracted Myosin. The purity of extracted myosin was estimated to be 90% by the densitometric analysis of SDS−PAGE gel (Figure 1). The appearance of smeared bands in the control group indicated degradation products accumulated prior to the myosin extraction. Because no notable changes were observed in the electrophoretic patterns of myosin by the incubation for up to 30 min at 55 °C, the extracted myosin was postulated to be free of contaminated proteinases and adequate for further analysis.

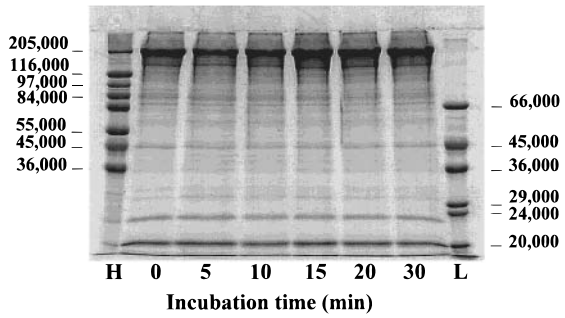

Figure 1. SDS−PAGE pattern of the purified ATF myosin incubated for various time periods at 55 °C. H and L designate high and low molecular weight protein standards, respectively.

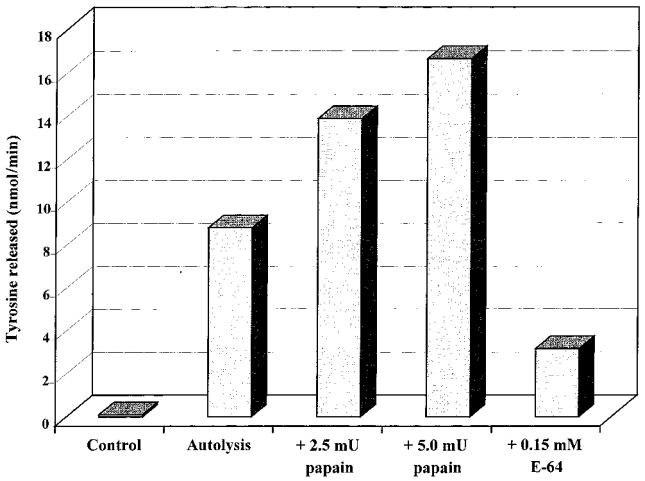

Figure 2. Extent of proteolysis of ATF mince caused by autolysis and the addition of papain during incubation under various conditions: (1) at 0 °C (control); (2) autolyzed during heating from 25 to 80 °C at 1.3 °C/min; (3) autolyzed during heating from 25 to 80 °C at 1.3 °C/min in the presence of 2.5 mU of papain; (4) autolyzed during heating from 25 to 80 °C at 1.3 °C/min in the presence of 5 mU of papain; (5) autolyzed during heating from 25 to 80 °C at 1.3 °C/min in the presence of 0.15 mM E-64. H and L designate high and low molecular weight protein standards, respectively.

Autolysis and Effect of Papain on Fish Mince. ATF mince was used as a substrate to compare the extent of proteolysis caused by papain and the extent of autolysis mediated by endogenous muscle proteinases. Autolytic degradation of fish mince, estimated as tyrosine released in oligopeptides, was ∼9 nmol/min during linear heating from 25 to 80 °C at the average of 1.3 °C/min (Figure 2). By adding papain at the concentrations used in the study, 2.5 and 5 mU/mg of protein, the extents of proteolysis caused by papain at the above ratios were determined to be about 1.6 and 1.9 times higher than that of autolysis of mince incubated under the same condition, respectively. Autolytic activity was substantially reduced by adding E-64, a cysteine protease inhibitor. The remaining activity was assumed to be due to the activities of other classes of proteinases.

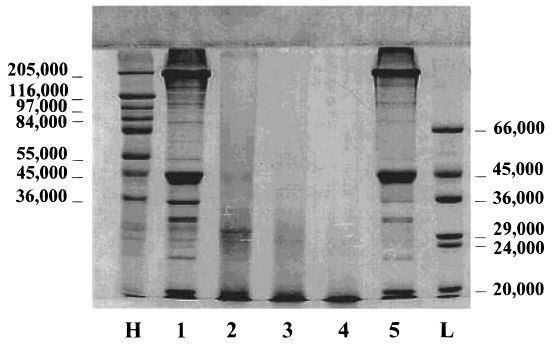

Figure 3. SDS−PAGE pattern of ATF mince incubated under various conditions: (1) at 0 °C (control); (2) heated from 25 to 80 °C at 1.3 °C/min; (3) heated from 25 to 80 °C at 1.3 °C/min in the presence of 2.5 mU of papain; (4) heated from 25 to 80 °C at 1.3 °C/min in the presence of 5 mU of papain; (5) heated from 25 to 80 °C at 1.3 °C/min in the presence of 0.15 mM E-64. H and L designate high and low molecular weight protein standards, respectively.

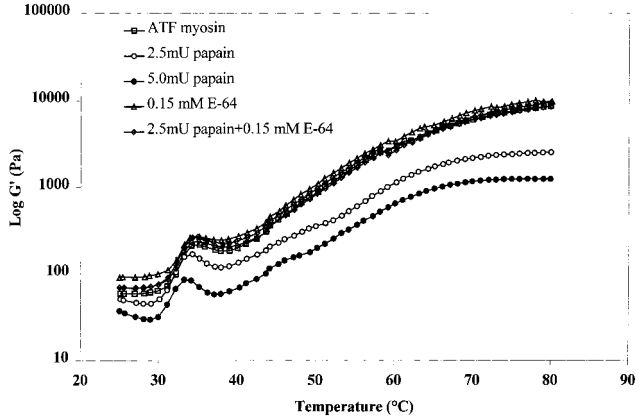

Figure 4. Change in storage modulus (G') during thermal gelation of ATF myosin added with papain and/or E-64.

Electrophoretic analyses of the degradation products indicated the extensive proteolysis of myofibrillar proteins occurred by the endogenous proteinases present in the ATF mince or the papain added (Figure 3). Most bands of the major myofibrillar proteins, that is, myosin heavy chain (MHC) ($M_r \sim 200000$), tropomyosin, troponin, and actin ($M_r \sim 45000$) disappeared after prolonged incubation with and without papain added. The disappearance of low molecular weight protein bands of mince added with papain, compared to the control, indicated the higher extent of proteolysis caused by papain. Those protein bands were presumed to undergo further proteolytic degradation until they were too small to be retained on the polyacrylamide gels. Only negligible degradation of some proteins was observed in mince with no obvious degradation of MHC when E-64 was added.

Effect of Papain on Heat-Induced Gelation of Myosin. Changes in G' as a function of temperature indicated ATF myosin formed gel in three stages: (1) gel setting, (2) gel weakening, and (3) gel strengthening (Figure 4). A similar pattern of gelation was also shown for myosin with added papain. The two systems, however, showed differences in the apparent onset temperatures. The first-derivative plots showed that the apparent onset temperatures at which those stages were observed were shifted to significantly to lower temperatures in the presence of papain ($P < 0.05$). It was also evident that myosin, when added with papain, exhibited a lower G' in all stages of gel development. To confirm that the adverse effects were due to proteolytic degradation, E-64, a specific cysteine proteinase inhibitor, was added to the myosin sample. Myosin added with E-64, regardless of both the presence and absence of papain, exhibited three stages of gel development, and the obtained G' values were higher than those of myosin alone. Besides the inhibitory action on proteinases, E-64 has an epoxy residue which may enhance gel formation of myosin by forming polymers. Because no differences were observed between the gelation characteristics of myosin alone and myosin added with papain in the presence of E-64, the loss in the gel-forming ability of myosin was postulated to be due to the proteolytic activity of papain.

More than 80% of G' was developed in the gel-strengthening stage. Therefore, the rate of G' development was determined by linear regression analysis, and final G' values were measured at the end of heating to evaluate gel formation of myosin under various conditions (Figure 5). Among the conditions tested, addition of papain resulted in the most severe reduction of both the rate of G' development and the final G' value developed. The values were reduced proportionally to the amount of papain added. When E-64 was added, myosin gels developed with papain showed the same rate of G' development and final G' value as those without papain.

Effect of Papain on Phase Angle. Addition of papain increased the phase angle (tan δ) throughout the heating process (Figure 6). The myosin added with 5.0 mU of papain showed the highest tan δ followed by that added with 2.5 mU of papain and the control without papain. The myosin added with E-64 showed the lowest tan δ. Corresponding to the changes in G' observed (Figure 4), the decrease in the phase angle of myosin with and without added papain reflected the transition of viscous myosin sol to elastic myosin gel as heating proceeded. The phase angle is derived from both G' and G'', which represent the elastic and viscous behaviors, respectively. For a completely elastic material, the phase angle is 0°, and for a purely viscous fluid, the phase angle is 90° (Egelansdal et al., 1995). Therefore, the results indicated that myosin gels formed in the presence of papain were less rigid than myosin gels formed by itself or in the presence of E-64.

Effect of Papain on Frequency Sweep Curves. Frequency sweep curves showed a linear relationship between the log G' and the log of the frequency of myosin gels formed under various conditions (Figure 7). Consistent with the results observed from thermal scanning experiments, the myosin gel formed by itself showed the highest log G' in the frequency sweep scan, whereas that added with papain exhibited the lowest log G'. The G' was shown to be dependent on the amount of papain added, thus showing the lowest log G' when the highest concentration of papain was added. Addition of E-64 resulted in an increase in log G' similar to the control value ($P > 0.05$). Generally, G' increased with the increase in frequency. For a totally elastic system, the G' values would be independent of frequency (Arntfield et al., 1989). Therefore, the slight dependence of frequency observed with the network reflected its viscoelastic nature. Because the protein gel network is generally developed at or above the denaturation temperature during the heating phase and continues during the cooling phase, frequency sweep of the gel can aid in the evaluation of the final gel properties, providing an

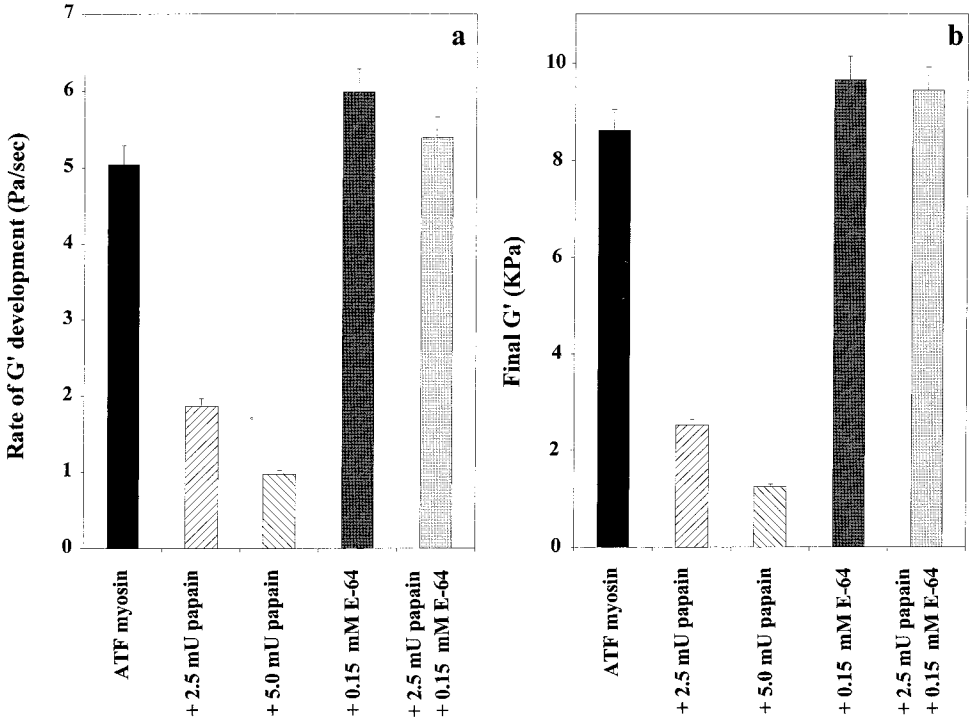

Figure 5. Developing rate and final storage modulus (G') of ATF myosin gels formed in the presence of papain and/or E-64. Rate was estimated by linear regression of the obtained data within the temperature range at ~20−80% of G' developed, and final G' was determined at 80 °C.

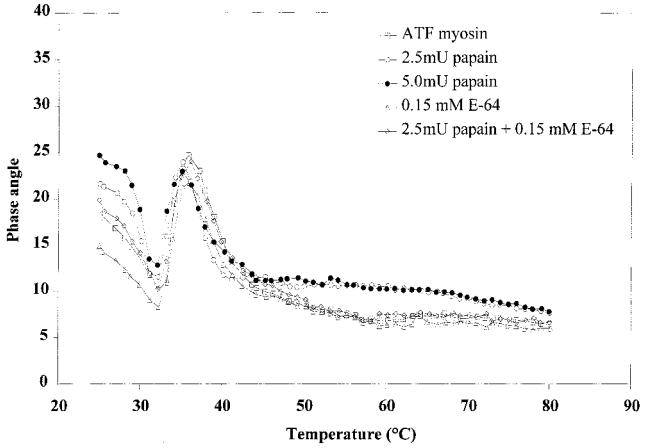

Figure 6. Change in phase angle (tan δ) during thermal gelation of ATF myosin in the presence of papain and/or E-64.

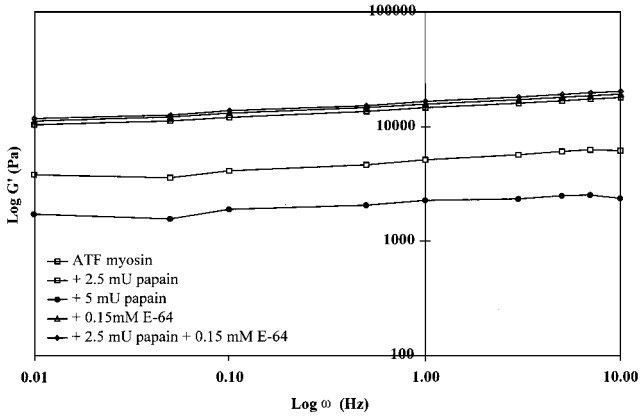

Figure 7. Change in the storage modulus (G') as a function of the oscillatory frequency (ω) of myosin gels formed in the presence of papain and/or E-64.

overview of the rheological behavior as a function of frequency (Cai and Arntfield, 1997).

Electrophoretic Study of Myosin Gels Formed after and during Dynamic Testing. The myosin gels obtained after dynamic testing is completed were subjected to electrophoretic analysis (Figure 8). The gel formed by itself showed the same pattern of protein bands as that of nonheated myosin suspension, indicating no degradation of myosin during heating. However, extensive degradation was observed in myosin gels formed in the presence of papain at the both concentrations tested. Adding E-64 reversed the effects of papain and protected myosin heavy chain (MHC) from proteolytic degradation. The results indicated that the degradation of ATF myosin, particularly that of MHC, was the main factor contributing to the decrease in the textural development of the gel.

To investigate the temperature range at which myosin was most susceptible to proteolysis, myosin added with 2.5 mU of papain was sampled periodically during heating on a dynamic rheometer. The electrophoretic analysis of myosin gels formed is shown in Figure 9. Myosin suspension was linearly heated from 25 °C to various temperatures of 30−60 °C under the same condition used in dynamic testing. By heating to 30 °C, no significant loss of MHC band was observed. However, a substantial decrease in MHC band was observed when it was heated to 40 °C or above. The results indicated myosin underwent substantial degradation between 30 and 40 °C before totally degrading at higher temperatures. It is surprising, however, to see that even such severely degraded products were still actively involved in gel formation.

Effect of Papain on Thermal Denaturation of Myosin. Different patterns in endothermic peaks and troughs were observed with DSC thermograms of myosin and that added with papain (Figure 10). Addition of papain significantly decreased the enthalpy required to induce myosin denaturation. Even though the onset

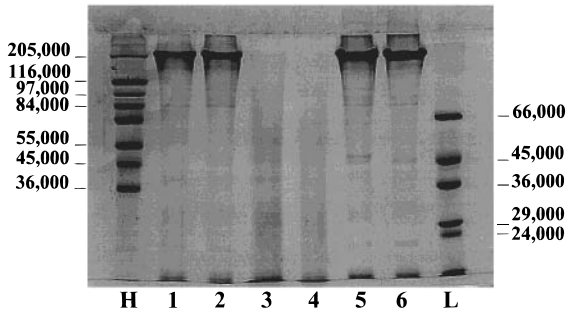

Figure 8. SDS−PAGE pattern of ATF myosin gels formed under various conditions during frequency sweep scan: (1) nonheated myosin suspension; (2) myosin (control); (3) myosin added with 2.5 mU of papain; (4) myosin added with 5 mU of papain; (5) myosin added with E-64 in the presence of 2.5 mU of papain; (6) myosin added with only E-64. H and L designate high and low molecular weight protein standards, respectively. The gels were heated linearly to 80 °C at 1 °C/min and cooled to 25 °C.

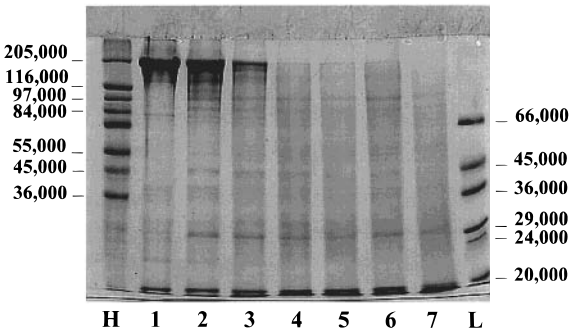

Figure 9. SDS−PAGE pattern of ATF myosin added with 2.5 mU of papain and linearly heated from 25 °C to temperatures 30, 40, 45, 50, 55, and 60 °C (lanes 2−6): (1) nonheated myosin suspension. H and L designate high and low molecular weight protein standards, respectively.

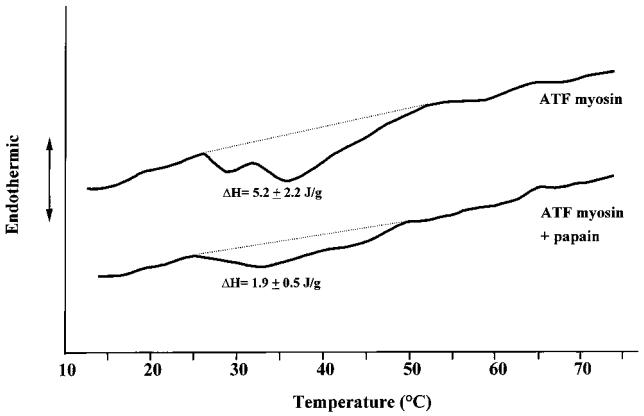

Figure 10. DSC endotherm of ATF myosin in the presence and absence of 2.5 mU of papain. Samples were prepared in 0.6 M KCl, 20 mM sodium phosphate buffer, pH 7.0, and heated from 2 to 80 °C at a rate of 10 °C/min.

(T_{onset}) and maximun transition temperatures (T_{max}) were not shown to be statistically different ($P > 0.05$), the differences indicated changes in the thermal property of myosin in a way that made it more sensitive to heat because less energy was required for denaturation.

Kinetic studies were conducted based on the Arrhenius model to determine how proteolysis affects myosin denaturation. The kinetic parameters were calculated by the variable program rate method (Ozawa, 1970) based on the experimental fact that T_{max} is sensitive to heating rate (β) while the conversion at the reaction peak remains constant (Duswalt, 1974). A trend of upward shifts in T_{max} of myosin transition was observed as the heating rate increased (data not shown). Linear correlation of the plot between $-\ln(\beta/T^2_{max})$ and $10^3/T_{max}$ was obtained with the coefficient >0.95 in which the slopes were found to be significantly different ($P < 0.05$). The activation energy (E_a) and the pre-exponential factor of the Arrhenius equation (Z) for denaturation of myosin added with papain were significantly lower than those of myosin (Table 1). The results indicated that myosin in the presence of papain has a tendency to undergo thermal denaturation more easily than myosin alone. However, with the higher rate of constant (K_d) calculated at any temperatures selected, myosin was shown to denature more rapidly than myosin added with papain. The effects of temperature on the rates of myosin denaturation are shown in Figure 11. With the rise in temperature, there was an increase in reaction rate. The Q_{10} factor, defined as the ratio of rate for each 10 °C rise in temperature, of myosin is higher than that of myosin added with papain. The results indicated that papain decreased the thermal sensitivity of myosin to undergo the denaturation process. Even though the activation energy of the denaturation process was lowered by papain addition, myosin added with papain was found to denature at a slower rate than myosin alone.

Effect of Papain on α-Helical Content of Myosin. A rapid decrease in the initial α-helical content of ATF myosin was observed by the addition of papain prior to heating. Because the decrease was observed only after papain addition, it was probably caused by proteolysis rather than thermal denaturation of myosin. By heating linearly from 9 to 65 °C at 1.5 °C/min, ATF myosin, both in the presence and in the absence of papain, showed a decrease in helicity as temperature increased (Figure 12). The melting temperature (T_m), at which 50% of the initial helical structure is lost, of myosin added with papain was significantly lower than that of myosin alone (34 °C). In addition, the first-derivative plots also indicate that the α-helical content of myosin with papain added started to decrease at the same temperature (~10 °C) as myosin alone but proceeded into fewer stages before completely unfolding at 65 °C (Figure 13).

DISCUSSION

The myosin model used in this study indicated three major changes associated with proteolysis during heat-induced gelation. They included (1) an upward shift of onset temperature (T_{onset}) at which gel setting, gel weakening, and gel strengthening were observed; (2) a significant decrease in the rate of gel development during gel strengthening; and (3) a significant decrease in the rigidity of the formed gel. Heat-induced gelation of ATF myosin involves both denaturation and aggregation processes (Visessanguan et al., 2000). Because the onset temperature in dynamic test indicates the occurrence of a viscoelastic gel matrix, which normally occurs at or above the denaturation temperatures of protein, a temperature shift reflects the change in the thermal properties and stability of myosin during the process of denaturation. Furthermore, changes in the gel-developing rate reflect the ability to form aggregates during the aggregation process, which eventually determines the properties of the final gels formed.

Thermal denaturation of myosin is an endothermic process in which myosin changes its conformation to a

Table 1. Denaturation Kinetic Constant of Myosin Heated in the Presence and Absence of Papain

				Kd (min^{-1})			
	T_{max} (°C)	E_a (kcal/mol)	Z (min^{-1})	60 °C	50 °C	40 °C	30 °C
control	36.3 ± 1.2	12.0	1.76×10^8	2.17	1.24	6.79×10^{-1}	3.58×10^{-1}
+ papain	38.6 ± 5.3	10.6	1.19×10^7	1.44	8.78×10^{-1}	5.20×10^{-1}	2.97×10^{-1}

[a] T_{max}, peak temperature at 10 °C/min; E_a, activation energy; Z, preexponential factor of Arrhenius equation; K_d, rate constant at selected temperatures. Values of E_a and Z were calculated from the slope in (β/T^2_{max}) versus $10^{-3}/T_{max}$ according to the dynamic method. Two or three samples of myosin were analyzed at eight different rates of heating. Line correlation coefficients were >0.95. Slopes were significantly different ($P < 0.05$).

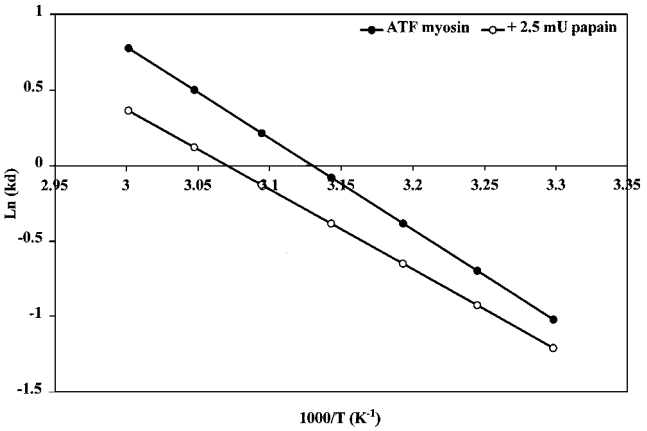

Figure 11. Arrhenius plot for determination of denaturation constants of myosin in the presence and absence of papain using data presented in Table 1.

more disordered structure without rupturing peptide bonds involved in the primary structure (Cheftel et al., 1985). However, in the presence of papain, the denaturation of myosin was complicated by the rupture of peptide bonds, producing degradation products that are heterogeneous in size and structure. Papain has a broad specificity of substrates; however, it has a marked preference for binding onto basic amino acid residues, such as Arg and Lys (Cleveland et al., 1977), which are relatively rich in the rod portion of myosin. Under a condition, limited myosin degradation can be induced by papain to cleave myosin into head and rod subfragments (Rattrie and Regenstein, 1977), which can be further hydrolyzed into smaller fragments or peptides unless enzymatic activity is stopped by adding a protease inhibitor.

Because degradation products are different in molecular composition and conformation, they are thought to be different in thermal properties as well. The differences in the shape of the heat flow curve and a significant decrease in enthalpy were observed as a result of papain addition. It is believed that the irregular shape of the thermogram was from composite of endothermic changes derived from each component of myosin that was partially degraded by papain and/or simultaneously denatured by heat. For intact myosin, each endothermic peak is derived from each domain structure, which unfolds at different temperatures depending on its thermal stability (Ogawa et al., 1993). Fragmentation of myosin resulted in reduction of molecular size and destruction of structural complexity; therefore, a lesser amount of energy was required to unravel the residual molecular structures. Unfolding of myosin has been shown to relate to the disruption of α-helical structure. As shown by CD studies, the significant decrease in enthalpy required for myosin denaturation in the presence of papain was probably due to an abrupt decrease in α-helical content. Because it occurred prior to heating, an abrupt decrease was thought to result from the partial hydrolysis of myosin molecules that probably caused unfolding of the helical structure. Myosin, especially at the tail region, is highly susceptible to proteolysis (Privalov, 1982; Lopez-Lacomba et al., 1989).

Kinetic studies of myosin denaturation indicated papain probably facilitated the denaturation process by reducing the activation energy. However, the resultant myosin was less sensitive to heat and denatured at a slower rate than intact myosin. The intact structure of myosin allowed each structural domain to unfold step by step and reflected the cooperative nature of the transition from the native conformation to the least structured state. Thus, the results imply the importance of the intact structure of myosin in the denaturation process.

Because denaturation and aggregation are continuous processes, changes in denaturation characteristics may also affect subsequent aggregation. Denaturation of the least stable domain at low temperature enables myosin molecules to associate with one another via head to head interactions possibly through disulfide bonds, so that interactions of uncoiled tail can follow at higher temperature (Sharp and Offer, 1992). The decrease in rate of G' development implied that addition of papain also resulted in a decrease in the gelling ability of myosin. It was postulated from the electrophoretic pattern of myosin gel formed in the presence of papain that the detrimental effects of proteolysis resulted from the degradation of MHC portions into smaller fragments. Even though cross-links and deposition of small fragments may occur, the resulting gel structures are much weaker than those of intact myosin.

The gel property of myosin is highly correlated to the length of double-stranded α-helical tail (Ishioroshi et al., 1982). Proteolysis resulted in the reduction in molecular weight and the loss of structural domains, which are probably essential for molecular interaction and binding. The gel-forming ability of myosin is associated with the surface hydrophobicity displayed by the unfolded domains of the MHC and the temperature at which such domains unfold (Wicker et al., 1986). Unfolding of the α-helical region in ATF myosin resulted in the exposure of hydrophobic and sulfhydryl groups, which are subsequently involved in the formation of intermolecular bonding during the aggregation process (Visessanguan et al., 2000). The extent of aggregation of fish myosin seems to depend on the amount of hydrophobic surface exposed on the heated molecules (Chan et al., 1992; Wicker et al., 1986). An increase in surface hydrophobicity is found to be important for gelation and other functional properties of proteins. It is postulated that the cross-linking ability of the MHC may be localized at one or more discrete portions of the molecule (Wicker et al., 1986). The involvement of hydrophobic interaction is a prerequisite for the formation of large myosin

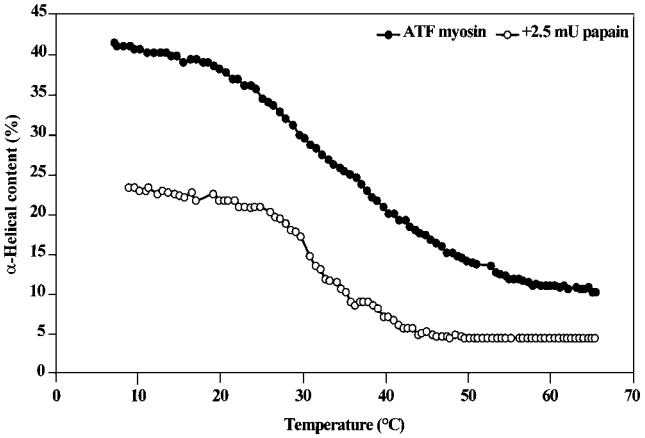

Figure 12. Changes in α-helical content of ATF myosin as a function of temperature in the presence and absence of 2.5 mU of papain.

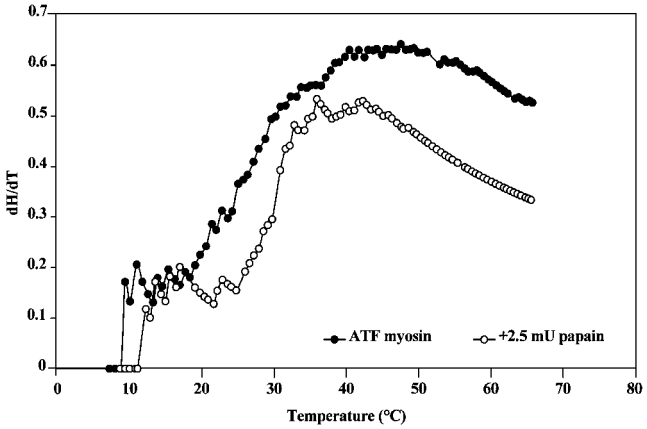

Figure 13. First-derivative plot of α-helical content of ATF myosin as a function of temperature in the presence of 2.5 mU of papain.

aggregates and an elastic gel. Consequently, anything that affects the surface hydrophobicity of myosin molecules may also affect the rheological properties of the muscle gel (Gill et al., 1992).

The lower gel modulus of myosin gels added with papain indicated the overall effect of proteolysis as a result of changes in myosin denaturation and aggregation. Similar to the detrimental effects on surimi gel strengths caused by endogenous muscle proteinases, this model system not only explains how proteolysis affects gelation but also emphasizes the importance of the structural integrity of myosin. The structural integrity facilitates the sequential unfolding of myosin and promotes the ordered formation of protein−protein interactions, which are essential for the formation of highly elastic protein gels. Studies of the various fragments of myosin obtained by limited proteolysis have shown that gels prepared from myosin showed the highest rigidity over those prepared from rod, HMM, and S1 subfragments under the identical pH, ionic strength, and protein concentration (Ishioroshi et al., 1982). It is well-known that maximal gel strength cannot be obtained from denatured myosin prior to the initiation of gelation (Niwa, 1992).

CONCLUSION

Proteolytic degradation of myosin resulted in the reduction in molecular weight and the loss of structural domains, which are essential for molecular interaction and binding. Myosin fragments were less stable to heat but denatured or aggregated at a slower rate than intact myosin. Even though cross-links and deposition of small fragments may occur, the resulting gel structures are much weaker than those of intact myosin, leading to lower gel modulus. These facts indicated that proteolysis affects myosin gelation by modifying their denaturation and aggregation properties.

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