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Effect of Low and High pH Treatment on the Functional Properties of Cod Muscle Proteins

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The functional properties of cod myosin and washed cod mince (myofibrillar protein fraction) treated at high (11) and low (2.5) pH were investigated after pH readjustment to 7.5. The solubility of refolded myosin was essentially the same as the native myosin. The pH-treated myofibrillar proteins had increased solubility over the whole ionic strength range studied. Acid and alkali treatment gave myosin and myofibrillar proteins improved emulsification properties, which were correlated with an increase in surface hydrophobicity and surface/interfacial activity. Enhanced gel strength was observed with acid- and alkali-treated myosin compared to native myosin, while the same treatment did not significantly improve the gel strength of acid- and alkali-treated myofibrillar proteins. The acid- and alkali-treated protein samples unfolded and gelled at a lower temperature than did the native proteins, suggesting a less conformationally stable structure of the refolded proteins. Functional studies show that acid and alkali treatment, which leads to partial unfolding of myosin may improve functional properties of cod myosin and myofibrillar proteins, with the greatest improvement being from the alkali treatment. The results also show that improvements in functionality were directly linked to the extent of partial unfolding of myosin on acid and alkali unfolding and refolding.

KEYWORDS: myosin; cod myosin; myofibrillar proteins; molten globule; pH; functional properties; solubility; emulsification; gelation

INTRODUCTION

The myofibrillar proteins play the most critical functional role in muscle foods, as they produce viscoelastic gel matrixes, entrap water, form strong cohesive membranes on lipid globules in emulsions, and are instrumental in forming strong flexible films at the air−water interface (*1*). Myofibrillar proteins are typically not soluble unless in high salt (>300 mM), and therefore salt is commonly added to meat-derived products so desirable functional attributes can be achieved. However, studies show that it is possible to solubilize myosin and the myofibrillar proteins at low ionic strength (*2*−*5*) and at low or high pH values (*6*).

Two processes have been developed in which functional protein isolates that are low in lipid and cellular membranes are produced from muscle using acid (pH 2−3) or alkaline (pH 10.5−11.5) solubilization of the proteins followed by isoelectric precipitation (pH 5.5) (*7*−*8*). Myosin is the main protein recovered in the processes, and the role acid and alkaline unfolding has on myosin's functionality is unknown. We have reported on the dissociation of the subunits of myosin and on the conformation of myosin at pH 2.5 and 11 and after subsequent adjustment to pH 7.5 (*9*). Our results showed that acid and alkali conditions each partially unfolded myosin in a different manner. These treatments led to the almost complete loss of myosins ATPase activity and more reactive sulfhydryl groups were exposed (*9*). It is of much interest to understand how the functionality of the myofibrillar proteins is affected after acid or alkaline treatment.

Myosin has been extensively investigated for its functional properties in foods. As the long rod of myosin is composed of many charged residues, the functionality of the protein is highly sensitive to changes in pH and ionic strength. The solubility properties of the protein are largely dictated by its rod, and thus, pH and ionic strength. Water-binding properties of myosin are also dictated by these properties. Myosin has been found to be the most effective emulsifier of all the muscle proteins (*10*) due to a prevalence of hydrophobic residues in the globular head region and the predominance of polar-charged groups in the fibrous rod. One of the most important functional properties of muscle proteins is their ability to form gels on heating, in which myosin is a key player (e.g., *11*−*12*). The mechanism by which myosin can form gels at high salt is strongly dictated by its dual fibrous-globular structure, in which head-to-head interactions are believed to initiate gel network formation on heating

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followed by cross-links of the rods forming a strand-like gel network (*1*). The nature and extent of gelation is highly pH- and salt- sensitive, pointing to the importance of electrostatic interactions of myosin.

Fish muscle proteins are used predominantly for their gelling ability in making products such as surimi, which is used to make kamaboko, the generic name given to a group of fish analogues such as crab sticks or fish sausage (*12*). Fish myosins are, however, typically characterized by a more unstable structure than their mammalian counterparts, as indicated by their lower thermal resistance to aggregation, higher tendency to lose light chains, increased tryptic susceptibility, and lower thermal inactivation of their ATPase (*13*−*17*). For this reason, they start to aggregate and gel at a lower temperature. Due to the instability of fish myosins, it is widely held that any denaturation in the protein will be detrimental to its ability to form gels, and it is commonly reported that functional ATPase activity is required for good gel quality (*18*−*19*). Three primary functional properties of acid treated, alkali treated and native myosin and myofibrillar proteins have been the focus in this study; solubility, emulsification properties and gelation behavior.

MATERIALS AND METHODS

Preparation of Cod Myosin and Myofibrillar Protein Fraction. The method described by Kristinsson (*20*) was employed to isolate myosin from cod muscle. To prepare protein fractions consisting mostly of myofibrillar proteins ground muscle was washed three times with distilled deionized water following a centrifugation step at 15 000*g* to remove soluble compounds and sarcoplasmic proteins (*21*). This fraction is referred to as myofibrillar protein fraction. Myosin and myofibrillar protein samples were kept on ice and used within 2 days for all functional studies. Protein concentration was determined using the method described by Lowry et al. (*22*)

Solubility of Myosin and Myofibrillar Protein Fraction. Myosin (1 mL solution; ∼30 mg myosin in distilled deionized water, pH 7) was added to distilled deionized water (29 mL) and the pH adjusted to 2.5, 7.5, and 11 with HCl or NaOH, and samples were gently homogenized for 1 min on ice in a Potter-Elvehjelm homogenizer. Myofibrillar protein solutions were prepared and treated the same way. The isolated myosin and myofibrillar proteins were studied at equal protein concentrations. Samples were kept at 2 °C for 30 min, and then the pH 2.5 and 11 samples were readjusted to pH 7.5 to give a protein concentration of 0.8 mg/mL. To glass tubes containing increasing amounts of dissolved KCl (final concentration of ∼0−600 mM), 3 mL of the native and refolded myosin solutions were added and thoroughly mixed to dissolve the salt. Samples were kept on ice for 1 h and were periodically vortexed at low speed. The glass tubes were then centrifuged at 2200*g* for 20 min in a Sorvall RT6000 refrigerated tabletop centrifuge at 4 °C. The supernatant was assayed for protein concentration using the method of Lowry et al. (*22*). Protein concentration in the supernatant after centrifugation was divided by the protein concentration of the protein solution before centrifugation, and solubility was reported as percent solubility.

Emulsification Properties of Myosin and Myofibrillar Protein Fraction. Proteins were unfolded at pH 2.5 and 11 for 30 min at a concentration of 5 mg/mL, and refolded to pH 7.5 by readjusting pH with addition of HCl or NaOH. The refolded and native proteins were tested for their ability to form emulsions according to the procedure by Pearce and Kinsella (*23*). Emulsions were prepared by mixing 45 mL protein solution to 12 mL corn oil (3.75 mg protein/mL emulsion) using a Polytron tissue homogenizer (Kinematica Gmbh, Switzerland), setting the rheostat at speed 20. The emulsion (0.05 mL) was then immediately mixed in 15 mL of 3.5 mM (0.1%) SDS solution and the absorbance read at 500 nm. This method essentially measures turbidity which is translated to an emulsification activity index (EAI)

$$\mathrm{EAI} = \frac{2 \cdot 2.303 \cdot A}{l \cdot C \cdot \phi} \cdot k$$

where A is the absorbance at 500 nm, l is the path length of the cuvette, C is the protein concentration in the aqueous solution (mg/mL), ϕ is the oil volume ratio (volume oil/total volume), and k is the dilution factor of the protein in the SDS solution. The units are m^2/g protein.

Emulsion stability was studied by adding 14 mL of the above emulsion in polyethylene tubes and placing them with cap on in refrigerated storage (5 °C) and following oil and aqueous phase separation periodically for 96 h. Emulsion stability was expressed as percent.

$$\text{Emulsion stability} = \frac{\text{Volume of cream layer}}{\text{Total volume of emulsion}} \cdot 100$$

Surface and Interfacial Properties of Myosin and Myofibrillar Protein Fraction. Surface and interfacial tension of myosin solutions were determined using a Krüss K 10 ST digital tensiometer (Krüss GmbH, Hamburg, Germany) employing a platinum−iridium ring. Solutions (30 mL) of native (pH 7.5), acid [pH 2.5 (30 min) → 7.5] and alkaline [pH 11 (30 min) → 7.5] treated myosin solutions (0.5 mg/mL) were added to a glass vessel which was then placed in a thermostated (10 °C) vessel in the tensiometer. For surface tension measurements the ring was lowered into the solution, the solution left to equilibrate for 1 h and the ring pulled up by the instrument which recorded the force needed to do this in mN/m. The instrument was zero adjusted in the air for the surface tension measurements. For interfacial tension measurements 40 mL of hexadecane (lower density phase) was carefully layered using a transfer pipet on top of the protein solution (higher density phase) which had the ring already inserted. The measurement was then performed just as for the surface activity after a 1 h equilibration period. Zero adjustment was done by measuring the hexadecane surface tension which was adjusted to zero prior to measuring the interfacial tension. The vessel speed was set on 3, and a temperature of 10 °C was kept throughout the measurement. To calculate the force needed to pull the ring through a surface or interface the following equation was employed

$$\sigma = \sigma^* \cdot F = \frac{P}{U} \cdot F$$

where σ = absolute surface tension in mN/m, σ^* = measured surface tension in mN/m, F = correction factor, P = maximal force on ring, $U = 2\pi (R_i + R_a)$, R_i = inner ring radius, R_a = outer ring radius.

Because liquids vary in density, which in turn varies with liquid temperature, the surface and interfacial tension could not be read directly from the instrument. The correction factor (F) takes the weight of the liquid that is lifted by the ring into consideration and is calculated based on the density of the solutions

$$F = 0.725 + \sqrt{0.4036 \cdot 10^{-3} \cdot \frac{\sigma^*}{(D - d)} + 0.0128}$$

where σ^* = measured surface tension in mN/m; and D, d = densities of both phases in g/cm_3. The densities of the protein solutions and hexadecane were measured on a Mettler-Toledo densitometer.

Surface Hydrophobicity of Myosin. Hydrophobicity of refolded myosin was studied from its ability to bind a hydrophobic dye, 8-anilino-1-naphthalene-sulfonic acid (ANS) as described previously (*9*). The fluorescence emission intensity of the myosin-ANS complex at 470 nm was plotted against myosin concentration (0−0.078 mg/mL).

Thermal Aggregation of Myosin. Myosin refolded from pH 2.5 and 11 to pH 7.5 (after 30 min unfolding in the absence of salt) and native myosin (at pH 7.5) was tested for their ability to aggregate on heating in 600 mM KCl. Myosin samples (0.45 mg/mL) were heated in a quartz cuvette at 1.5 °C/min (from 5 to 90 °C) using a Neslab temperature controlled water bath (Neslab Instruments, Portsmouth, NH). A thin layer of mineral oil was layered on top of the sample to prevent evaporation. Aggregation was monitored by measuring turbidity at 350 nm in a Hitachi-3110 double beam spectrophotometer (Hitachi Instruments, Inc., San Jose, CA), with buffer as a reference.

Viscoelastic/Gelation Properties of Myosin and Myofibrillar Protein Fraction. Viscoelastic changes of isolated myosin and the myofibrillar protein fraction during heating and cooling was determined

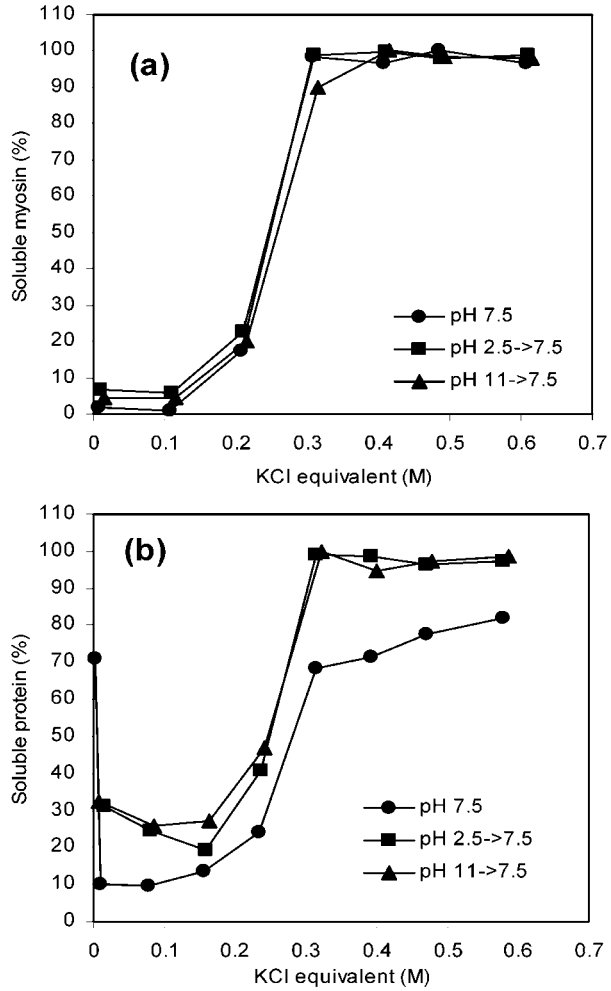

Figure 1. Solubility of native and acid/alkali treated cod myosin (a) and cod myofibrillar proteins (b). Protein concentration was 0.8 mg/mL.

using a Bohlin CS-10 rheometer (Bohlin Instruments, Inc., Cranbury, NJ), employing a pair of coaxial cylinders. A myosin pellett (5 mL) was mixed with one volume (5 mL) of distilled water, and the pH was adjusted to 2.5, 7.5, or 11 with HCl or NaOH and kept on ice for 30 min. To the samples two volumes (10 mL) of 1.2 M KCl, 40 mM Tris buffer was added and immediately mixed to give a protein concentration of ~35 mg/mL, and the pH was adjusted to 7.5 with HCl or NaOH. Samples were then homogenized at low speed (speed 1) in a few bursts of 5 s each on ice in a Tissue-tearor homogenizer to break up any protein aggregates, and the pH checked again and readjusted if necessary. Myofibrillar proteins were treated the same way to yield a sample of 35 mg/mL. Samples were then degassed for 3 min in a Microcal (Microcal, Northampton, MA) degasser to remove air bubbles which could have interfered with the rheometric readings. Samples were run in the rheometer which operated in an oscillation mode with the sample sheared at a constant frequency of 0.1 Hz with a maximum shear strain of 0.01. A thin layer of mineral oil was layered on top of the sample to prevent evaporation. The sample was heated from 15 to 80 °C at 1.5 °C/min and cooled from 80 to 15 °C using a computer controlled Bohlin waterbath. Viscoelastic changes on heating were studied by following changes in storage modulus (G′). The storage modulus characterizes the rigidity of the sample.

RESULTS

Solubility of Native and pH-Treated Cod Myosin and Myofibrillar Protein Fraction. Solubility of native and pH treated cod myosin and myofibrillar protein fraction. Native and refolded myosin exhibited almost identical solubility properties at pH 7.5 in increasing amounts of KCl (**Figure 1a**). All samples were largely insoluble below 200 mM (pH-treated samples were slightly more soluble at low ionic strength), and a sudden increase in solubility was observed in the range of 200−300 mM. Above 300 mM, samples were almost 100% soluble. The solubility of acid- and alkali-treated cod myofibrillar proteins behaved similarly as refolded myosin did, as they were almost 100% soluble at 300−600 mM KCl (**Figure 1b**). The pH-treated myofibrillar proteins however exhibited higher solubility below 200 mM KCl than isolated refolded myosin did, between 20 and 30%. On the other hand, the untreated myofibrillar proteins, although exhibiting a similar salt dependency solubility curve, were less soluble at most all salt concentrations than the pH-treated samples.

Emulsifying Properties of Native and pH-Treated Cod Myosin and Myofibrillar Protein Fraction. The emulsification properties of native and refolded myosin were tested by its ability to emulsify and stabilize corn oil in water. Acid and alkaline treatment significantly improved the ability of cod myosin to form an oil-in-water emulsion. Emulsification activity increased by a factor of 1.19 and 1.56 for the acid- and alkali-treated myosin, respectively, compared to native myosin ($P < 0.05$) (**Figure 2a** and **Table 1**). The acid- and alkaline-treated cod myofibrillar proteins had also improved ability to form emulsions ($P < 0.05$) with the alkaline treatment yielding the highest activity index (**Figure 2a**). Although the myofibrillar proteins were tested at the same protein concentration, they gave lower emulsification activity indices compared to isolated cod myosin. It is clear from **Figure 2b** that the improved emulsification activity of the acid/alkaline treated proteins also resulted in significantly improved ability to stabilize the emulsions ($P < 0.05$). A value of 50% represents complete phase separation of the oil and water phase. The untreated myosin had very poor ability to stabilize the oil-in-water emulsion, as the emulsion was almost completely broken down after only 20 min at 5 °C (**Figure 2b**). The emulsion stabilized by the refolded proteins on the other hand was more slowly degraded, and after 48 h the emulsion had stabilized at a value close to 70%.

The improved ability of refolded myosin to form and stabilize emulsions raised questions as to whether the surface and interfacial properties of the refolded proteins had been modified, which could explain the protein's behavior. The results show that both interfacial and surface tensions of the solutions containing the refolded myosin were significantly ($P < 0.05$) lowered compared to native myosin in the following sequence: pH 11−7.5 > pH 2.5−7.5 > pH 7.5 (**Table 2**). The increase in interfacial and surface activity was therefore in good agreement with improved emulsification activity and stability. To obtain a more complete picture on the nature of the enhanced emulsification ability and interfacial/surface activity of the refolded proteins, they were assayed for their surface hydrophobicity using the hydrophobic fluorescent probe ANS. Results indicate that the refolded myosins are more hydrophobic as ANS fluorescence was higher at all protein concentrations tested compared to that of the native myosin (**Figure 3**). Myosin refolded from pH 11 had the highest surface hydrophobicity, according to the ANS assay.

Viscoelastic/Gelling Behavior of Native and pH-Treated Cod Myosin and Myofibrillar Protein Fraction. Acid or alkali treatment of myosin modified the viscoelastic behavior of myosin, as shown by the change in storage modulus with heating and cooling (**Figure 4**a). The storage modulus started to increase at a lower temperature for the pH-treated myosins, indicating a lower gelation temperature. Aggregation of myosin was different for the refolded proteins compared to the native protein (**Figure**

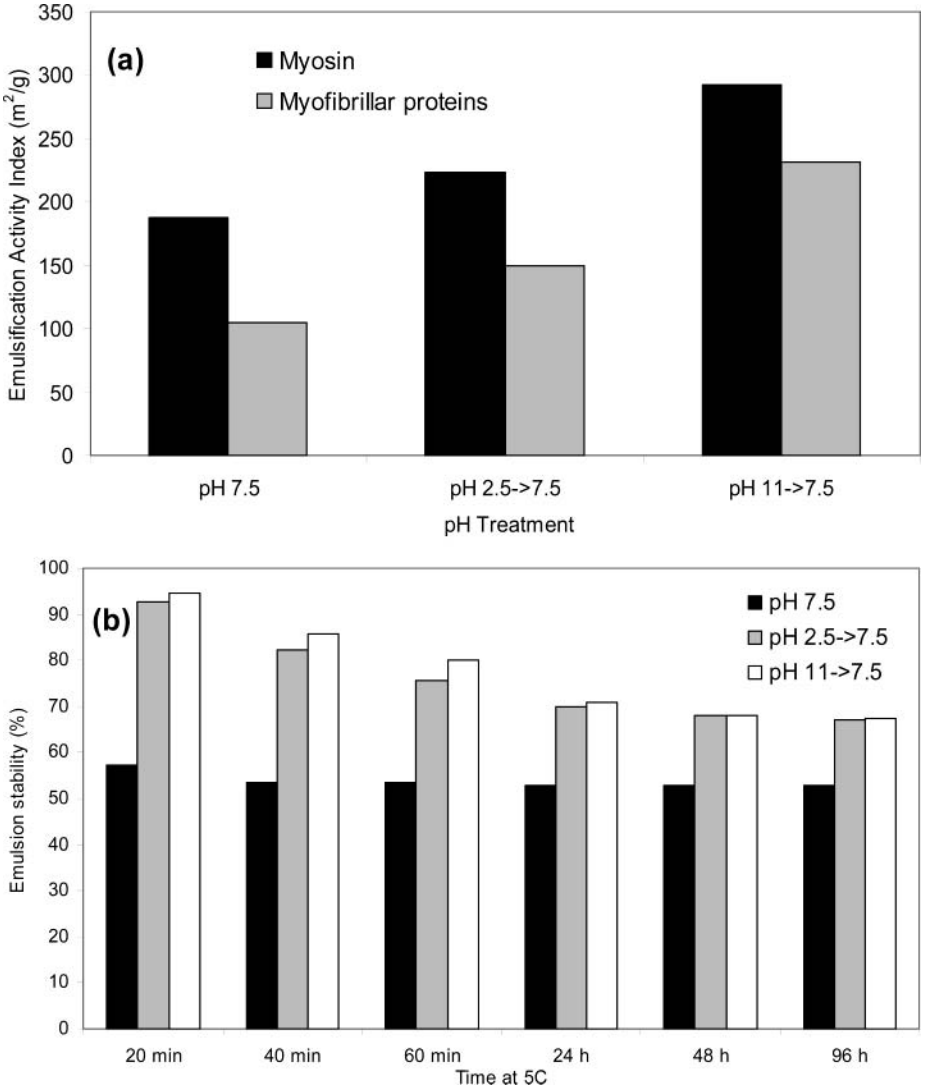

Figure 2. The ability of cod myosin and myofibrillar proteins to form and stabilize corn oil-in-water emulsions. (a) Emulsification activity index of native and refolded cod myosin and myofibrillar proteins. (b) Emulsion stability of native and refolded cod myosin. Emulsion pH was 7.5, and myosin/myofibrillar protein concentration was 3.75 mg/mL emulsion. Experiments were done in triplicate.

Table 1. Increases in Emulsification Activity Index (EAI) and ANS Binding of Refolded Cod Myosin Compared to Native Myosin[a]

pH treatment	increase in EAI	increase in ANS binding
pH 7.5 (control)	0	0
pH 2.5 → 7.5	1.19	1.21
pH 11 → 7.5	1.56	1.58

[a] The values represent the EAI or ANS binding values for the pH treated proteins divided by the value for the native protein at pH 7.5.

5). The native proteins started to aggregate between 35 and 40 °C and exhibited a cooperative thermal aggregation pattern. On the other hand, myosin refolded from pH 11 and 2.5 started to aggregate between 15 and 20 °C and 20 and 25 °C, respectively, and had a less cooperative thermal aggregation pattern, suggesting a less stable structure compared to the native proteins. After the onset of gelation, the storage modulus (G′) was higher for the pH-treated myosin compared to the native protein. Furthermore, the acid and alkali treated myosin gave stronger gels on cooling compared to native myosin, as indicated by the higher storage modulus after cooling to 15 °C, ∼1250 Pa for pH 7.5, ∼1500 Pa for pH 2.5−7.5, and ∼1900 Pa for pH 11−7.5 (**Figure 4a**).

Table 2. Surface and Interfacial Tension of Native and Refolded Myosin Solutions[a]

pH treatment	surface tension (mN/m)	interfacial tension (mN/m)
pH 7.5	56.03 ± 0.42^b	22.84 ± 0.36^b
pH 2.5 → 7.5	54.11 ± 0.25^c	20.78 ± 0.21^c
pH 11 → 7.5	51.88 ± 0.19^d	17.84 ± 0.33^d

[a] Myosin concentration was 0.5 mg/mL in 0.6M KCl, 20 mM Tris, pH 7.5. Triplicate experiments were performed. Surface tension without the protein was ca. 72 mN/m. [b−d] Samples with the same letter in a column are not statistically different ($P > 0.05$).

When myofibrillar proteins were subjected to the same gelation tests at an equal protein concentration as myosin (35 mg/mL), the onset of increase in G′ (i.e., gelation) was at a lower temperature for the acid- and alkali- treated myofibrillar proteins (**Figure 4b**), which is in agreement with that observed for myosin. There was no significant difference in gel strength on cooling, as samples had a final storage modulus of 115−130 Pa. The native sample was at 130 Pa, which was not statistically higher ($P > 0.05$) than the pH treatments. The gelling behavior was, however, different for both the acid- and alkali-treated myofibrillar proteins compared to the untreated

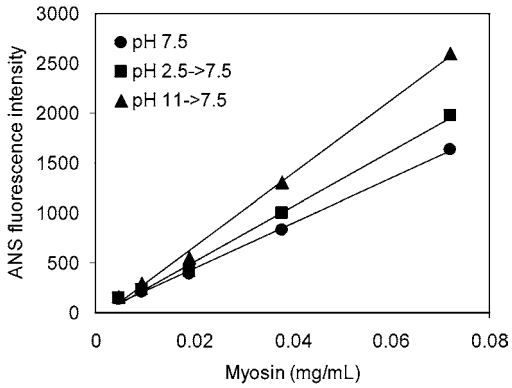

Figure 3. Surface hydrophobicity as a function of native and refolded myosin concentration from ANS binding/fluorescence. Fluorescence excitation was 390 nm, and emission wavelength was 470 nm. The temperature was 5 °C, and samples were measured 15 min after ANS (10 mM) was added.

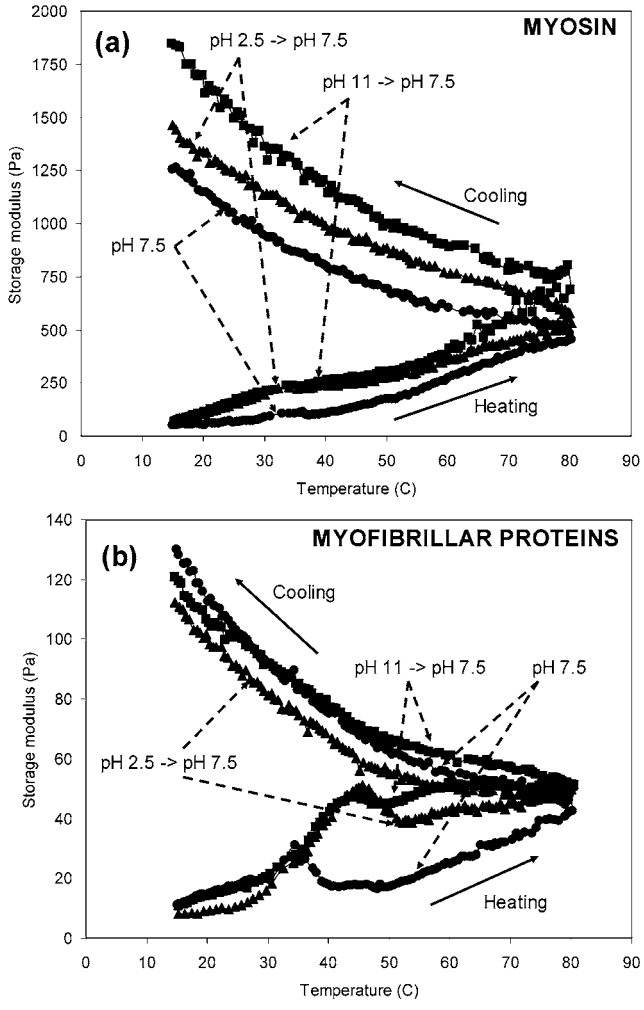

Figure 4. The viscoelastic behavior of cod (a) myosin and (b) myofibrillar proteins on heating and cooling as determined from the storage modulus. Protein concentration was 35 mg/mL and samples were heated from 15 to 80 °C at a rate of 1.5 °C/min and subsequently cooled to 15 °C.

proteins, as protein−protein interactions on heating developed very quickly and peaked at about 45 °C, followed by a slight decline and then an small, gradual increase to 80 °C (**Figure 4b**). The untreated protein, on the other hand, had an earlier peak (at ca. 35 °C), but onset of gelation came well after the

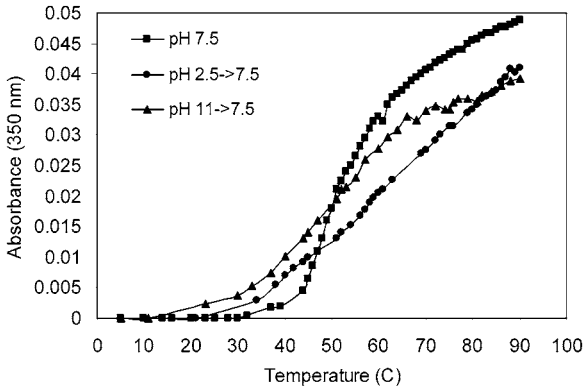

Figure 5. Thermal aggregation of native and refolded myosin as assessed by turbidity development at 350 nm. Protein concentration was ∼0.45 mg/mL, and samples were heated in a sealed cuvette at 1.5 °C/min.

peak (at ca. 50 °C) (**Figure 4b**). The acid- and alkali-treated myosin did not exhibit the peak in storage modulus on heating as the myofibrillar proteins did, which points to the participation of other proteins in the gelation of the myofibrils.

DISCUSSION

The myofibrillar proteins are categorized as salt soluble proteins, as they typically do not become soluble at physiological pH values unless salt concentration is relatively high, > 0.3 M. Recent studies have, however, shown that these proteins can become soluble at low ionic strengths or at very low or high pH (*3*, *24*). Good myofibrillar protein solubility is believed to be a prerequisite for many functional properties, including gelation and emulsification, and this is why salt is typically added to muscle-derived products to obtain desirable quality characteristics. Thermal denaturation of myofibrillar proteins leads to aggregation and decreased solubility (*1*). The acid and alkaline unfolding of cod myosin had no impact on the solubility characteristics of myosin refolded to pH 7.5. Even though hydrophobicity of the protein increased on refolding (**Figure 3**) and myosin was only partially refolded to its native state when pH was readjusted to pH 7.5 (*9*), it still exhibited almost identical solubility behavior to the native protein. This is likely due to the fact that the rod portion of the protein, which is known to dictate its solubility characteristics (*25*), was in a native configuration after acid and alkaline treatment, as previously demonstrated using circular dichroism analysis (*9*). The increase in hydrophobicity was likely from the head region, which on its own is soluble in both high and low ionic strength salt solutions, and has little bearing on the salt dependency solubility profile. The slightly increased solubility below 200 mM KCl for the acid- and alkali- treated myosin compared to untreated myosin may have been due to light chains, which dissociated on acid and alkali unfolding (*9*) and are soluble at low ionic strength (*25*).

It was interesting to see that the pH treatment significantly increased the solubility of the myofibrillar proteins in ∼0−600 mM KCl. Even at low ionic strength, a greater percentage of the pH-treated proteins was soluble compared to the untreated proteins. It has been demonstrated with chicken and mackerel muscle that their myofibrillar proteins, which are insoluble at low ionic strengths, become soluble in water after the removal of certain proteins, which presumably are structural elements of the muscle cell (*4*−*5*). High solubility at very low salt concentrations was seen in the current study for the untreated cod myofibrillar proteins, consistent with the results of Stefansson and Hultin (*3*) using minced whole cod muscle (**Figure 1**). The

ionic strength for the refolded protein solutions was not low enough to exhibit this high solubility at the point of no KCl addition, as residual salt was present from the refolding experiments. The acid and alkali treatment employed in this study is known to solubilize the muscle proteins (*6*). It is likely that this solubilization may involve some of the restraining proteins, which might explain why the acid and alkali treatments consistently had higher solubility than the untreated protein. That the untreated myofibrillar proteins had ~10% solubility could be due to certain proteins of the myofibrillar element which were extracted upon the large dilution of the myofibrillar proteins (0.8 mg/mL) and remained soluble at the relatively low centrifugal force (2200*g*) used.

The identical response to increasing amounts of salt of the refolded and native protein could suggest that myosin had the capability to form thick filaments at low ionic strength after refolding. This is because the characteristics of the salt solubility curve are believed to be largely due to the depolymerization of the thick filament (*25*). As the myosin rod was in its native configuration on refolding (*9*) and the rod is what makes myosin arrange itself into filaments, it is possible that it could have rearranged into thick filaments, but this remains to be confirmed.

The principal role of emulsifying agents is to reduce the interfacial tension at the oil−water interface and to form an interfacial film to protect droplets against coalescence (*26*). The ability of muscle proteins to emulsify fat in minced meat products is of much importance (*1*). The effectiveness of fish muscle proteins as emulsifiers in oil-in-water emulsions compared to other well-established emulsifying proteins has been demonstrated (*27*). Unfolding both cod myosin and myofibrillar proteins of cod at pH 2.5 or 11 led to significant improvement in both emulsion formation and stability (**Figure 2**). That myosin and myofibrillar proteins showed a similar trend in increased emulsification ability after pH treatments underlines how myosin plays an instrumental role in the emulsifying properties of the muscle proteins. This is supported by work demonstrating that myosin was a better emulsifier than actomyosin, sarcoplasmic proteins, and actin at the same protein concentration (*10*). This may also explain why the cod myofibrillar proteins were less effective emulsifiers than isolated cod myosin at the same protein concentration, because the presence of some of the other myofibrillar proteins could have negatively affected emulsification. The question is, what conformational change in myosin makes it a better emulsifier after acid and alkaline treatment? The ability of a protein to rapidly lower free energy of a newly created interface is controlled by (a) how rapidly it can adsorb to the interface and (b) how rapidly and easily it can undergo conformational rearrangement and reorientation at the interface. The latter is the rate-controlling step, as it determines the rate at which a protein can decrease the interfacial tension between oil and water (*28*). From earlier conformational studies (*9*), it was evident that the rod was essentially native after the acid and alkaline treatment, while the head appeared misfolded with lower tertiary interactions, increased hydrophobicity, and more reactive thiol groups. Li-Chan and co-workers (*29*) have demonstrated that emulsifying properties of a preparation of crude salt soluble muscle proteins correlated well with their surface hydrophobicity. Surface hydrophobicity accounted for over 70% of the variability seen with the emulsifying acitivity index in their studies. The results with cod myosin are in excellent agreement with their findings, as the relative increase in hydrophobicity and emulsification activity shows an almost perfect relationship (**Table 1**), suggesting that surface hydrophobicity can be a sensitive measure of the emulsification ability of myosin. It is therefore proposed that misfolding of the myosin head fraction as a result of alkaline and acid treatment, which led to increased surface hydrophobicity as more hydrophobic clusters became exposed (*9*), is likely the cause for the increased ability of refolded myosin to form and stabilize the oil-in-water emulsion. This hypothesis is supported by the fact that alkali-unfolded myosin was more misfolded and hydrophobic and in turn had better emulsifying properties than the acid-unfolded protein. The mechanism by which this occurs is possibly first due to the more rapid adsorption of the relatively hydrophobic globular head of the pH-treated protein to the nonpolar lipid globules. The higher the hydrophobicity, the more energetically favorable would be the interaction of the protein with the nonpolar oil surface. Once at the interface, the refolded protein, which has a structurally less stable (**Figure 5**) and more flexible head than native myosin would more easily unfold and thus more effectively enable its head to associate with the lipid phase, leaving its more polar residues, presumably the tail, to interact with the aqueous phase. That the refolded proteins are more effective at reducing interfacial (and surface) tension than the native protein was confirmed by the interfacial and surface tension experiments (**Table 2**), and this ability is likely due to its different molecular configuration.

The relationship between the ability of proteins to decrease interfacial and surface activity to their emulsifying properties is known (*30*). The process of conformational changes at the interface is hypothesized to be through loss in tertiary structure rather than secondary structure (*31*), as the interfacial energy at the oil−water interface is probably insufficient to overcome the activation energy barrier for complete unfolding (*27*). The refolded myosin would therefore be at an advantage over the native protein, as it already has partly unfolded tertiary structure but a native secondary structure, and thus its head is in a molten globular state (*9*). To support this idea, it has been found that cytochrome c in its molten globular state, where it lacks a well-defined tertiary structure, does in fact adsorb faster and unfold more easily at an air−liquid interface than the native protein (*32*). Similar findings have been reported for lactalbumin in its molten globular state (*33*). The characteristics of the globular head of myosin is therefore proposed to be instrumental in its emulsification properties. It is also possible that the dissociation of the light chains previously reported on acid and alkali pH treatment (*9*) may have aided in emulsification, as they could have left exposed hydrophobic patches on the myosin head. Also, the increased reactivity of the thiol (*9*) groups may have facilitated the formation of a stable protein network at the interface, thus aiding in the stabilization of the emulsion.

The mechanism of myosin and myofibrillar gelation at high ionic strength has been extensively investigated. However, there has been no previous report on the gelation behavior of acid- and alkali- treated cod myosin and myofibrillar proteins and connecting it with changes in conformation. It is a common view throughout the literature that good gelation properties are only achieved when starting out with a native configuration of the myosin molecule (*34*), and studies frequently report on the positive relationship between functional ATPase activity and gel quality (*18*−*19*). ATPase activity is mostly lost on acid and alkali treatment, and the protein does not regain its native configuration as the pH is readjusted to pH 7.5 (*9*). The current study shows that a native configuration and ATPase activity is not a prerequisite for gelation. A number of studies on the different components of myosin have led to a general theory on its gel forming mechanism. It is widely held that the onset

of gelation on heating involves unfolding and aggregation of the headgroups of myosin to other headgroups, likely via hydrophobic interactions and possibly aided by the formation of disulfide bonds between them. On further heating, the helical-coiled coil is thought to unfold, and network formation is initiated by hydrophobic interactions of exposed nonpolar residues of the unfolded rods (e.g., *11*, *35*). Considering the above, it may be hypothesized that conformational changes in the myosin head after acid and alkaline treatment is the reason myosin started to aggregate and form a gel matrix at a lower temperature than the native proteins (**Figures 4** and **5**). Presumably, the low-temperature aggregation/gelation of refolded myosin was due to increased protein−protein interactions of myosin, because the head region did not completely refold and was more hydrophobic and less thermally stable than the head region of the untreated myosin. It has been reported that an increase in hydrophobicity is necessary for the onset of gelation (*36*). Kato and Konno (*17*) found that the head is responsible for almost all of the ANS binding capacity. This contributes to the above suggestion that the misfolded and hydrophobic myosin head is responsible for the low-temperature onset of aggregation/gelation of the pH-treated myosin.

The role of disulfide bond formation between the myosin heads at the onset of gelation is controversial. Several authors have found that reactive thiols do contribute to the head-to-head aggregation via disulfide formation (*35*, *37*) and that the thiols on the rods are not involved (*38*). Both the acid- and alkali- treated proteins were found to have more exposed reactive thiols after refolding (*9*). This may in part also help to explain the earlier onset of gelation as the additional thiol groups could have aided in head-to-head interactions. The loss of the myosin light chains (*9*) did not negatively affect the gelling ability of the refolded protein, suggesting that the heavy chains are primarily responsible for the gelation. Samejima and co-workers (*10*) had previously demonstrated that the myosin-heavy chains and the whole molecule were remarkably similar in gelation behavior, which suggests that light chains have no effect. Sharp and Offer (*39*) also found that centrifuging a myosin gel left the light chains in the supernatant, again suggesting no involvement of light chains in gelation. The work with the refolded cod myosin is further evidence that the light chains themselves are probably unnecessary for proper gelation. On the other hand, the removal of the light chains may have aided in the aggregation at low temperatures, as they may have left exposed hydrophobic patches that may facilitate head to head associations (*39*). The earlier onset of gelation correlated well with the thermal unfolding/aggregation behavior of myosin (**Figure 5**). This is consistent with thermal unfolding being a prerequisite for gelation. Development in gel strength on heating has been found to correlate with increase in turbidity (*35*, *40*) and hydrophobicity (*1*, *36*), as seen here with cod myosin. The noncooperative thermal unfolding/aggregation seen with refolded myosin (**Figure 5**) bears similarities to that seen for proteins in the molten globule state (*41*). The head region of myosin was likely in a molten globular conformation after refolding (*9*). This suggests that much of the modified functionality of myosin may be due to this region.

The rod section of myosin has been found to be predominantly responsible for the rigidity (i.e., formation of a strong gel network) development of the gel (*35*). The substantial increase seen in storage modulus (i.e., gel strength) on cooling in this study was therefore likely due to the interactions between myosin rods. The ability of refolded cod myosin to form a strong gel on cooling was thus likely due to the fact that its rod was in a native configuration after refolding (*9*) and still would have the ability to form a strong and stable protein network. The increased gel strength after cooling for the pH 2.5 and pH 11 treated protein compared to native myosin, respectively, can possibly be explained by the improved ability of the headgroups to initiate the protein network, as discussed above. O′Neill and co-workers (*42*) found that while disulfide bridging was not a prerequisite for the formation of a gel network it significantly increased the mechanical strength and cohesiveness of actomyosin gels. The stronger gel could therefore be a result of increased disulfide bonding.

The earlier onset of protein−protein interactions, and thus gelation, was also seen with the pH-treated myofibrillar proteins versus untreated proteins. Although the onset of gelation was at a lower temperature for the pH-treated myofibrillar proteins versus the untreated proteins, it was about 5−10 °C higher compared to isolated myosin. This may be due to the presence of actin in the myofibrillar protein fraction, as actin has a higher unfolding temperature than myosin, and adding it to myosin has been reported to delay the onset of gelation (*43*). At the same total protein concentration, myosin produced over a 10-fold stronger gel than the myofibrillar proteins (**Figure 4**) are. This may indicate that other proteins in myofibrils may be hindering the ability of myosin to form gels or may suggest that the relation of myosin concentration to gel strength is not linear, but undergoes a rapid increase from 17.5 (the approximate concentration of myosin in the myofibrillar protein sample) to 35 mg/mL. Other workers have reported on the lowering of gel strength when adding actin to myosin (*44*), while Yasui and co-workers (*45*) reported that a positive effect of actin on gel formation has an optimal ratio of actin/myosin. As the myofibrillar proteins are composed of many proteins, which may be in their filamentous or dissociated state, depending on solution conditions, the effects of each of the proteins on each other is expected to be very complex, and the study here cannot elucidate why the myofibrillar proteins form weaker gels than pure myosin. Results nevertheless show that myosin forms stronger gels compared to the myofibrillar proteins at the same total protein concentration. However, although the refolded cod myofibrillar protein fraction clearly started to gel at a significantly lower temperature than the untreated proteins, the gels formed by either were of similar strength on cooling, suggesting that under the conditions studied here, the conformational and structural changes experienced at pH 2.5 and 11 and during pH readjustment to pH 7.5 may not be of much importance to the gelation characteristics of the gel.

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