JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Conformational and Rheological Changes in Catfish Myosin as Affected by Different Acids during Acid-Induced Unfolding and Refolding

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Changes in the conformation of catfish (*Ictalurus punctatus*) myosin due to (i) anions, (ii) acid pH, and (iii) salt addition were determined using tryptophan fluorescence, hydrophobicity measurements, differential scanning calorimetry, and circular dichroism. The relationship between conformation and storage modulus (G') of acid-treated myosin was studied. Three acids, HCl, H_2SO_4, and H_3PO_4, were used for unfolding myosin at three acidic pH conditions, 1.5, 2.0, and 2.5. Unfolded myosin was refolded to pH 7.3. Denaturation and unfolding of myosin was significantly ($p < 0.05$) lower when salt (0.6 M NaCl) was present during acid unfolding than in the absence of salt. When salt was added before unfolding, the α-helix content of myosin treated at pH 1.5 was significantly lower than that treated at pH 2.5. When salt was added after refolding, the α-helix content of myosin was unaffected by different pH treatments. The G' of myosin increased with an increase in myosin denaturation. The G' of myosin was significantly ($p < 0.05$) higher when salt was added to myosin after refolding than before acid unfolding. Among the different anion treatments, the G' of acid-treated myosin decreased in the order $Cl^- \sim SO_4^{2-} > PO_4^{3-}$. Among the different pH treatments, the G' of myosin treated at pH 1.5 was significantly ($p < 0.05$) higher than myosin treated at pH 2.5. The conditions that would result in maximum myosin denaturation and maximum G' were unfolding of myosin at pH 1.5 using Cl^- (from HCl) followed by refolding at pH 7.3 and subsequent addition of 0.6 M NaCl.

KEYWORDS: Myosin; catfish; acid unfolding; anion; pH shift; pH; salt; conformation; gelation

INTRODUCTION

The functional properties of myofibrillar proteins are important for the physical and textural attributes of meat and muscle food products (*1*, *2*). Myosin, a major myofibrillar protein, is responsible for a number of properties such as gelation, water holding, and emulsification. For this reason, myosin has been given special attention in conformational and functional studies.

A recent process involving solubilization of myofibrillar proteins using acid (*3*, *4*) is used for preparing protein isolates from muscle food sources such as fish. In brief, the process involves solubilizing muscle proteins under low pH conditions (pH 2.0−3.5) and removing insoluble muscle components, such as connective tissue, bones, and fat, by centrifugation. The solubilized myofibrillar proteins are then recovered using isoelectric precipitation at a pH around 5.5. Some advantages of this process are (i) high recovery of muscle proteins, (ii) utilization of underutilized raw material, and (iii) removal of membranes and lipids that are susceptible to oxidation. Myofibrillar proteins subjected to low pH conditions will undergo denaturation. It is generally believed that denaturation would adversely affect the functionality of proteins (*5*, *6*). Hence, one may expect lower functionality for the protein isolates prepared using the above-mentioned acid process. However, results have shown that the acid-treated myofibrillar proteins have improved gelation properties as compared to traditional protein isolation processes (*7*). In this work, we attempt to understand the structure−functional relationship of channel catfish (*Ictalurus punctatus*) myosin and how various acid conditions could affect myosin structure and its functionality, particularly its gelation properties.

Under extreme pH conditions, proteins would unfold due to energetically favorable conditions. The extent of unfolding and the configuration of proteins have been found to be protein-dependent (*8*). It has been found that acid-induced unfolding is primarily dependent on the pH of the medium while acid-induced refolding is dependent on the nature of anions associated with the acid species (*9*). Hence, refolded proteins may attain different conformations depending on the type of acid used. The stabilizing and destabilizing effects of various anions on the conformation of proteins are given by the Hofmeister series (*10*), where the destabilizing effect of various anions increases in the order $PO_4^{3-} < SO_4^{2-} < Cl^-$. Normally, hydrochloric acid (HCl)

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10.1021/jf063184v CCC: $37.00 © 2007 American Chemical Society
Published on Web 04/17/2007

is used to adjust the pH for acid solubilization. The effect of other acids such as sulfuric acid (H_2SO_4) and phosphoric acid (H_3PO_4) on the unfolding and refolding of myosin and the resultant effect on the functional properties of myosin have not been studied. Goto et al. (*8*) showed that extreme pH conditions may result in refolding of proteins into a compact conformation with properties similar to a molten globule. As acid solubilization of myofibrillar proteins normally involves pH 2.5, we wanted to test the effect of lower pH values, 1.5 and 2.0, on the conformational changes of myosin and how it relates to the changes in protein functionality such as gelation. Proteins possess a net charge at pH values other than their normal isoelectric pH. The net positive or negative charge on a protein molecule could play an important role in folding or unfolding due to electrostatic attraction or repulsion. Salt (NaCl) is usually added to solubilize myosin. The addition of salt could have a neutralizing or shielding effect on the net protein charge (*11*, *12*). During the recovery of protein isolates using acid solubilization, salt is usually added after the adjustment of pH to the isoelectric point of the proteins, that is, after protein refolding. We wanted to study the effect of adding salt before unfolding and after refolding as well as the absence of salt on the conformational and gelation properties of myosin.

MATERIALS AND METHODS

Materials. Fillets of catfish (*I. punctatus*) were purchased locally in Gainesville, FL, and transported to the laboratory on ice. Chemicals were purchased from Fisher Scientific (Fair Lawn, NJ). All reagents were of ACS grade.

Methods. *Preparation of Myosin.* Myosin was prepared from the white muscle tissue of catfish fillets by the using a method similar to Kristinsson (*13*). All solutions used for myosin preparation were kept cold at 10 °C. Catfish white muscle was minced once through an Oster heavy duty food grinder (model 4726-1, Sunbeam, Delray Beach, FL). The mince was mixed with 10 volumes of solution A (0.10 M potassium chloride, 1 mM phenyl methyl sulfonyl fluoride, 0.02% sodium azide, and 20 mM tris-HCl buffer, pH 7.5) and homogenized using a Bio homogenizer (M133/1281-0, Bio Spec Products Inc., Bartlesville, OK) at high speed for 1 min. The homogenate was incubated for 60 min at 0−4 °C and centrifuged at 1000*g* for 10 min in a Sorvall RC-5B refrigerated centrifuge (DuPont Instruments, Newtown, CT). The sediment was suspended with five volumes of solution B [0.45 potassium chloride, 5 mM β-mercaptoethanol, 0.2 M magnesium acetate, 1 mM ethylene glycol bis(β-amino ethyl ether)tetraacetic acid, and 20 mM tris maleate buffer, pH 6.8], mixed with adenosine triphosphate to a final concentration of 10 mM, and incubated for 90 min at 0−4 °C. The mixture was then centrifuged at 10000*g* for 15 min. The supernatant was diluted slowly with 25 volumes of distilled water and kept at 10 °C for 60 min. The supernatant was centrifuged at 12000*g* for 10 min, and the pellet was resuspended with solution C (0.5 M potassium chloride, 5 mM β-mercapto ethanol, and 20 mM tris HCl buffer, pH 7.5). The resuspended pellet was incubated for 10 min at 0−4 °C, diluted with 2.5 volumes of 1 mM bicarbonate and 10 mM magnesium chloride, and incubated overnight at 0−4 °C. Myosin pellets, obtained by centrifugation at 22000*g* for 15 min, were washed with 40 volumes of distilled water, and the myosin purity was checked using 4−20% linear gradient precast sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) gels. Myosin bands were identified using high molecular weight SDS-PAGE standards. Samples of myosin were over 93% pure as determined using densitometry. The protein contents of myosin samples were determined using the Biuret reaction (*14*). Myosin samples were kept on ice and typically used within 4 days of preparation. This storage period did not change their electrophoretic patterns.

Unfolding of Myosin under Acidic Conditions. Catfish myosin (initial pH ~ 6.2) was unfolded under different acidic conditions and refolded by bringing the pH to 7.3 using sodium hydroxide (NaOH). Myosin was unfolded and refolded within 5 min. A preparation of myosin homogenate in 20 mM Tris-HCl was subjected to 24 different acidic pH treatments involving (i) three acidic pH values, (ii) three different anions, (iii) the presence or absence of salt, and (iv) salt added before or after pH adjustment. The acidic pH values used were 1.5, 2.0, and 2.5. The different anions used for pH adjustments were Cl^- (hydrochloric acid, HCl), SO_4^{2-} (sulfuric acid, H_2SO_4), and PO_4^{3-} (phosphoric acid, H_3PO_4). Studies were done in the absence or presence of 0.6 M sodium chloride (NaCl). When salt was used, it was added either before unfolding or after refolding. In the case of PO_4^{3-} (phosphoric acid), only pH values of 2.0 and 2.5 were studied. This was due to aggregation of myosin at pH 1.5. When salt was added after the folding of myosin, the pH of myosin homogenate was readjusted back to 7.3 if necessary.

Rheology Studies. The viscoelastic changes of catfish myosin during heating and cooling were studied using an AR 2000 Advanced Rheometer (TA instruments, New Castle, DE) with an aluminum double concentric cylinder geometry (outer/inner diameter, 21.9/20.4 mm). A homogenate of myosin (3.5 mg/mL) was prepared in Tris-HCl buffer. The homogenate was subjected to the 24 different acidic pH treatments as described above. The viscoelastic property of myosin, namely, storage modulus G' (elasticity), was studied using dynamic oscillation experiments as a function of temperature. The myosin samples were heated from 5 to 80 °C and then cooled back to 5 °C at 2 °C/ min. A constant oscillation frequency of 0.1 Hz was used. G' at the initial (5 °C), heated (80 °C), and cooled (5 °C) temperatures was used for studying the gelation properties of myosin. All rheology studies were done in duplicate. Three different controls were used. One control was a homogenate of myosin adjusted to pH 7.3 in the absence of salt. The other two controls were myosin homogenates adjusted to pH 7.3 with 0.6 M salt added before unfolding or after refolding.

Tryptophan Fluorescence. A myosin protein concentration of 50 μg/mL was used for measuring intrinsic tryptophan fluorescence. Two different acid types, HCl and H_3PO_4, were tested. Three controls, each adjusted to pH 7.3, were used. The first control was prepared in 20 mM Tris-HCl buffer. The second control was prepared in 20 mM Tris-HCl buffer with 0.6 M NaCl added before the pH adjustment to 7.3, and the third control was prepared with the salt added after the pH adjustment to 7.3. Changes in tryptophan fluorescence were measured by exciting myosin at 297 nm and recording the emission spectra between 300 and 400 nm in a Perkin-Elmer LS 45 Luminescence Spectrophotometer (Norwalk, CT).

Surface Hydrophobicity. The hydrophobicity of myosin using extrinsic fluorescence method was measured using 6-propionyl-2-(dimethylamino) naphthalene (Prodan) as the external probe. Two different acid types, HCl and H_3PO_4, were used for the studies. A Prodan stock solution of 1.11 mM was prepared in methanol. The concentration of Prodan in the stock solution was determined spectrophotometrically at 360 nm using molar absorption coefficients of 1.8×10^4 M^{-1} cm^{-1} (*15*). Five hundred microliters of Prodan stock solution was diluted to 30 mL with methanol and used for fluorescence studies (A). A stock solution of myosin with a protein concentration of 1.5 mg/mL was used. Four different concentrations of myosin were prepared by diluting 0.5, 1.0, 1.5, and 2.0 mL of myosin stock solution to 4 mL with 20 mM Tris-HCl buffer. The samples were subjected to various pH treatments with 0.6 M salt added before or after the pH treatment. The myosin samples were then taken in vials covered with aluminum foil, vortexed with 20 μL of A and incubated for 15 min. The relative fluorescence intensity was measured by using excitation at 365 nm and measuring the emission spectra between 380 and 560 nm. A mixture of buffer and Prodan was used as a blank. The initial slope of the net fluorescence intensity vs protein concentration was calculated using linear regression analysis and used as an index of protein hydrophobicity. All analyses were done in duplicate.

Circular Dichroism Studies. Changes in the secondary and tertiary conformations of myosin were studied by recording circular dichroism spectra of the protein using an AVIV 215 spectropolarimeter (AVIV instruments Inc., Lakewood, NJ) at 5 °C. Secondary structure determination was performed by scanning pH-treated myosin samples (~30 μg/ mL) between 260 and 190 nm in a 1 cm quartz cell. The tertiary structure was studied by scanning myosin samples (1 mg/mL) between 350 and 260 nm in a 1 cm quartz cell. The resolution was set at 0.2 nm, the bandwidth was set at 1 nm, the sensitivity was set at 20 mdeg,

the response was set at 1 s, and the scanning speed was 20 mdeg/min. The spectrum was obtained in duplicate, and the duplicates were averaged into one spectrum. Secondary and tertiary spectra of denatured myosin were obtained by scanning protein samples prepared in 6 M guanidine-hydrochloride (Gua-HCl). All studies were done at 5 °C. The α-helix content in the secondary structure was analyzed using Dichroweb (*16*).

Microdifferential Scanning Calorimetry (DSC). The denaturation and thermal transition of myosin were studied using a differential scanning microcalorimeter (VP-DSC, MicroCal, LLC, Northampton, MA). Samples of myosin with a protein concentration of 10 mg/mL in 20 mM tris-HCl buffer were prepared. The pH of the samples was adjusted using HCl or H_3PO_4 to 2.0 and readjusted back to pH 7.3. Sodium chloride (0.6 M) was added either before or after the pH adjustment to 7.3. The samples were degassed at 5 °C under vacuum for 7 min. The thermal transition of myosin was studied between 5 and 80 °C with a temperature ramp of 1.5 °C/min. Tris-HCl buffer (20 mM) containing 0.6 M sodium chloride at pH 7.3 was used as a blank.

Statistical Analyses. Statistical analyses on the samples were performed using JMP Statistical Discovery Software (version 5.0). Dunnet's multiple comparison test was used for comparing the effect of different treatments to the control. Analysis of variance was employed to examine the difference among treatments at the $P < 0.05$ level. Tukey's multiple comparison tests were used to compare the differences among the means.

RESULTS

Effect of Acidic pH, Anions, and Salt on Myosin Gelation. The effect of acidic pH conditions on the viscoelastic properties of myosin was studied. Catfish myosin homogenate in tris-HCl was unfolded under three pH conditions, using three different acids and three different salt treatments. Sodium hydroxide was used for refolding myosin to pH 7.3. The acid-treated myosin was heated from 5 to 80 °C and cooled to 5 °C. The storage modulus (G') of acid-treated myosin was measured at three temperatures, 5 (initial temperature), 80 (final heated temperature), and 5 °C (cooled temperature). G' values of myosin subjected to different acid treatments are shown in **Figure 1a** (HCl), **b** (H_2SO_4), and **c** (H_3PO_4).

At the beginning of the heating cycle, the temperature of acid-treated myosin was 5 °C. At this temperature, myosin subjected to pH 1.5 treatment had a significantly ($p < 0.05$) higher G' as compared to the control and samples treated at pH 2.0 and 2.5. There was no significant difference ($p > 0.05$) among the type of anions. Among the different salt treatments, G' decreased in the following order: no salt ∼ salt added after refolding > salt added before unfolding. When the heating cycle reached the maximum temperature of 80 °C, myosin treated with HCl and H_2SO_4 at pH 1.5 had a significantly ($p < 0.05$) higher G' than controls. Also, samples treated with HCl at pH 2.0 and 2.5 had a higher G' ($p < 0.08$) than the controls. Among the different salt treatments, G' decreased in the following order: no salt > salt added after refolding > salt added before unfolding. Among the different anions, myosin treated with PO_4^{3-} had a lower G' as compared to myosin treated with Cl^- and SO_4^{2-}.

At the end of the heating cycle, acid-treated myosin was cooled back to 5 °C. At this temperature, samples treated with HCl to pH 1.5 and 2.0 and with H_2SO_4 to pH 1.5 had a significantly ($p < 0.05$) higher G' as compared to the control, while samples treated with H_3PO_4 to pH 2.0 had a significantly lower ($p > 0.05$) G' as compared to the control treatments. Among the different anions, the decreasing order of G' was HCl ≥ H_2SO_4 > H_3PO_4. Within the different acid types, G' values of samples treated with HCl to pH 1.5 were higher than those treated to pH 2.5 and G' values of H_3PO_4 samples treated to pH 2.5 were higher than those treated to pH 2.0. The different salt treatments affected G' for H_2SO_4 treatment but not for HCl or H_3PO_4 treatments. For H_2SO_4, the decreasing order of G' was salt added after refolding > salt added before unfolding ∼ absence of salt.

DSC. DSC was used to investigate the influence of anions and the order of salt addition on the thermal stability and endothermic transitions of myosin. Myosin was unfolded using acids, HCl or H_3PO_4, at pH 2.0 and refolded using sodium hydroxide at pH 7.3. HCl and H_3PO_4 were chosen since myosin treated with Cl^- and PO_4^{3-} showed the maximum and minimum G' values, respectively. Among the three pH treatments, pH 2.0 was chosen since it could represent the midpoint of the three pH treatments. Salt (0.6 M NaCl) was added either before unfolding or after refolding of myosin. Two controls were used, one for each order of addition of salt.

The endotherms of acid-treated myosin showed major transitions between 30 and 50 °C. When salt was added after refolding (**Figure 2a**), the control treatment showed two transition temperature peaks (T_p), at 37.7 and 44.9 °C. The calorimetric enthalpies (ΔH) corresponding to these peaks were 48.7 and 34.5 kcal/mol, respectively (**Table 1**). When myosin was treated with Cl^-, T_p values for the two peaks were similar to those of the control (37.9 and 45.2 °C), whereas ΔH decreased to 36.4 and 33.9 kcal/mol, respectively. For PO_4^{3-} treatment, ΔH decreased further to 12.5 and 28.8 kcal/mol, while T_p of the first peak increased to 38.7 °C. An additional peak was also observed at around 34.1 °C for the PO_4^{3-} treatment (**Figure 2a** and **Table 1**).

When salt was added before unfolding (**Figure 2b**), the control treatment showed two T_p values, one at 37.8 and another at 44 °C. ΔH values corresponding to these T_p values were 62.1 and 55.7 kcal/mol, respectively. Acid treatments lead to a decrease in enthalpy. ΔH values at the two domains for Cl^- treatment were 24.3 and 42.9 kcal/mol, while ΔH values for PO_4^{3-} treatment were 25.4 and 36.1 kcal/mol. T_p of the first peak for Cl^--treated myosin was similar to that of the control (37.5 °C), while T_p of PO_4^{3-}-treated myosin increased to 38.5 °C. For PO_4^{3-} treatment, an additional peak was also observed at around 34.3 °C (**Figure 2b** and **Table 2**).

Tryptophan Fluorescence of Acid-Treated Myosin. The effect of various acid treatments on the unfolding of myosin molecule was studied using tryptophan fluorescence. Tryptophan fluorescence of acid-treated myosin was measured by exciting myosin at 297 nm and recording the emission spectra between 300 and 400 nm. Three different salt treatments, salt added before unfolding of myosin, salt added after refolding, and absence of salt, were studied.

The presence or absence of salt had a significant effect on the tryptophan absorbance of the myosin molecules. Tryptophan fluorescence decreased in the following order ($p < 0.05$): salt added before unfolding (**Figure 3a**) > absence of salt (**b**) > salt added after refolding (**c**). For the Cl^- treatments, the intensity of tryptophan fluorescence increased significantly ($p < 0.05$) from pH 1.5 to 2.0 and then decreased from 2.0 to 2.5. When PO_4^{3-} was used, there was no significant difference ($p > 0.05$) between the different pH treatments. At pH 2.0 and 2.5, when salt was absent or added last, the fluorescence intensity of Cl^--treated myosin was significantly higher ($p < 0.05$) than PO_4^{3-}-treated myosin. The reverse was true for the addition of salt after refolding.

Surface Hydrophobicity of Acid-Treated Myosin. The hydrophobicity of acid treated myosin was measured using an external fluorescence probe, Prodan. Acid treatment was performed using two anions, Cl^- and PO_4^{3-}. Fluorescence was

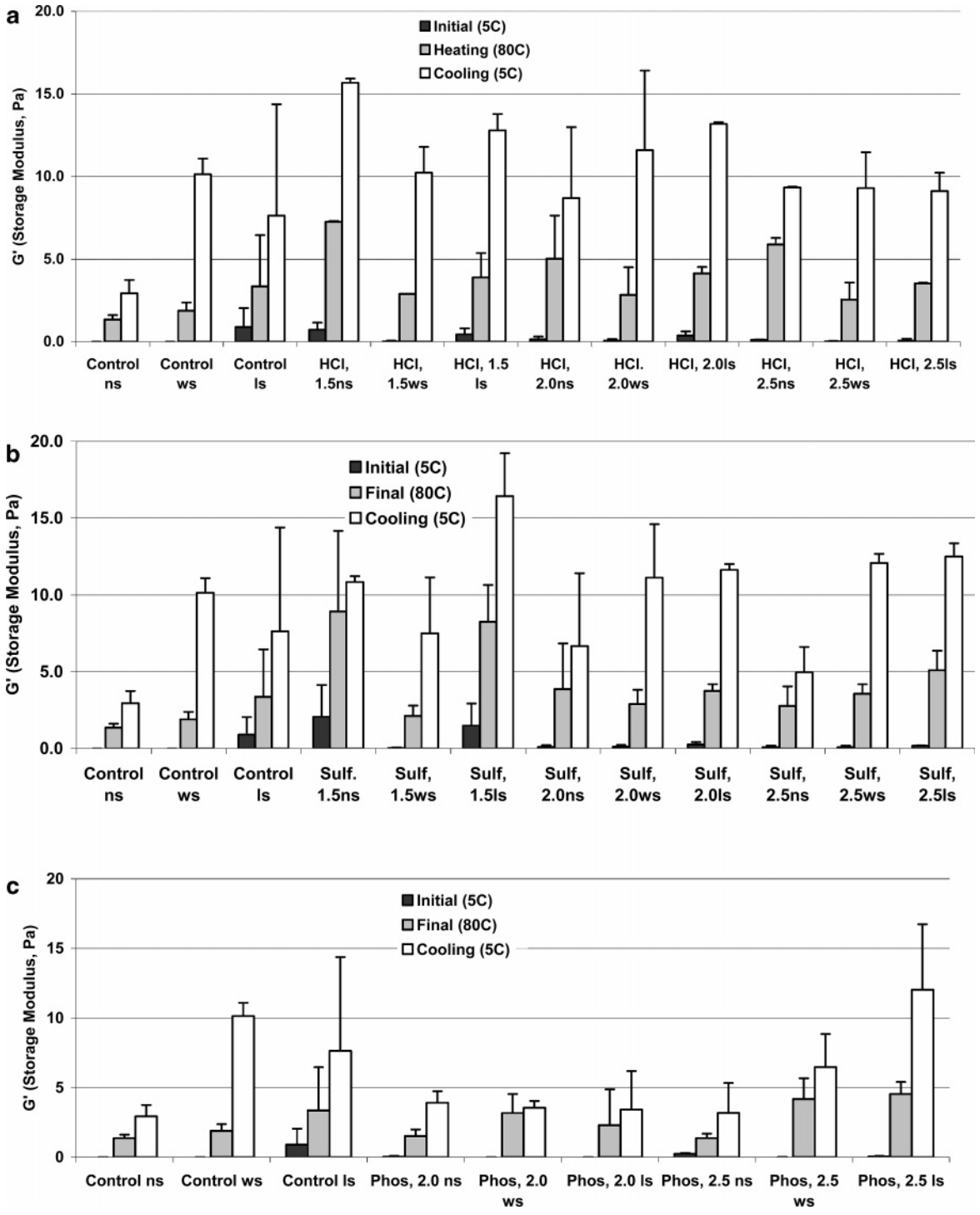

Figure 1. (**a**) Storage modulus (*G′*) of acid-treated myosin (3.5 mg/mL in tris-HCl buffer) heated from an initial temperature of 5 °C to a final temperature of 80 °C and cooled back to 5 °C. The treatment of myosin involves unfolding under different acid conditions and refolding by adjusting the pH to 7.3. Hydrochloric acid (HCl) was used for unfolding myosin at pH 1.5, 2.0, and 2.5. Sodium hydroxide (NaOH) was used for refolding. The unfolding of myosin was done under three salt conditions: absence of salt, salt (0.6 M sodium chloride) added before unfolding, and salt (0.6 M sodium chloride) added after refolding. Three controls with the pH adjusted to 7.3 in the absence of salt, salt added before pH adjustment, and salt added after pH adjustment were used. HCl, hydrochloric acid; ns, no salt treatment; ws, 0.6 M NaCl added before unfolding; and ls, 0.6 M NaCl added after refolding. (**b**) Storage modulus (*G′*) of acid-treated myosin (3.5 mg/mL in tris-HCl buffer) heated from an initial temperature of 5 °C to a final temperature of 80 °C and cooled back to 5 °C. The treatment of myosin involves unfolding under different acid conditions and refolding by adjusting the pH to 7.3. Sulfuric acid (H_2SO_4) was used for unfolding myosin at pH 1.5, 2.0, and 2.5. Sodium hydroxide (NaOH) was used for refolding. The unfolding of myosin was done at conditions similar to part **a**. Controls were similar to part **a**. Sulf, sulfuric acid; ns, no salt treatment; ws, 0.6 M NaCl added before unfolding; and ls, 0.6 M NaCl added after refolding. (**c**) Storage modulus (*G′*) of acid-treated myosin (3.5 mg/mL in tris-HCl buffer) heated from an initial temperature of 5 °C to a final temperature of 80 °C and cooled back to 5 °C. The treatment of myosin involves unfolding under different acid conditions and refolding by adjusting the pH to 7.3. Phosphoric acid (H_3PO_4) was used for unfolding myosin at pH 2.0 and 2.5. Sodium hydroxide (NaOH) was used for refolding. The unfolding of myosin was done at conditions similar to part **a**. Controls were similar to part **a**. Phos, phosphoric acid; ns, no salt treatment; ws, 0.6 M NaCl added before unfolding; and ls, 0.6 M NaCl added after refolding.

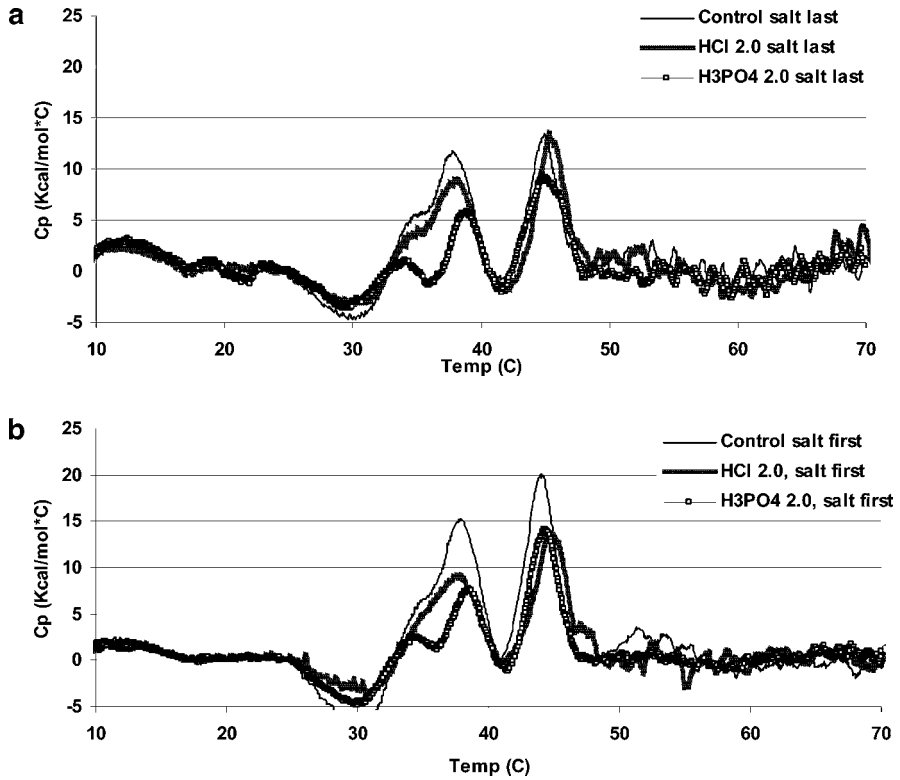

Figure 2. (**a**) DSC endotherms of acid-treated myosin, with salt (0.6 M NaCl) added after refolding. The scan rate was 1.5 °C/min between 5 and 80 °C. Acids used for unfolding were HCl and H_3PO_4. Unfolding was done at pH 2.0, and refolding was done at pH 7.3. (**b**) DSC endotherms of acid-treated myosin, with salt (0.6 M NaCl) added before unfolding, followed by folding at pH 7.3. The scan rate was 1.5 °C/min between 5 and 80 °C. Acids used for unfolding were HCl and H_3PO_4, and unfolding was done at pH 2.0.

Table 1. Transition Peak Temperatures (T_p) and Enthalpy Change (ΔH) for DSC Endotherms of Acid-Treated Myosin with Salt Added after Refolding[a]

	temperature (T_p, °C)			ΔH (kcal/mol)		
sample	T_p (1)	T_p (2)	T_p (3)	for T_p (1)[b]	for T_p (2)[c]	for T_p (2)[d]
control	NA	37.7	44.9	NA	48.7	34.5
Cl^--treated myosin	NA	37.9	45.2	NA	36.4	33.9
PO_4^{3-}-treated myosin	34.1	38.7	44.9	1.18	12.52	28.81

[a] NA, not applicable. [b] Indicates ΔH under peak T_p (1). [c] Indicates ΔH under peak T_p (2). [d] Indicates ΔH under peak T_p (3).

measured by excitation at 365 nm and by measuring the emission spectra between 380 and 560 nm. Two salt treatments, salt added before unfolding and after refolding, were studied.

There was no significant difference ($p > 0.05$) between the two salt treatments and between the two anion treatments on the Prodan values of myosin. However, when salt was added after refolding (**Figure 4a**), acid-treated myosin showed a higher surface hydrophobicity than the control ($p < 0.05$), while for the addition of salt before unfolding (**Figure 4b**), there was no significant ($p > 0.05$) difference between the control and the acid treatments.

Effect of Anions and Salt on the Secondary and Tertiary Structure of Myosin. The change in secondary and tertiary structure of myosin due to acid treatments was measured using circular dichroism. Changes in the secondary structure were measured between 190 and 260 nm in the far-UV absorbance range. The effects of anions, pH, and salt addition were studied. The α-helix content of myosin denatured using 6 M guanidine hydrochloride was 4% (**Figure 5a**).

The secondary structure of acid-treated myosin was significantly ($p < 0.05$) affected by the order of salt addition (**Figure 5a**,**b**). When salt was added before readjusting to pH 7.3, the α-helix content of the control was 32% as compared to 24% for the addition of salt after refolding (**Table 3**). Acid treatments decreased the α-helix content of myosin. The α-helix contents of myosin treated with Cl^- at pH 1.5 were 8 and 9%, and for myosin treated with PO_4^{3-} at pH 2.0, the contents were 8 and 5% for the addition of salt before unfolding and after refolding, respectively. When salt was added first, an increase in the pH of acid treatments significantly ($p < 0.05$) increased the α-helical content of myosin. For Cl^- and PO_4^{3-}, α-helicity increased from 8 to 25% for an increase in pH from 1.5 to 2.5 (**Table 3**). When salt was added last, there was no significant difference ($p > 0.05$) in the α-helicity between the different pH treatments.

The tertiary structure of myosin was measured between 260 and 350 nm in the near-UV absorbance range. Guanidine hydrochloride (6 M) was used for complete denaturation of myosin. When salt was added before unfolding, myosin underwent lesser denaturation as compared to samples where salt was added after refolding (**Figure 5c**,**d**). Also, when salt was added before unfolding, there was no significant difference ($p > 0.05$) between Cl^- and PO_4^{3-} treatments (**Figure 5c**). When salt was added after refolding, Cl^--treated myosin was significantly more ($p < 0.05$) denatured than PO_4^{3-}-treated myosin (**Figure 5d**). Myosin was denatured to a greater degree by lower pH treatments (pH 1.5−2.5) than by higher pH treatments.

DISCUSSION

Thermal gelation is an important attribute of myofibrillar proteins in muscle foods (*2*). Myosin, a major myofibrillar

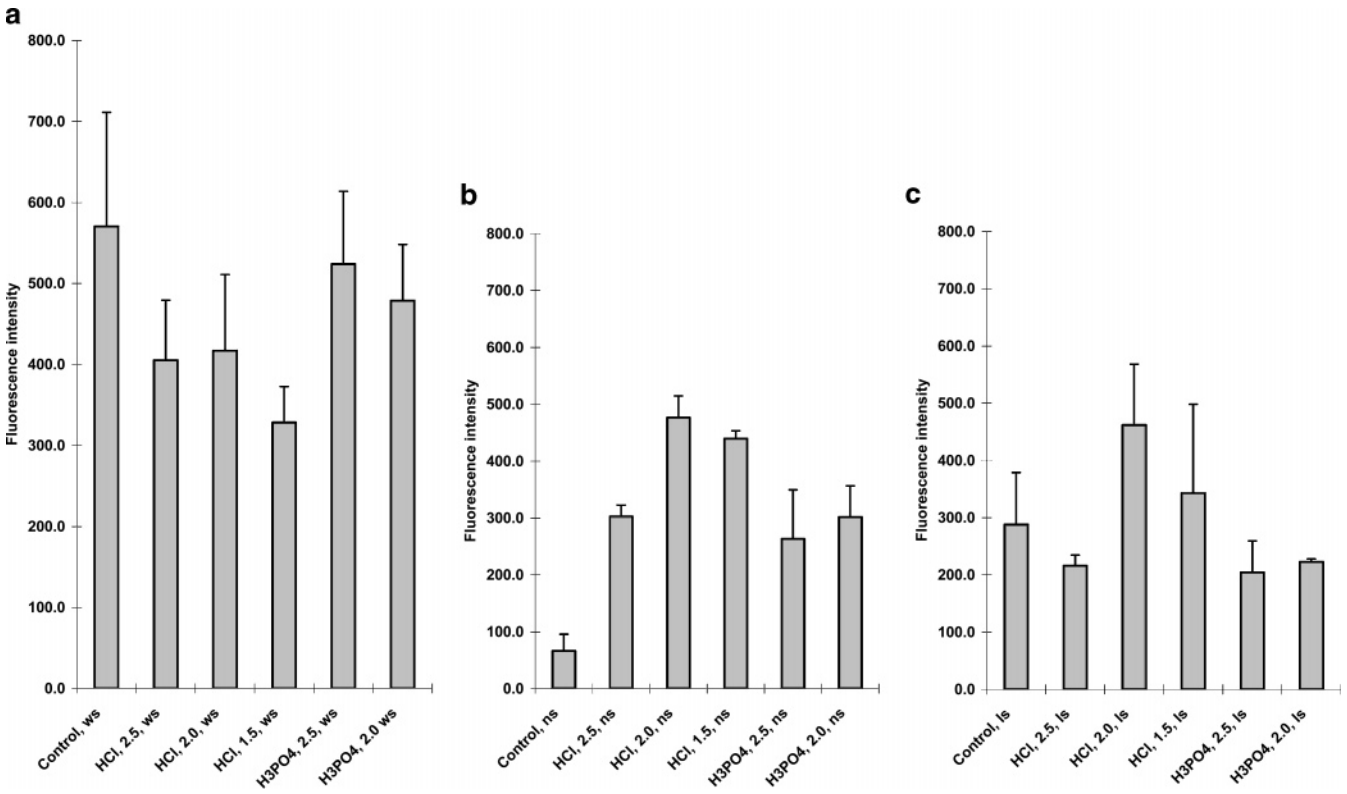

Figure 3. (**a**) Intrinsic tryptophan fluorescence of myosin obtained with an excitation wavelength of 297 nm (the protein concentration was 50 μg/mL). Myosin was subjected to acid unfolding using HCl and H_3PO_4, with 0.6 M NaCl added before acid unfolding. Refolding was achieved at pH 7.3; ws, 0.6 M NaCl added before refolding. (**b**) Intrinsic tryptophan fluorescence of myosin obtained with an excitation wavelength of 297 nm (the protein concentration was 50 μg/mL). Myosin was subjected to acid unfolding using HCl and H_3PO_4, and refolding was achieved at pH 7.3; ns, no salt was used during the acid treatment. (**c**) Intrinsic tryptophan fluorescence of myosin obtained with an excitation wavelength of 297 nm (the protein concentration was 50 μg/mL). Myosin was subjected to acid unfolding using HCl and H_3PO_4, with 0.6 M NaCl added after refolding. Refolding was achieved at pH 7.3; ls, 0.6 M NaCl added after refolding.

Table 2. Transition Peak Temperatures (T_p) and Enthalpy Change (ΔH) for DSC Endotherms of Acid-Treated Myosin with Salt Added before Unfolding

	temperature (T_p, °C)			ΔH (kcal/mol)		
sample	T_p (1)	T_p (2)	T_p (3)	for T_p (1)[b]	for T_p (2)[c]	for T_p (3)[d]
control	NA	37.8	44	NA	62.1	55.7
Cl^--treated myosin	NA	37.5	44.6	NA	24.3	42.9
PO_4^{3-}-treated myosin	34.3	38.5	44.3	2.8	25.4	36.1

[a] NA, not applicable. [b] Indicates ΔH under peak T_p (1) [c] Indicates ΔH under peak T_p (2) [d] Indicates ΔH under peak T_p (3)

protein involved in gel formation, is an unstable molecule and is known to slowly form insoluble aggregates at 0 °C, even at neutral pH conditions (*17*). It is widely reported that the structural disruption and denaturation of fish muscle proteins could adversely affect the functionality of proteins and their ability to form gels (*5*, *18*). Subjecting myosin to acidic pH values could have a significant effect on the conformation and functionality of the protein. Under low pH conditions, denaturation and loss of gelling properties of myofibrillar proteins have been reported by a number of researchers (*19*, *20*). However, using a new process for making protein isolates, Hultin and Kelleher (*4*) showed an improved gelation behavior of acid-treated muscle protein. Using the same acid treatment process, Kristinsson and Hultin (*21*) showed improved emulsification properties for myosin and myofibrillar proteins from Atlantic cod (*Gadus morhua*). The enhanced property of muscle protein was attributed to partial unfolding of myosin during acid unfolding and refolding. In our present work, we attempt to understand the mechanism behind the modified rheological properties of acid-treated myosin prepared from channel catfish. It was shown by Goto et al. (*8*) that at low ionic strength, native proteins could be unfolded by acidic pH, and under high acid concentrations, proteins could refold into a compact conformation with properties similar to a molten globule. This was attributed to the shielding effect by excess anions (*9*). In this work, we wanted to study the effect of different pH treatments on the unfolding and refolding of myosin molecules. We also wanted to determine the effect of salt (NaCl), that is, the Debye−Huckel effect, and the effect of various anions, that is, the Hofmeister effect, on myosin.

Myosin is comprised of two globular head sections attached to a rodlike tail section. The hydrophobicity of myosin arises mainly from head regions, while the rod region contributes mostly to the α-helical secondary structure. Sharp et al. (*22*) had earlier shown the involvement of head assembly in the heat-induced setting of myosin gels. Samejima et al. (*23*) showed the participation of a myosin rod in gel formation. It was concluded that gelation involves the interaction of myosin head assemblies while the network formation involved the interaction of rod assemblies.

In our studies, myosin was unfolded by lowering the pH and refolded by raising the pH back to 7.3. Unfolding at low pH and readjustment to pH 7.3 were expected to cause partial denaturation of myosin. When myosin becomes denatured, it could lead to the exposure of hydrophobic groups that were previously buried inside the protein structure. Prodan, an external fluorescent probe, was used to determine the surface hydro-

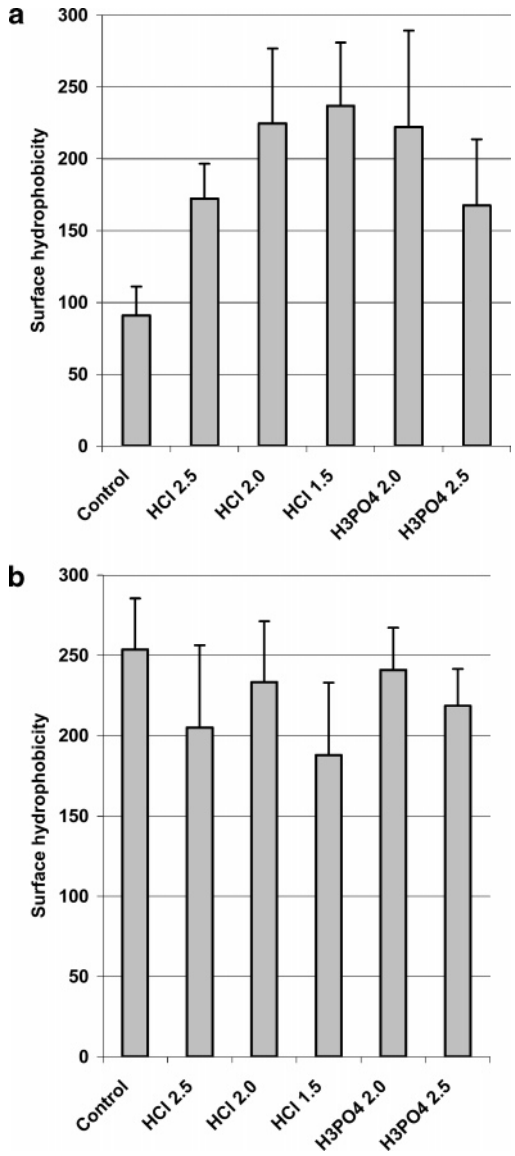

Figure 4. (**a**) Surface hydrophobicity of myosin measured using the fluorescence probe Prodan. Myosin was refolded at pH 7.3 followed by the addition of 0.6 M NaCl. (**b**) Surface hydrophobicity of myosin measured using the fluorescence probe Prodan. Myosin was unfolded in the presence of 0.6 M NaCl and refolded at pH 7.3.

phobicity of myosin at 5 °C. When salt was added to myosin before acid unfolding (**Figure 4b**), there was no significant difference ($p > 0.01$) in hydrophobicity between the control and the different acid treatments. However, when salt was added to myosin after refolding, acid-treated myosin showed a significantly higher ($p < 0.01$) hydrophobicity as compared to the control (**Figure 4a**). Typically, the total charge on a protein molecule would be affected by acid treatments, while the interaction between charged protein surfaces would be affected by salt due to shielding effects (*24*). Salt (0.6 M NaCl) added to myosin before acid treatment could protect myosin from acid denaturation and unfolding. When salt is absent during acid treatment, myosin would undergo more unfolding. The exposure of the hydrophobic interior due to unfolding would increase the hydrophobicity of acid-treated myosin as compared to the control. As pH affects the total charge on a protein molecule, the lower the pH would be the more unfolded myosin would be. Hence, in the absence of salt, myosin treated at pH 1.5 showed greater hydrophobicity than myosin treated at pH 2.5 (**Figure 4a**).

The α-helix content of acid treated myosin was determined at 5 °C using circular dichroism. The α-helix content of myosin was significantly higher ($p < 0.05$) for the addition of salt before unfolding than for the addition of salt after refolding (**Table 3**). The difference in the α-helix content of myosin could be due to the stabilizing effect of salt against protein denaturation and unfolding. When salt was added before acid unfolding, the pH of acid treatments had a significant effect ($p < 0.05$) on the α-helix content of myosin. When salt was added before unfolding, higher pH values gave a higher α-helical content than lower pH values. According to circular dichroism data, the degree of denaturation of myosin rod was of the order pH 1.5 > 2.0 > 2.5 (**Figure 5a**,**b**). The type of anions did not have any significant effect ($p > 0.05$) on the α-helix content of myosin.

Thermal gelation of myosin was studied by measuring the storage modulus (G'). At the beginning of the heating cycle (5 °C), myosin treated at pH 1.5 had a significantly higher G' as compared to the control and other treatments (**Figure 1a**−**c**). As indicated by secondary structure studies conducted at 5 °C, myosin underwent denaturation and unfolding at pH 1.5, since the refolded myosin had lost part of its native secondary structure. Partially unfolded myosin may form better gels and thus may exhibit a high G'. Also at 5 °C, G' of acid-treated myosin decreased in the following order: absence of salt ∼ salt added after refolding > salt added before unfolding. The addition of salt before unfolding may stabilize and protect myosin from denaturation as compared to the addition of salt after refolding, thus causing less interactions between myosin, and hence a lower G'.

During the heating cycle, myosin was heated from 5 to 80 °C and then cooled to 5 °C. When subjected to heating (80 °C) and cooling (5 °C), acid-treated myosin formed gels with higher G' values as compared to the control samples. This suggests that the partially refolded myosins are able to form better interactions and form stronger gel matrixes. The effect of heat on acid-treated myosin was studied using DSC. DSC measured thermal transitions in the partially denatured myosin molecule. The refolded myosin could retain some amount of its unfolded conformational state due to denaturation by acid. Acid-treated myosin subjected to heating from 5 to 80 °C showed two sharp endothermic peaks (T_p) at around 38 and 44 °C. Similar endotherms were obtained by Togashi et al. (*25*), who studied Pollock light meromyosin and myosin, and by Cross et al. (*26*), who did DSC studies on rabbit skeletal myosin rod. The different acid treatments changed the thermal sensitivity of myosin toward denaturation. The enthalpy change (ΔH) associated with thermal transition was measured as the area under the peaks and could be used as a measure of protein stability. The ΔH of acid-treated myosin with salt added before unfolding (**Table 2**) was significantly higher ($p < 0.05$) than myosin with salt added after refolding (**Table 1**). This indicates that the addition of salt to myosin before acid treatment provides greater stability against denaturation than the addition of salt after acid treatment, although the effect of salt addition sequence on gelation was not as clear.

DSC studies indicated major thermal transitions (T_p), occurring between 35 and 50 °C (**Figure 2a**,**b**). Lichan et al. (*27*, *28*) determined that progressive heating of myosin protein concentrates would lead to gradual exposure of hydrophobic residues. Chan et al. (*29*) reported that an increase in the exposure of hydrophobic residues during heat denaturation of myosin resulted in increased aggregation and gelation. ΔH for both orders of salt addition decreased in the following order:

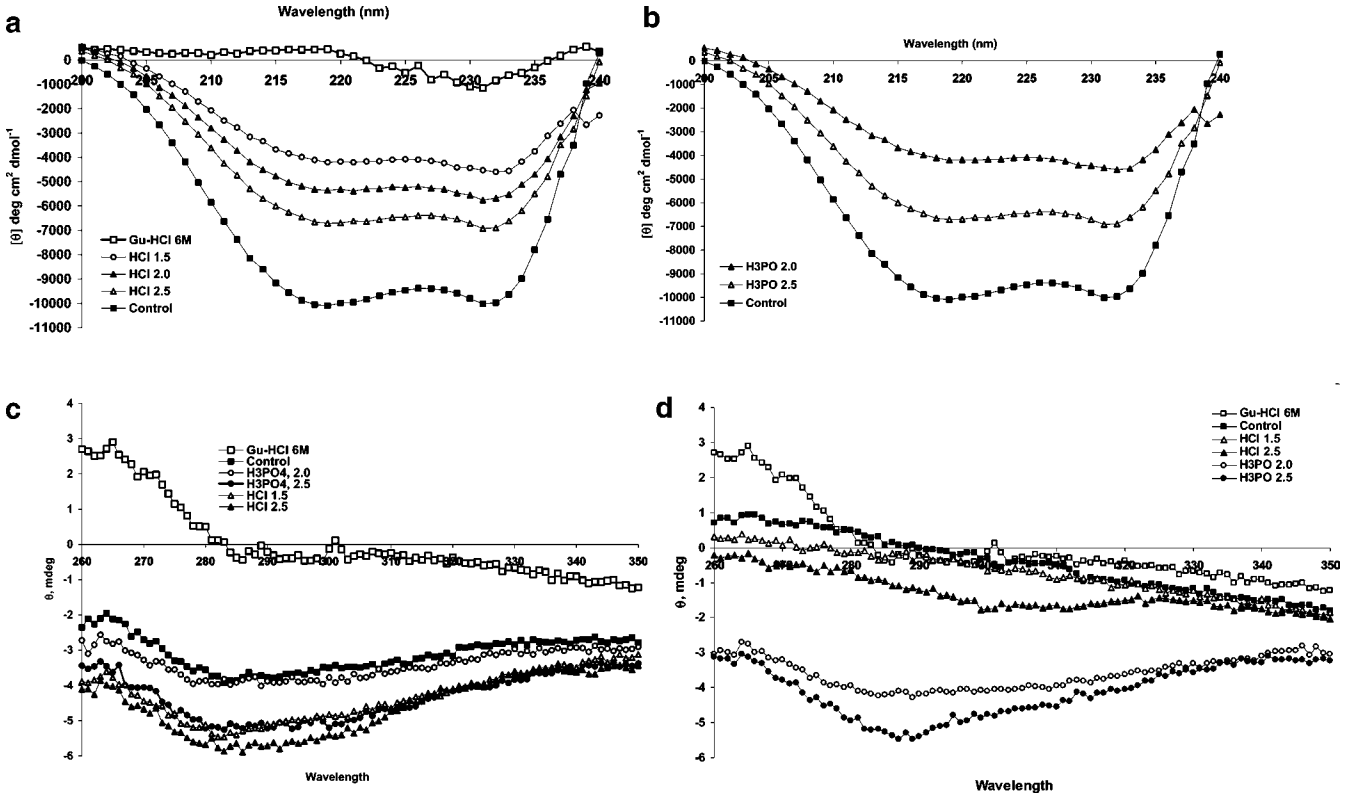

Figure 5. (**a**) Far-UV circular dichroism spectra of HCl-treated myosin with 0.6 M NaCl added before unfolding. Conformational changes in the secondary structure were measured using circular dichroism as mean residue ellipticity [θ]. The pH of myosin was adjusted to 1.5 (○), 2.0 (▲), or 2.5 (△) in the presence of 0.6 M NaCl and readjusted to 7.3. Myosin adjusted to pH 7.3 in the presence of 0.6 M NaCl was used as the control (○). Guanidine hydrochloride, Gu-HCl (6 M), was used for complete myosin denaturation (□). (**b**) Far-UV circular dichroism spectra of H_3PO_4-treated myosin with 0.6 M NaCl added before unfolding. The conformational change in the secondary structure was measured using circular dichroism as mean residue ellipticity [θ]. The pH of myosin was adjusted to 2.0 (▲) or 2.5 (△) in the presence of 0.6 M NaCl and readjusted to 7.3. Myosin adjusted to pH 7.3 in the presence of 0.6 M NaCl was used as the control (■). (**c**) Near-UV circular dichroism spectra of acid-treated myosin with 0.6 M NaCl added before unfolding. Conformational changes in the tertiary structure were measured from the circular dichroism spectra between 250 and 360 nm. The pH of myosin was adjusted using HCl to 1.5 (△) and 2.5 (▲) and using H_3PO_4 to 2.0 (○) and 2.5 (●) in the presence of 0.6 M NaCl and readjusted to 7.3. Myosin adjusted to pH 7.3 in the presence of 0.6 M NaCl was used as the control (■). Guanidine hydrochloride, Gu-HCl (6 M), was used for complete myosin denaturation (□). (**d**) Near-UV circular dichroism spectra of acid-treated myosin with 0.6 M NaCl added after refolding. Conformational changes in the tertiary structure were measured using the circular dichroism spectra between 250 and 360 nm. The pH of myosin was adjusted using HCl to 1.5 (△) and 2.5 (▲) and using H_3PO_4 to 2.0 (○) and 2.5 (●) and readjusted to 7.3. Myosin adjusted to pH 7.3 followed by the addition of 0.6 M NaCl was used as the control (■). Guanidine hydrochloride, Gu-HCl (6 M), was used for complete myosin denaturation (□).

Table 3. α-Helix Content of Myosin Subjected to Various Acid Treatments

	% α-helix	
treatment[a]	salt added before unfolding	salt added after refolding
control	32	24
HCl, pH 1.5	8	9
HCl, pH 2.0	14	9
HCl, pH 2.5	25	10
H_3PO_4, pH 2.0	8	5
H_3PO_4, pH 2.5	25	6

[a] Two acids used were HCl and H_3PO_4. HCl was used to adjust the pH to 1.5, 2.0, or 2.5 and then to readjust it back to 7.3. H_3PO_4 was used to adjust the pH to 2.0 or 2.5 and then to readjust it back to 7.3. Salt (0.6 M NaCl) was added either before unfolding (salt first) or after refolding (salt last). The α-helix content of denatured myosin (using 6 M guanidine hydrochloride) was 4%.

control > Cl^- > PO_4^{3-} treatment. The ΔH value, calculated from the area under the transition peak, is correlated with the content of ordered secondary structure of a protein (*30*). Myosin treated with Cl^- and PO_4^{3-} showed lower ΔH values on heating as compared to the control, which is likely due to denaturation and unfolding of myosin by acid treatments. Among the two anions, PO_4^{3-} treatment showed lower ΔH as compared to Cl^- treatment, indicative of greater denaturation of myosin by PO_4^{3-} treatment (**Tables 1** and **2**). Our studies on the secondary structure and hydrophobicity of myosin indicate that denatured myosin forms better gels with a higher G' as compared to less denatured control samples. Although DSC studies showed lower ΔH for PO_4^{3-}-treated myosin, we found that Cl^--treated myosin formed gels with a higher G' than PO_4^{3-}-treated myosin. When myosin was heated to 80 °C, the G' of myosin decreased in the following order: $SO_4^{2-} \sim Cl^- > PO_4^{3-}$. When myosin was cooled to 5 °C, the G' of myosin decreased in the following order: $Cl^- \geq SO_4^{2-} > PO_4^{3-}$. Also, when salt was added after refolding, Cl^-- and SO_4^{2-}-treated myosin had greater G' values than PO_4^{3-}-treated myosin, indicating a greater denaturation effect with Cl^-. The effect of various anions on the conformation of proteins is given by Hofmeister series (*10*), where the destabilizing effect of various anions decreases in the following order: $Cl^- > SO_4^{2-} > PO_4^{3-}$. Chaotropic anions such as Cl^- disrupt the water structure around protein molecules resulting in denaturation, whereas kosmotropic anions stabilize the water structure around the protein molecule. An excess amount of

kosmotropic anions could result in salting-out due to the removal of water molecules surrounding the protein. When H_3PO_4 at pH 1.5 was used, refolded myosin aggregated and precipitated. This could be due to salting-out of myosin with this particular anion. Hence, we used pH 2.0 and 2.5 for the acid treatments of myosin with H_3PO_4. DSC measurements on PO_4^{3-}-treated myosin showed a T_p at around 34.2 °C, while T_p at this temperature range for the control and Cl^- treatments was weak or nearly absent. The additional T_p observed for the PO_4^{3-}-treated myosin could be due to thermal unfolding of myosin domains (**Tables 1** and **2**). PO_4^{3-}-treated myosin showed a lower ΔH as compared to Cl^- treatment. However, according to the Hofmeister series, Cl^- is more chaotropic than PO_4^{3-}. One explanation for this anomaly between the Hofmeister series and the DSC endotherms could be that Cl^- may cause myosin to refold into a molten globular conformational state. Goto et al. (*9*) had earlier shown that the anions associated with strong acids could refold proteins into the globular state. Cl^- anions are not only present in the acid used for pH adjustment but also present in the added salt, that is, 0.6 M NaCl. Refolding of acid-denatured myosin in the presence of Cl^- may lead to higher ΔH values than PO_4^{3-}. The conformation of the refolded myosin would be different from the native state (*31*). However, the disruption of protein structure due to the chaotropic anion, Cl^-, may result in a significantly higher G' as compared to PO_4^{3-}-treated myosin.

In myosin, the secondary structure could be used as a measure of conformational change in the rod region of myosin while the tertiary structure arises primarily from the globular head region of myosin. Circular dichroism studies on the tertiary structure (**Figure 5c**,**d**) of myosin showed that the addition of 0.6 M NaCl before acid treatment had a protective effect against denaturation as compared to the addition of salt after acid treatment. Salt may prevent the unfolding of myosin by binding and stabilizing the net positive charge acquired during the lowering of pH. When salt was added after refolding, myosin treated with Cl^- was significantly more denatured ($p < 0.05$) than PO_4^{3-}-treated myosin. This could be due to the chaotropic effect of Cl^- on myosin. The secondary structure and tertiary structures of Cl^-- and PO_4^{3-}-treated myosin with salt added after refolding (**Table 3**) showed significant ($p < 0.05$) denaturation with a decrease in pH, whereas the tertiary structures of Cl^-- and PO_4^{3-}-treated myosin showed nonsignificant ($p > 0.05$) increases in denaturation with a decrease in pH (**Figure 5c**,**d**). This may indicate that low pH could more significantly alter the conformation of myosin in the rod portion than in the head region. The presence or absence of salt during acid treatment could affect the conformation of both the myosin rods and the myosin head region.

Tryptophan residues could be used as a tool for monitoring changes in the local structure and dynamics of a protein molecule (*32*, *33*). In myosin, a decrease in tryptophan fluorescence intensity could be an indication of denaturation and exposure of indole side chain of tryptophan to the aqueous environment (*34*). Tryptophan fluorescence in acid-treated myosin decreased in the following order: salt added before unfolding > absence of salt > salt added after refolding (**Figure 3a**−**c**). This could be due to the protective ability of salt to bind and stabilize myosin from unfolding; thus, we agree with the other conformational methods used.

In conclusion, salt had an impact on the provided conformational stability of myosin during acid denaturation. When salt was present during acid unfolding and refolding, myosin underwent less denaturation as compared to the addition of salt after refolding. When salt was added after refolding, myosin exhibited a higher G' as compared to the addition of salt before unfolding. Salt added before unfolding may possibly bind and stabilize myosin from denaturation. Myosin was denaturated more as the pH was decreased. Among the different acid treatments, HCl at pH 1.5 or 2.0 and H_2SO_4 at pH 1.5 formed gels with higher G' values as compared to H_3PO_4. According to the Hofmeister series, the chaotropic behavior of anions decreased in the following order: $Cl^- > SO_4^{2-} > PO_4^{3-}$. Among the different anions, G' decreased in the order $Cl^- \sim SO_4^{2-} > PO_4^{3-}$, which indicates that G' increases with the degree of myosin denaturation.

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Received for review November 5, 2006. Revised manuscript received March 7, 2007. Accepted March 16, 2007. This work was supported by the Cooperative State Research, Education and Extension Service, US Dept. of Agriculture by Grant 2004-35503-14119 of the USDA NRI Competitive Grants Program.

JF063184V