Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 107 (2008) 385–398
Food Chemistry
www.elsevier.com/locate/foodchem

Conformational and rheological changes in catfish myosin during alkali-induced unfolding and refolding

Sivakumar Raghavan *, Hordur G. Kristinsson

Laboratory of Aquatic Food Biomolecular Research, Department of Food Science and Human Nutrition, University of Florida, Gainesville, FL 32611, United States

Received 1 June 2007; received in revised form 10 August 2007; accepted 14 August 2007

Abstract

Changes in the conformation of catfish (*Ictalurus punctatus*) myosin due to (i) cations (ii) alkaline pH and (iii) salt addition were determined using circular dichroism, tryptophan fluorescence, differential scanning calorimetry and hydrophobicity studies. The relation between conformation and storage modulus (G') of alkali treated myosin was studied. Two types of bases, NaOH and KOH were used for unfolding myosin under three alkaline conditions, pH 11.0, 11.5 and 12.0. Myosin, unfolded under alkali conditions was immediately refolded by adjusting pH back to 7.3. Subjecting myosin to alkaline conditions and subsequent readjustment to pH 7.3 increased the G' of thermally treated myosin. G' was affected by the presence or absence of salt during alkali treatments. When salt was present during alkali unfolding of myosin, the added salt stabilized the conformation of myosin against alkali unfolding and denaturation. In the absence of salt or when salt was added after refolding, myosin showed significantly higher denaturation and high G' on heating and cooling. Among the different alkaline pH values, myosin treated at pH 11.0 showed higher G'. The type of anions influenced the conformation of myosin and the strength of gels. Treatment of myosin with KOH resulted in greater denaturation and higher gelling ability (G') compared to NaOH.

Keywords: Myosin; Catfish; Alkali unfolding; Cation; pH; pH shift; Salt; Conformation; Gelation

1. Introduction

Myofibrillar proteins play an important role in the physical and textural attributes of meat and muscle food products (Acton, Ziegler, & Burge, 1983; Asghar, Samejima, & Yasui, 1985; Xiong, 1994). Among the different myofibrillar proteins, myosin 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. Conformational changes and gel forming capabilities of a protein could be affected by a number of factors such as temperature, pH, hydrophobicity, ionic strength and type of ions (Phillips, Whitehead, & Kinsella, 1994). When pH of myosin is adjusted above or below its isoelectric point, myosin would gain a net negative or positive charge, which could result in repulsion among protein molecules hindering the formation of strong gels (Zayas, 1997). Disruption of protein structure could expose hydrophobic residues, which may affect gel strength. The addition of ions could shield the repulsive forces among protein molecules and could promote gelation (Mulvihill & Kinsella, 1988).

A new process involving solubilization of myofibrillar proteins using alkali (Hultin & Kelleher, 2000; Ingadottir, 2004; Kristinsson, Theodore, Demir, & Ingadottir, 2005) is used for preparing protein isolates from muscle food sources such as fish. In brief, the process involves solubilizing muscle proteins under high pH conditions (pH 10.5–11.5) and removing insoluble muscle components, such as connective tissue, bones and fat by centrifugation.

* Corresponding author. Tel.: +1 (352) 392 1991; fax: +1 (352) 392 9467.
E-mail addresses: siva.kr@gmail.com (S. Raghavan), hordur@ufl.edu (H.G. Kristinsson).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.08.037

The solubilized myofibrillar proteins are then recovered using isoelectric precipitation at pH around 5.5. Some advantages of this process are (a) high recovery of muscle proteins, (b) utilization of under-utilized raw material and (c) removal of membranes and lipids which are susceptible to oxidation. Myosin is an unstable molecule and is known to slowly form insoluble aggregates even under neutral pH conditions at 0 °C (Connell, 1960). Subjecting myosin to alkaline (high) pH values could have a significant effect on the conformation and functionality of the protein. In general, myofibrillar proteins subjected to high pH conditions would under go denaturation. Good gelation properties are normally associated with the native conformation of myosin molecules (Visessanguan & An, 2000). Numerous studies have reported positive relationship between functional ATPase activity and gel quality (Katoh, Nozaki, Komatsu, & Arai, 1979; Ooizumi, Hashimoto, Ogura, & Arai, 1981). It is generally believed that denaturation would adversely affect the functionality of proteins (Konno, Yamanodera, & Kiuchi, 1997; Visessanguan & An, 2000). Hence, one may expect lower functionality for protein isolates prepared using the above mentioned alkali process. However, Kristinsson and Liang (2006) have demonstrated improved functionality and gelation properties for alkali treated muscle proteins compared to traditional protein isolation processes. In our present work, we attempt to use conformational studies to understand the mechanism behind the improved rheological properties of alkali treated myosin.

The structure and functional of proteins could also be influenced by the type of anions and cations (Hochachka & Somero, 1984). The Hofmeister series (Kunz, Henle, & Ninham, 2004) ranks various cations based on their ability to precipitate a mixture of hen egg-white proteins. The series is of the order, $Mg^{+2} > Li^{+} > Na^{+} = K^{+} > NH_4^{+}$. Nowadays, Hofmeister series are used to rank the ability of cations to stabilize (kosmotropic) or destabilize (chaotropic) proteins. In general, the ability of cations to stabilize proteins decreases in the order (Hochachka & Somero, 1984), $NH_4^{+} > K^{+} \geqslant Na^{+} > Li^{+} > Mg^{+2}$. However, the position of various cations in the series would vary depending on the nature of protein, pH and temperature. Normally, sodium hydroxide (NaOH) is used for the alkali treatment of myofibrillar proteins. In our studies, we wanted to compare the effect of two cations, Na^{+} and K^{+}, on the conformation and functionality of channel catfish (*Ictalurus punctatus*) myosin.

Goto, Calciano, and Fink (1990, 1989) had earlier shown that extreme pH conditions and salt could refold an unfolded protein into a compact conformation similar to a molten globule. As alkali treatment would involve the use of high pH values, we wanted to study the effect of different alkaline pH (11.0, 11.5 and 12.0) on the conformation of catfish myosin and how these conformational changes would relate to the gelation behavior (storage modulus, G') of myosin. Proteins would possess a net charge at pH values other than their normal isoelectric pH. The net positive or negative charge on a protein molecule would play an important role on folding or unfolding due to electrostatic attraction or repulsion. While pH determines the total charge on a protein, salt determines the extent of interaction among those charges. The ability of a salt to shield charges on a protein molecule is known as Debye–Huckel effect. Sodium chloride (NaCl) is usually added to solubilize myosin. The addition of NaCl could have a neutralizing or shielding effect on the net protein charge (Arakawa & Tokunaga, 2004; Melander & Horvath, 1977). During the recovery of protein isolates using alkaline solubilization, salt is usually added to the proteins after adjusting the pH of the proteins to their isoelectric point, i.e. after protein have refolded. In our research, 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 catfish myosin.

2. Materials and methods

2.1. 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.

3. Methods

3.1. Preparation of myosin

Myosin was prepared from the white muscle tissue of catfish fillets by using a method similar to Kristinsson (2001). All solutions used for myosin preparation were kept cold at 4 °C. Catfish white muscle was minced once through an Oster heavy duty food grinder (model 4726-1, Sun beam, 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) tetra acetic 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 10,000*g* for 15 min. The supernatant was diluted slowly with 25 volumes of distilled water and kept at 4 °C for 60 min. The supernatant was centrifuged at 12,000*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 22,000*g* for 15 min were washed with 40 volumes of distilled water and myosin purity was checked using SDS-PAGE electrophoresis. Myosin bands were identified using high molecular weight SDS-PAGE standards. Samples of myosin were 93% pure as determined by densitometry. The protein content of myosin samples was determined using the Biuret reaction (Gornall, Bardawill, & David, 1949). Myosin samples were kept on ice and used within 4 days of preparation. This storage period did not change their electrophoretic pattern.

3.2. Unfolding myosin under alkali conditions

Catfish myosin (initial pH ~ 6.2) was unfolded under different alkaline conditions and immediately refolded by bringing the pH to 7.3 using hydrogen chloride. A preparation of myosin homogenate in 20 mM Tris–HCl was subjected to 18 different alkaline pH treatments involving (a) two different cations (b) three alkaline values for each cation, and (c) absence of salt and addition of salt before or after alkali pH adjustment for each alkaline value. The concentration of salt used during alkaline treatments was 0.6 M NaCl. The alkaline pH values used were 11.0, 11.5 and 12.0. The different cations used for pH adjustments were K^+ (from KOH), Na^+ (from NaOH). Studies were done in the absence or presence of 0.6 M sodium chloride (NaCl). When salt was used, it was added to myosin either before unfolding or after folding. When salt was added after the folding of myosin, the pH of the myosin homogenate was readjusted back to 7.3 if necessary. Three different controls were used. One control was a homogenate of myosin adjusted to pH 7.3 in the absence of salt. The second and third controls were myosin homogenates with 0.6 M salt added before or after the adjustment of pH to 7.3.

3.3. Rheology studies on myosin

Changes in storage modulus of catfish myosin during heating and cooling was studied using an AR 2000 Advanced Rheometer (TA instruments, New Castle, DE) with aluminum double concentric cylinder geometry (outer/inner diameter 21.9/20.4 mm). A myosin protein concentration of 3.5 mg/mL of homogenate was prepared in Tris–HCl buffer. Myosin was subjected to 18 different alkali treatments as described above. The viscoelastic properties 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), final (80 °C) and re-cooled (5 °C) temperatures were used for studying the gelation properties of myosin. All rheology studies were done in duplicates.

3.4. Tryptophan fluorescence studies

A myosin protein concentration of 50 μg/mL was used for measuring intrinsic tryptophan fluorescence. Two different alkali types, NaOH and KOH, 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 pH adjustment to 7.3 and the third control was prepared with the salt added after pH adjustment to 7.3. Changes in tryptophan fluorescence were measured by exciting alkali treated myosin at 297 nm and recording the emission spectra between 300 and 400 nm in a Perkin–Elmer LS 45 Luminescence Spectrophotometer (Norwalk, CT).

3.5. Surface hydrophobicity

Hydrophobicity of myosin was measured using 6-propionyl-2-(dimethylamino) naphthalene (PRODAN) as the external fluorescent probe. Two different alkali types, NaOH and KOH, 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 $1.8 \times 10^4\ M^{-1}\ cm^{-1}$ (Weber & Farris, 1979). Five hundred μL 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 using by 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 versus protein concentration was calculated using linear regression analysis and used as an index of protein hydrophobicity. All analyses were done in duplicates.

3.6. Protein conformational studies

Changes in the secondary and tertiary conformation of alkali treated myosin were studied using circular dichroism in 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. Tertiary structure was studied by scanning myosin

samples (1 mg/mL) between 350 and 260 nm in a 1 cm quartz cell. Resolution was set at 0.2 nm, bandwidth at 1 nm and sensitivity at 20 mdeg, response 1 s and scanning speed was 20 mdeg/min. Spectrum was obtained in duplicates and were averaged into one spectra. Secondary and tertiary spectra of denatured myosin were obtained by scanning protein samples prepared in 6 M guanidine hydrochloride. All studies were done at 5 °C. The alpha-helix content in the secondary structure was analyzed using DICHROWEB (Lobley, Whitmore, & Wallace, 2002).

3.7. Differential scanning calorimetry (DSC)

Denaturation and thermal transition of alkali treated myosin was studied using differential scanning micro calorimeter (VP-DSC, MicroCal, LLC, Northampton, MA). Myosin samples with a protein concentration of 10 mg/mL in 20 mM Tris–HCl buffer were used for DSC studies. The pH of the myosin samples was adjusted using NaOH or KOH to 11.5 and readjusted back to pH 7.3. Sodium chloride (0.6 M) was added either before or after 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 was used as blank.

3.8. Statistical analyses

Statistical analyses on the samples were performed using JMP™ Statistical Discovery Software (version 5.0). Analysis of variance was employed to examine the difference among treatments. Tukey's honestly significant test was used to compare the differences among treatment means.

4. Results

4.1. Effect of alkaline pH, cations and salt on the gel forming properties of myosin

The effect of alkaline pH conditions on the storage modulus (G') of catfish myosin was studied. Catfish myosin homogenate was subjected to three alkaline pH treatments, using two different bases and three different salt treatments. G' of myosin for the two different bases are shown in Fig. 1a (NaOH) and 1b (KOH).

At the beginning of the heating cycle the temperature of the alkali treated myosin was 5 °C. At this temperature, myosin subjected to Na^+ and K^+ treatments were not significantly different from each other or from the control ($p > 0.05$). However, when myosin was treated with KOH, the pH 11.0 treatment gave a higher G' than the pH 12.0 treatment ($p = 0.08$).

When the heating cycle reached the maximum temperature of 80 °C, several of the treatments were significantly different from the control. When salt was added after refolding, G' of myosin treated with NaOH or KOH at pH 11.0 was significantly higher ($p < 0.05$) than the control. In the absence of salt, myosin treated with NaOH at pH 11.0, 11.5 and 12.0 and with KOH at pH 11.0 had significantly higher G' ($p < 0.05$) than the control treatments. However, when salt was present during alkali unfolding, there was no significant difference ($p > 0.05$) between the different alkali treatments and the control. When myosin was treated with KOH (Fig. 1b), G' decreased in the order, pH 11 > 11.5 ~ 12.0. The storage modulus of myosin was affected by salt treatments. For NaOH and KOH treated myosin, G' decreased in the order, no salt > salt added before alkali unfolding ~ salt added after refolding.

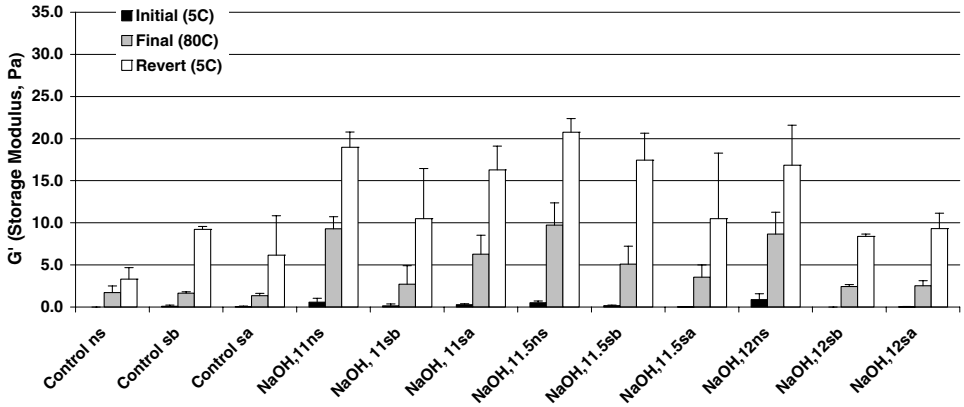

Fig. 1a. Storage modulus (G') of alkali treated myosin (3.5 mg/mL in Tris–HCl buffer) heated from an initial temperature of 5 °C to a final temperature of 80 °C and then cooled back to 5 °C. Treatment of myosin involves unfolding under different alkali conditions and refolding by adjusting pH to 7.3. Sodium hydroxide (NaOH) was used for unfolding myosin at pH 11.0, 11.5 and 12.0. Hydrochloric acid (HCl) was used for refolding. Unfolding of myosin was done under three salt conditions, (a) absence of salt, (b) salt (sodium chloride, 0.6 M) added before unfolding (c) salt (sodium chloride, 0.6 M) added after refolding. 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 pH adjustment to 7.3 and the third control was prepared with the salt added after pH adjustment to 7.3. NaOH, sodium hydroxide ns, no salt treatment sb, NaCl, 0.6 M was added before unfolding sa, NaCl, 0.6 M was added after refolding.

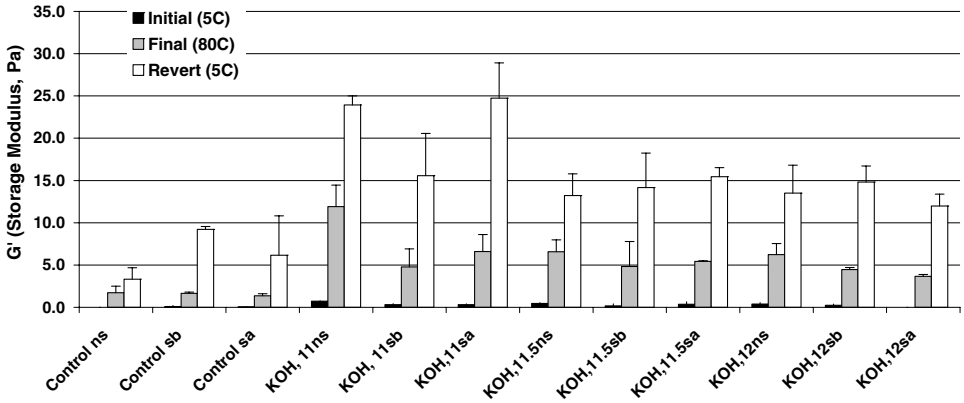

Fig. 1b. Storage modulus (G') of alkali treated myosin (3.5 mg/mL in Tris–HCl buffer) heated from an initial temperature of 5 °C to a final temperature of 80 °C and then cooled back to 5 °C. Treatment of myosin involves unfolding under different alkali conditions and refolding by adjusting pH to 7.3. Potassium hydroxide (KOH) was used for unfolding myosin at pH 11.0, 11.5 and 12.0. Hydrochloric acid (HCl) was used for refolding. Unfolding of myosin was done under three salt conditions (a) absence of salt, (b) salt (sodium chloride, 0.6 M) added before unfolding (c) salt (sodium chloride, 0.6 M) added after refolding. Three controls were prepared as described in Fig. 1a. KOH, potassium hydroxide; ns, no salt treatment; sb, NaCl, 0.6 M was added before unfolding; sa, NaCl, 0.6 M was added after refolding.

At the end of the thermal cycle, myosin was cooled back to 5 °C. At this temperature, myosin treated with alkali in the absence of salt had significantly higher G' ($p < 0.05$) compared to the control. Also, myosin treated with KOH at pH 11.0 with salt added after refolding had significantly higher ($p < 0.05$) G' than the control. Storage modulus of myosin was affected by different pH treatments. When myosin was treated with KOH, G' decreased in the order, pH 11.0 > pH 11.5–12.0. However, G' of myosin treated with NaOH (Fig. 1a) decreased in the order, pH 11.0–11.5 > 12.0. When the effect of salt was studied, G' of NaOH treated myosin decreased in the order, absence of salt > salt added first ~ salt added last. In general, G' of myosin treated with KOH was higher ($p = 0.09$) than that treated with NaOH. In the absence of salt, G' of myosin decreased in the order $K^+ > Na^+$ ($p < 0.05$).

4.2. Micro-differential scanning calorimetry (DSC) studies on alkali treated myosin

DSC was used to investigate the influence of cations and order of salt addition on the thermal stability and endothermic transitions of myosin. Myosin was unfolded using NaOH or KOH at pH 11.5, and refolded using HCl at pH 7.3. Conformational studies performed on myosin such as tryptophan fluorescence and hydrophobicity studies (see below), showed either a decreasing or increasing trend among the three pH treatments, 11.0, 11.5 and 12.0. Hence, pH 11.5 was chosen for DSC studies since it represented the midpoint of the three pH treatments. Salt (0.6 M NaCl) was added either before unfolding or after refolding of myosin. Two controls, one for each order of salt addition were used.

The endotherms of alkali treated myosin showed two major transitions peaks (T_m) between 30 and 50 °C. The first T_m occurred at around 38 °C for both myosin samples where salt was added before unfolding and after refolding. When salt was added after refolding, the second T_m occurred between 44.5 and 45.0 °C (Fig. 2a and Table 1), whereas for the addition of salt before refolding, the second T_m occurred between 44.0 and 44.5 °C (Fig. 2b and Table 2). Calorimetric enthalpy (ΔH) corresponding to each T_m was calculated as area under the peak. When salt was added to myosin before alkali unfolding, ΔH of the control was significantly higher ($p < 0.05$) than the corresponding ΔH of myosin with salt added after refolding. ΔH of Na^+ treated myosin with salt added before unfolding was higher than the corresponding ΔH with salt added after refolding while for K^+ treated myosin this trend between ΔH and salt addition was reversed (Tables 1 and 2).

4.3. Tryptophan fluorescence studies on alkali treated myosin

The effect of alkaline treatments on the unfolding of myosin was studied using tryptophan fluorescence. The presence or absence of salt had a significant effect on the tryptophan absorbance of myosin. Tryptophan fluorescence decreased in the order ($p < 0.05$), salt added first (Fig. 3a) > absence of salt (Fig. 3b) > salt added last (Fig. 3c). Also, in the absence of salt, tryptophan fluorescence of Na^+ treated myosin increased in the order, pH 11.0 < 11.5 < 12.0. In the presence of salt, there was no significant difference among myosin molecules treated with different cations.

4.4. Hydrophobicity studies on alkali treated myosin

Hydrophobicity of alkali treated myosin was measured using an external fluorescence probe, PRODAN (Fig. 4a and 4b). For myosin treated with Na^+, there was no significant difference ($p > 0.05$) between the two salt treatments. However for the K^+ treatment, the fluorescence intensity of

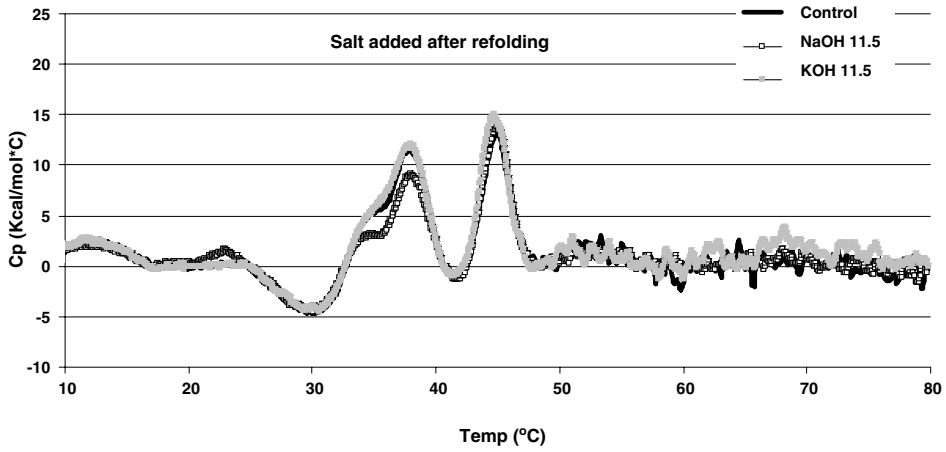

Fig. 2a. DSC endotherms of alkali treated myosin with salt (0.6 M NaCl) added after refolding. Scan rate was 1.5 °C/min between 5 and 80 °C. Alkalis used for unfolding were NaOH and KOH. Myosin was unfolded at pH 11.5 and refolded at pH 7.3. A control was prepared in 20 mM Tris–HCl buffer, with 0.6 M NaCl added after pH adjustment to 7.3.

Table 1
Transition peak temperatures (T_m) and enthalpy change (ΔH) of alkali treated myosin unfolded at pH 11.5, measured using Differential Scanning Calorimetry

Sample	Temperature, T_m (°C)		ΔH (kcal/mol)	
	T_m (1)	T_m (2)	For T_m (1)[a]	For T_m (2)[b]
Control	37.7	44.9	48.7	34.5
Na^+ treated myosin	37.9	45.0	34.7	35.5
K^+ treated myosin	37.8	44.6	50.8	38.5

Salt (0.6 M NaCl) was added to myosin after refolding at pH 7.3. Control was prepared in 20 mM Tris–HCl buffer with 0.6 M NaCl added after pH adjustment to 7.3.

[a] Indicates ΔH under peak T_m (1).

[b] Indicates ΔH under peak T_m (2).

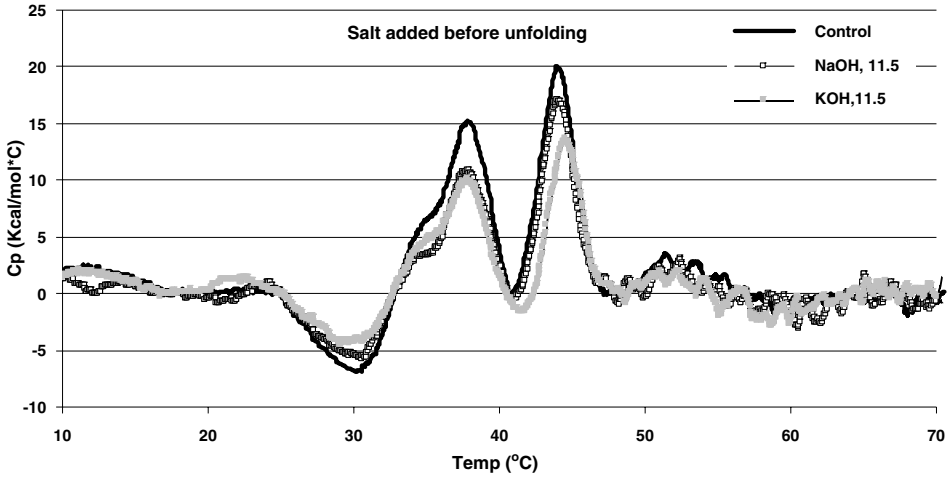

Fig. 2b. DSC endotherms of alkali treated myosin with salt (0.6 M NaCl) added before unfolding. Scan rate was 1.5 °C/min between 5 and 80 °C. Alkalis used for unfolding were NaOH and KOH. Myosin was unfolded at pH 11.5 and refolded at pH 7.3. A control was prepared in 20 mM Tris–HCl buffer, with 0.6 M NaCl added before pH adjustment to 7.3.

myosin with salt added before unfolding was significantly higher ($p < 0.05$) than those obtained with salt added after refolding. When salt was added to myosin before alkali unfolding, there was no significant difference ($p > 0.05$) between the control and different treatments. However, when salt was added to myosin after refolding, the alkali treated myosin samples had significantly higher fluorescence intensity ($p < 0.05$) than the control. Also, Na^+ treated myosin had significantly higher fluorescence intensity than K^+ treated myosin.

Table 2
Transition peak temperatures (T_m) and enthalpy change (ΔH) of alkali treated myosin unfolded at pH 11.5, measured using Differential Scanning Calorimetry

Sample	Temperature, T_m (°C)		ΔH (kcal/mol)	
	T_m (1)	T_m (2)	For T_m (1)[a]	For T_m (2)[b]
Control	37.8	44	62.1	55.7
Na^+ treated myosin	37.9	44.0	40.7	45.8
K^+ treated myosin	37.8	44.5	40.5	32.7

Salt (0.6 M) was added to myosin before unfolding. Control was prepared in 20 mM Tris–HCl buffer with 0.6 M NaCl added before pH adjustment to 7.3.
[a] Indicates ΔH under peak T_m (1).
[b] Indicates ΔH under peak T_m (2).

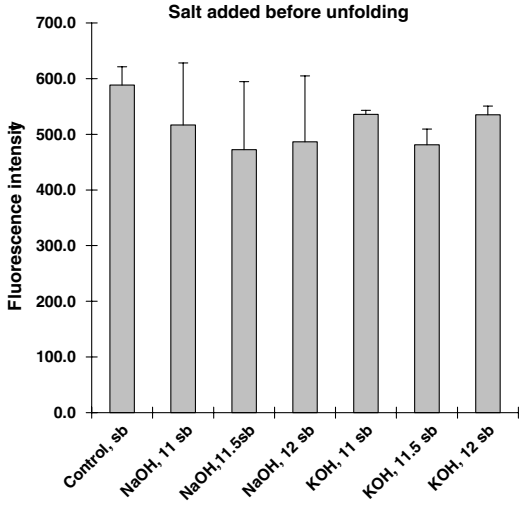

Fig. 3a. Intrinsic tryptophan fluorescence obtained with an excitation wavelength of 297 nm (protein concentration was 50 μg/mL). Myosin was unfolded using NaOH and KOH in the presence of 0.6 M NaCl and refolded at pH 7.3 using HCl. Maximum fluorescence intensity was measured. A control was prepared in 20 mM Tris–HCl buffer, with 0.6 M NaCl added before pH adjustment to 7.3. sb = 0.6 M NaCl added before unfolding.

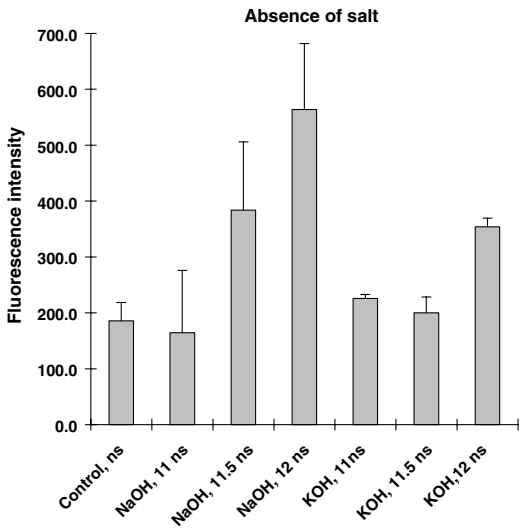

Fig. 3b. Intrinsic tryptophan fluorescence obtained with an excitation wavelength of 297 nm (protein concentration was 50 μg/mL). Myosin was unfolded using NaOH and KOH and refolded at pH 7.3 using HCl. Maximum fluorescence intensity was measured. A control was prepared in 20 mM Tris–HCl buffer in the absence of salt with pH adjusted to 7.3. ns, no salt was used during the acid treatment.

4.5. Effect of cations and salt on the secondary and tertiary structure of myosin

The changes in myosin conformation due to alkali treatment were measured using circular dichroism. The secondary structure of alkali treated myosin was significantly ($p < 0.05$) affected by the order of salt addition (Fig. 5a–5d). When salt was added before adjusting the pH to 7.3, the α-helix content of the control was 83% compared to 46% for the addition of salt after pH adjustment (Table 3). Alkali treatments decreased the α-helix content of myosin. When salt was added before unfolding, the α-helix content of Na^+ treated myosin was around 45% while that of K^+ treated myosin was between 59% and 63%. When salt was added after refolding, the α-helix content of Na^+ treated myosin was between 42% and 45% (similar to the treatment where Na^+ was added before unfolding) while that of K^+ treated myosin was between 13% and 27%. Irrespective of the order of salt addition, the secondary structure of NaOH treated myosin was not significantly ($p > 0.05$) affected by the various pH treatments. When salt was added before unfolding, NaOH treated myosin had lower ($p > 0.05$) α-helix content than KOH treated myosin. However, when salt was added to myosin after refolding, K^+ treated myosin had significantly lower amount of α-helix content than Na^+ treated myosin. When guanidine hydrochloride (0.6 M) was used for complete myosin denaturation, the α-helix content of denatured myosin was determined to be 4 % (Fig. 5a and Table 3).

The tertiary structure of myosin was measured between 260 and 350 nm in the near-UV absorbance range. Myosin was denatured using 0.6 M guanidine hydrochloride and used as a reference. The tertiary structure of myosin showed significant denaturation ($p < 0.05$) when salt was added to myosin after alkali refolding (Fig. 5f) compared to the addition of salt before alkali unfolding (Fig. 5e). When salt was added to myosin after refolding, the various pH and cation treatments had no significant effect ($p > 0.05$) on the tertiary structures of myosin. When salt was added before unfolding, the degree of denaturation of the tertiary structure increased in the order, Na^+, pH 12.0 < K^+, pH 11.0 < control ⩽ K^+, pH 12.0 < Na^+, pH 11.0.

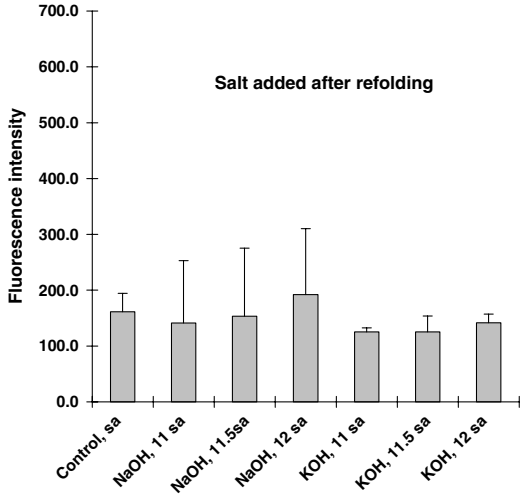

Fig. 3c. Intrinsic tryptophan fluorescence obtained with an excitation wavelength of 297 nm (protein concentration was 50 μg/mL). Myosin was unfolded using NaOH and KOH and refolded at pH 7.3 using HCl. Salt (0.6 M NaCl) was added after refolding. Maximum fluorescence intensity was measured. A control was prepared in 20 mM Tris–HCl buffer, with 0.6 M NaCl added after pH adjustment to 7.3. sa = 0.6 M NaCl added after refolding.

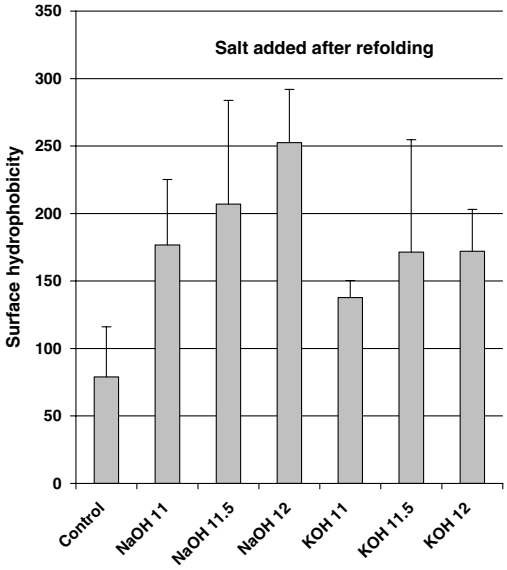

Fig. 4b. Surface hydrophobicity of myosin measured using the fluorescence probe PRODAN. Myosin was refolded at pH 7.3 followed by the addition of 0.6 M NaCl. A control was prepared in 20 mM Tris–HCl buffer with 0.6 M NaCl added after pH adjustment to 7.3.

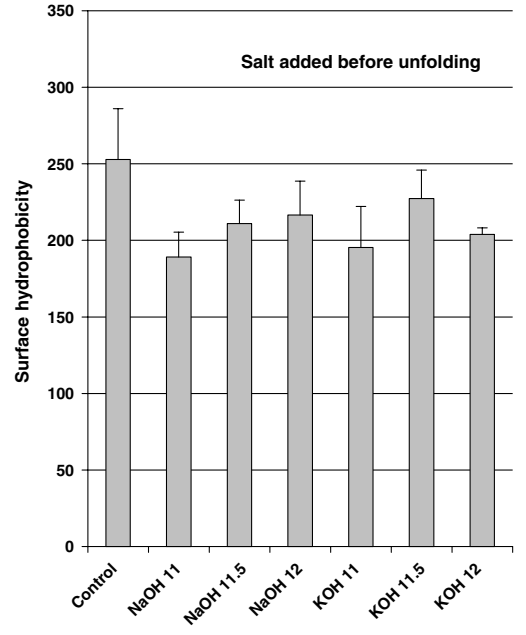

Fig. 4a. Surface hydrophobicity of myosin measured using the fluorescence probe PRODAN. Myosin was unfolded in the presence of 0.6 M NaCl and refolded at pH 7.3. A control was prepared in 20 mM Tris–HCl buffer, with 0.6 M NaCl added before pH adjustment to 7.3.

5. Discussion

Myosin comprises of two globular head sections attached to a rod-like tail section. Sharp and Offer (1992) had earlier shown the involvement of the head assembly in the heat induced setting of myosin gels. Samejima, Ishioroshi, and Yasui (1981) showed the participation of myosin rod in gel formation. It was concluded that the onset of gelation involves the interaction of myosin head assemblies while network formation involves the interaction of rod assemblies. Changes in the conformation of head and tail section of myosin could affect its functionality. Conformational changes in pH treated myosin could be studied using various biomolecular techniques such as circular dichroism, DSC, tryptophan fluorescence etc. Each of these techniques may provide information about changes occurring in the globular head region or in the tail region of myosin. Earlier studies on myosin have indicated that the α-helix content of myosin rod is >95% (King & Lehrer, 1989), while the globular head fraction has a much lower percentage of α-helix (Rayment et al., 1993). As the rod portion of myosin represents a majority of the secondary structure of myosin spectra (King & Lehrer, 1989), changes in the α-helix content would represent changes in the rod region of myosin, while changes in the tertiary structure would represent changes in the head region of myosin. Myosin also contains a number of tryptophan residues in its rod and head region. Hence, tryptophan fluorescence could be used as a tool to monitor changes in the local structure and dynamics of myosin. King and Lehrer (1989) had earlier determined that the rod region would contribute approximately 27% of the total fluorescence of the entire myosin molecule. In myosin, a decrease in tryptophan fluorescence could be an indication of denaturation and exposure of indole side chain of tryptophan to the aqueous environment (Li & King, 1996). Unfolding of myosin could also lead to the exposure of hydrophobic residues from the interior of the protein leading to an increase in hydrophobicity. Changes in the hydrophobicity of alkali treated myosin could be studied using an external fluorescent probe, PRODAN.

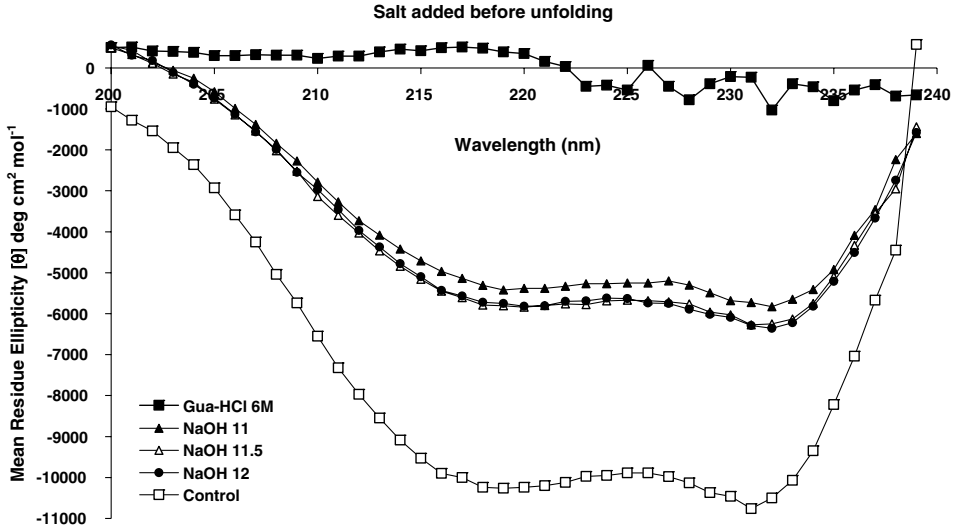

Fig. 5a. Far-UV circular dichroism spectra of NaOH treated myosin with 0.6 M NaCl added before unfolding. Conformational change in secondary structure was measured using circular dichroism as mean residue ellipticity. Myosin was unfolded at 11.0 (▲), 11.5 (△) or 12.0 (●) and refolded at pH 7.3. A control was prepared in 20 mM Tris–HCl buffer with 0.6 M NaCl added before pH adjustment to 7.3 (□). Guanidine hydrochloride (Gu–HCl, 6 M), was used for complete myosin denaturation (■).

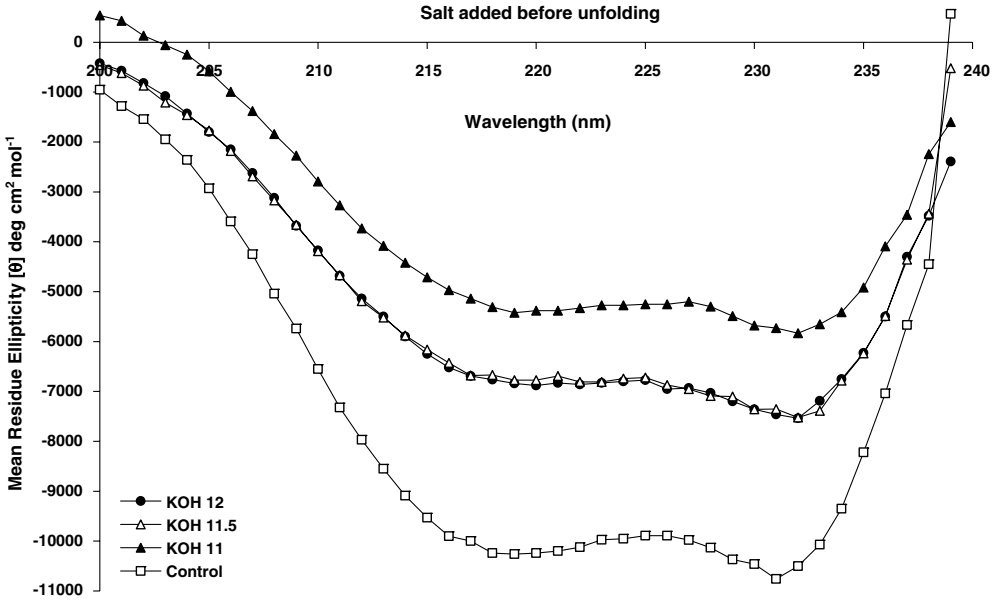

Fig. 5b. Far-UV circular dichroism spectra of KOH treated myosin with 0.6 M NaCl added before unfolding. Conformational change in secondary structure was measured using circular dichroism as mean residue ellipticity. Myosin was unfolded at pH 11.0 (▲), 11.5 (△) or 12.0 (●) and refolded at pH 7.3. Myosin with pH adjusted to 7.3 (as described in Fig. 5a) was used as control (□). Guanidine hydrochloride (Gu–HCl, 6 M), was used for complete myosin denaturation (■).

When tryptophan fluorescence intensity of alkali treated myosin was studied, the intrinsic tryptophan fluorescence decreased in the order, salt added before alkali unfolding > absence of salt > salt added after refolding (Fig. 3a–3c) indicating increased denaturation and unfolding of myosin and exposure of tryptophan residues, in the absence of salt. A high tryptophan fluorescence intensity in alkali treated myosin for the addition of salt before unfolding could be due to the ability of salt to bind and reduce the unfolding of myosin. Hydrophobicity measurements on myosin showed a trend similar to that of tryptophan fluorescence. When salt was present during alkali unfolding of myosin, the hydrophobicity of alkali treated myosin was similar to the control (Fig. 4a). However, when salt was added to alkali treated myosin after refolding (Fig. 4b), myosin showed significantly higher ($p < 0.05$) hydrophobicity than the control treatments. This effect could be due to the protective effect of salt on myosin against alkali unfolding. Salt could bind to the surface charges on myosin molecules and stabilize them from denaturation and unfolding. When

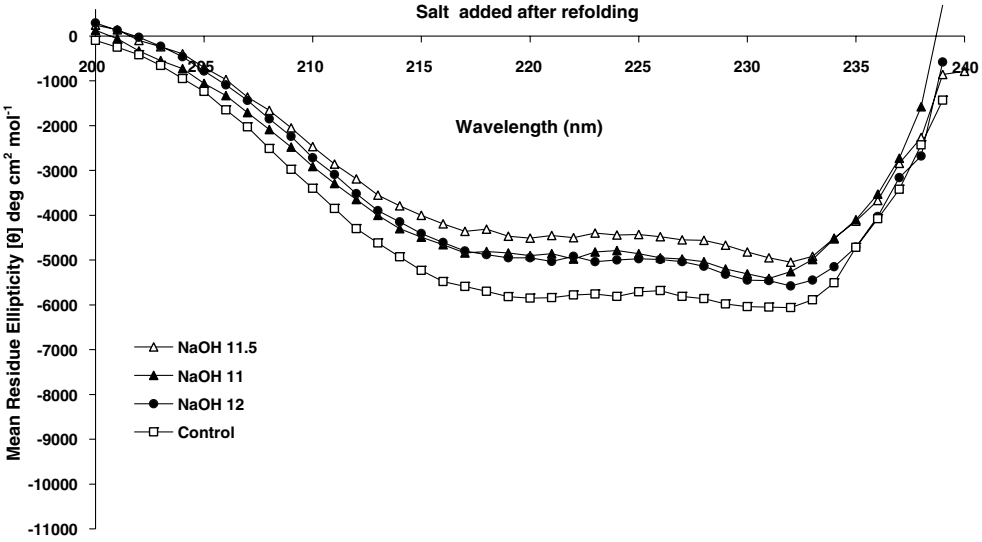

Fig. 5c. Far-UV circular dichroism spectra of NaOH treated myosin with 0.6 M NaCl added after refolding. Conformational change in secondary structure was measured using circular dichroism as mean residue ellipticity. Myosin was unfolded at 11.0 (▲), 11.5 (△) or 12.0 (●) and refolded at pH 7.3. A control was prepared in 20 mM Tris–HCl buffer with 0.6 M NaCl added after pH adjustment to 7.3 (□).

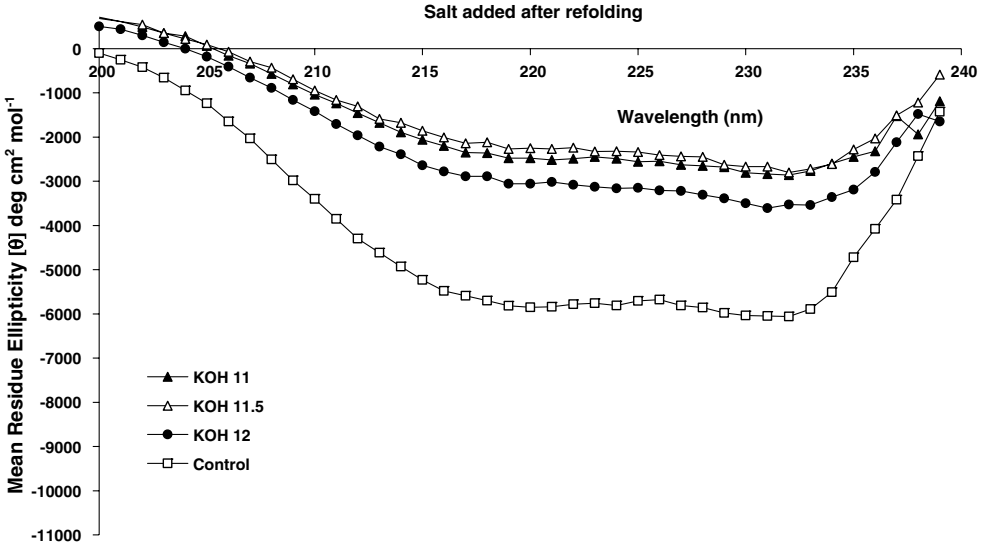

Fig. 5d. Far-UV circular dichroism spectra of KOH treated myosin with 0.6 M NaCl added after refolding. Conformational change in secondary structure was measured using circular dichroism as mean residue ellipticity. Myosin was unfolded at pH 11.0 (▲), 11.5 (△) or 12.0 (●) and refolded at pH 7.3. Myosin with pH adjusted to 7.3 (prepared as described in Fig. 5c) was used as control (□).

alkali treated myosins with salt added before unfolding and after refolding were compared side-by-side, the hydrophobicity of myosin with salt added before unfolding was higher than with salt added after refolding (Fig. 4a and 4b). This observation was contrary to the ability of salt to stabilize myosin. One reason for the low hydrophobicity of myosin with salt added last could be due to refolding of protein molecules at alkaline pH values. Goto and Fink (1989) had earlier shown that proteins would undergo unfolding at high pH and a further increase in pH could make protein refold into molten globular conformations. When salt is absent during alkali treatment (Fig. 4b), myosin may refold into a molten globular conformation. Refolding of myosin may bury the tryptophan residues and make it inaccessible for measurement leading to lower hydrophobicity (Fig. 4a). In the total absence of salt, myosin treated at pH 11.0 showed significantly lower ($p < 0.05$) fluorescent intensity of myosin compared to pH 12.0. A decrease in fluorescence intensity could be either due to unfolding and denaturation of myosin at pH 11.0 or due to refolding of myosin at pH 11.0 in to a molten globular conformation. When hydrophobicity was measured in the absence of salt (salt added after refolding, Fig. 4b), the surface hydrophobicity increased from pH 11.0 to 12.0. This could be either due to greater degree of myosin denaturation at pH 12.0, leading to the exposure of hydrophobic

Table 3
α-Helix content of myosin subjected to various alkali treatments

Treatment[A]	% α-Helix[B]	
	Salt added first	Salt added last
Control	83 a	46 b
NaOH, pH 11.0	45 b	45 b
NaOH, pH 11.5	45 b	42 b
NaOH, pH 12.0	45 b	45 b
KOH, pH 11.0	63 c	15 d
KOH, pH 11.5	59 c	13 d
KOH, pH 12.0	59 c	27 e

[A] NaOH or KOH was used to adjust pH to 11.0, 11.5 or 12.0 and then readjusted back to pH 7.3. Salt (0.6 M NaCl) was added either before unfolding (salt first) or after refolding (salt last). α-Helix content of denatured myosin (using guanidine hydrochloride) was 4 %. Two controls were prepared in 20 mM Tris–HCl buffer with 0.6 M NaCl added (a) before and (b) after, pH adjustment to 7.3.

[B] Columns having different alphabets were statistically different ($p < 0.05$).

residues or due to the refolding of denatured myosin at pH 11.0, leading to burial of hydrophobic residues. A hypothesis which would explain both tryptophan and hydrophobicity results would be that, myosin not only undergoes significant denaturation at pH 11.0 compared to pH 12.0 but also refolds into a molten globular conformation at pH 11.0.

Both myosin head (Sharp & Offer, 1992) and rod (Samejima et al., 1981) sections are known to participate in gel formation. Hence, differences in the degree of denaturation of myosin head and rod sections could affect the gelation behavior of myosin. Myosin showed a significantly higher ($p < 0.05$) degree of tertiary structure when salt was present during alkali unfolding of myosin compared to the addition of salt after alkali treatment (Fig. 5e and 5f). This could be due to the ability of salt to screen the electrostatic repulsive forces and stabilize the head region of myosin. However, when salt was added before alkali unfolding, the two alkali treatments, NaOH at pH 11.0 and KOH at pH 12.0, showed higher degree of denaturation than the control treatments. We were not able to explain this observed discrepancy in the tertiary structure of myosin. The secondary structure of myosin arises primarily from its rod region. Among the two cations, K^+ and Na^+, the secondary structure of KOH treated myosin showed a significantly ($p < 0.05$) lower denaturation when salt was present before alkali unfolding than after (Fig. 5b and 5d), while no difference was observed in the α-helix content of NaOH treated myosin for the different order of salt additions (Fig. 5a and 5c and Table 3). Also, when salt was present during alkali unfolding, KOH treated myosin had a significantly higher α-helix content than NaOH treated myosin and the reverse was true when salt was added after refolding. While studying the role of cations on the stabilization of malate dehydrogenase, Ebel, Faou, Kernel, and Zaccai (1999) observed that cations of high charge density such as Na^+ could efficiently stabilize proteins at low salt concentrations compared to cations such as K^+. They attributed the stabilization efficiency of cations to their effect on surface tension and to the interaction of cations to sites present on the folded protein. A similar observation was reported by Yamasaki, Yano, and Aoki (1991) while studying bovine serum albumin, which they called as reverse Hofmeister effect. We observed an effect similar to the reverse Hofmeister effect in alkali treated myosin, when salt was absent or added last after alkali treatment (Table 3). Na^+ due to its small size and high charge density

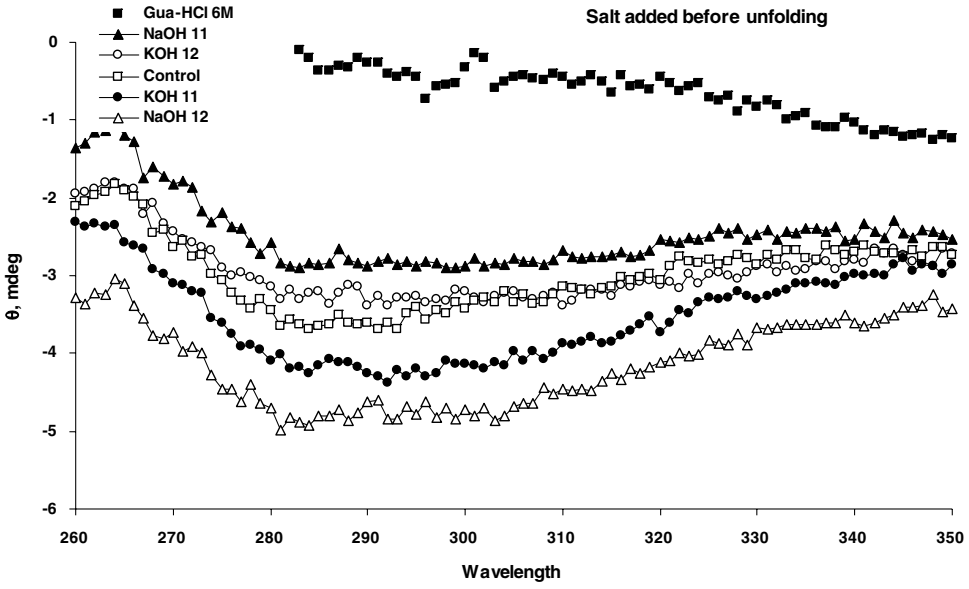

Fig. 5e. Near-UV circular dichroism spectra of alkali treated myosin with 0.6 M NaCl added before unfolding. Conformational change in the tertiary structure was measured using circular dichroism between 250 and 360 nm. Myosin was unfolded using (a) NaOH at pH 11.0 (▲) or 12.0 (△) (b) using KOH at pH 11.0 (●) or 12.0 (○), and refolded at pH 7.3. A control was prepared in 20 mM Tris–HCl buffer with 0.6 M NaCl added before pH adjustment to 7.3 (□). Guanidine hydrochloride (Gua-HCl, 6 M), was used for complete myosin denaturation (■).

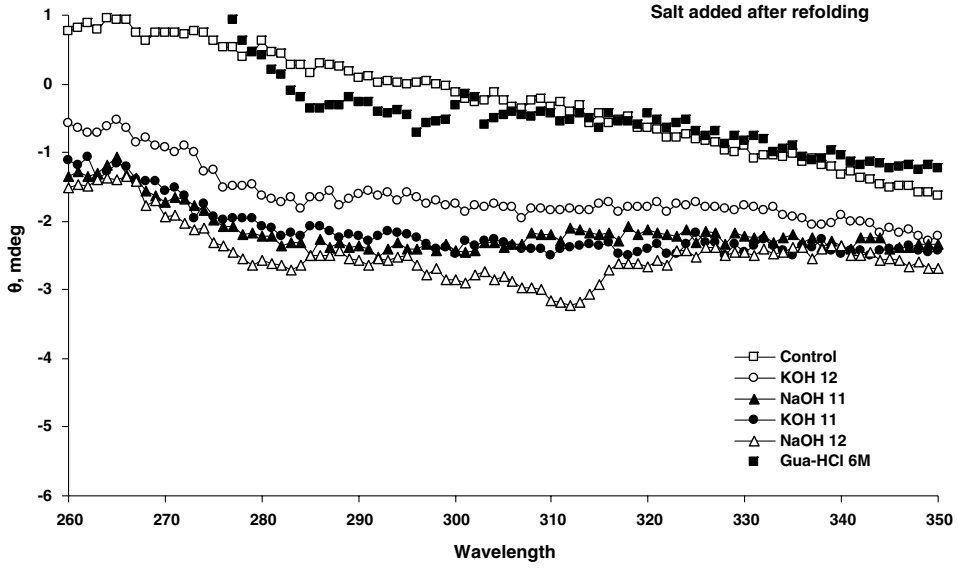

Fig. 5f. Near-UV circular dichroism spectra of alkali treated myosin with 0.6 M NaCl added after refolding. Conformational change in the tertiary structure was measured using circular dichroism between 250 and 360 nm. Myosin was unfolded using (a) NaOH at pH 11.0 (▲) or 12.0 (△) (b) using KOH at pH 11.0 (●) or 12.0 (○), and refolded at pH 7.3. A control was prepared in 20 mM Tris–HCl buffer with 0.6 M NaCl added after pH adjustment to 7.3 (□). Guanidine hydrochloride (Gua-HCl, 6 M), was used for complete myosin denaturation (■).

stabilized myosin and prevented it from unfolding compared to K^+ treatment. However, in the presence of salt, Na^+ becomes more chaotropic and destabilized myosin compared to K^+ treatment.

Unfolding and refolding of myosin by alkali treatments was expected to result in partial denaturation of myosin. DSC was used to measure thermal transitions in the partially denatured myosin. During heating, myosin would undergo structural unfolding, and hence a partially denatured myosin (by alkali treatments) would be more sensitive to heat compared to native myosin. Conversion of myosin from native or partially denatured state to a fully denatured state would be accompanied by a significant uptake of heat seen as endothermic peaks in a DSC curve. The thermal sensitivity of myosin could be related to the enthalpy change (ΔH) required for thermal denaturation and to transition peak temperatures (T_m). DSC studies on alkali treated myosin heated between 5 and 80 °C showed two sharp endothermic peaks at 37 °C and 45 °C. Similar DSC endotherms were obtained by Togashi, Kakinuma, Nakaya, Ooi, and Watabe (2002) who studied pollock myosin and light meromyosin and by Cross, Bardsley, Ledward, Small, and Sobieszek (1984) who did DSC studies on rabbit skeletal myosin rod. These DSC endotherms may correspond to the thermal denaturation and unfolding states of myosin. The addition of salt during alkali treatments changed the thermal sensitivity of myosin towards denaturation. ΔH of myosin with salt added before unfolding (Fig. 2b and Table 2) was significantly higher ($p < 0.05$) than ΔH of myosin with salt added after refolding (Fig. 2a and Table 1) indicating greater structural integrity for myosin in the presence of salt. When salt was absent or added after refolding (Table 1), ΔH of K^+ treated myosin was higher than Na^+ treated myosin. Earlier, while explaining the secondary structure of alkali treated myosin in the absence of salt, we hypothesized reverse Hofmeister effect to explain higher α-helix content in Na^+ treated myosin compared to K^+ treatment. As a reverse trend was observed with ΔH values of alkali treated myosin, it is possible that the rod and head section of myosin behave differently with different cations. K^+ is comparatively more kosmotropic than Na^+, and hence K^+ could provide greater thermal stability to alkali treated myosin in the absence of salt than Na^+ treatment.

Thermal gelation is an important attribute of myofibrillar proteins in muscle foods (Asghar et al., 1985). In general, when alkali treated myosin was heated to 80 °C or cooled to 5 °C, G' decreased in the order, absence of salt > salt after refolding ~ salt before unfolding > control (Fig. 1a and 1b). This decreasing order was similar to the order of alkaline denaturation of myosin. It may be concluded that denaturation of myosin could result in the formation of stronger gels. Our studies indicated that salt (0.6 M NaCl) could neutralize and shield the negative charges generated at high pH, thereby protecting myosin from denaturation during alkali unfolding (MacLean, Qian, & Middaugh, 2002). Although conformational studies showed that myosin with salt added before unfolding underwent lesser degree of denaturation than myosin with salt added after refolding, there was no significant difference ($p > 0.05$) in G' between these salt treated myosins. It is possible that myosin could not be sufficiently denatured by just changing the order of salt addition to obtain a significant difference in G'. When alkali treated myosin samples were heated to 80 °C and then cooled to 5 °C, the G' of myosin samples was significantly higher than the control, which would indicate that alkali denaturation would improve the viscoelastic properties of myosin.

Rheology studies showed that when salt was added after refolding, G' of K^+ treated myosin was significantly higher ($p < 0.05$) than Na^+ treated myosin. Conformational studies on myosin by the measurement of α-helix content indicates significant ($p < 0.05$) denaturation for K^+ treated myosin in the absence of salt compared to Na^+ treated myosin. According to Hofmeister effect, K^+ is a better stabilizer of proteins than Na^+ (Cacace, Landau, & Ramsden, 1997). The greater sensitivity and denaturation of K^+ treated myosin compared to Na^+ treatment was the reverse of Hofmeister effect. A reverse Hofmeister effect among anions is based on electroselectivity series (Salman Muzammil, 2000). Electroselectivity series has been attributed to the valency, charge and size of anions. A similar effect may exist among cations. Na^+ being smaller than K^+, could interact and bind strongly with the negative charge of myosin (at high pH values) thereby decreasing the degree of unfolding in myosin. Denaturation may expose the hydrophobic interior of myosin which could result in greater gelation and gel strength. As Na^+ could stabilize myosin from denaturation, G' of Na^+ treated myosin may be lower than K^+ treated myosin. In the absence of salt, K^+ treated myosin showed lower tryptophan fluorescence and hydrophobicity (Figs. 3b and 3c and 4b) than Na^+ treated myosin which might indicate that K^+ treated myosin may undergo refolding into a molten globular conformation after alkali unfolding (Goto & Fink, 1989). Among the various alkaline pH values, the conformation of myosin showed significant ($p < 0.05$) denaturation and high G' at pH 11.0 compared to pH 11.5 or 12.0. Kim, Park, and Choi (2003) had earlier determined the gel strength of fish proteins at different alkaline pH values. They also reported higher gel strength at pH 11.0 and poorer gel strength at pH 12.0. In our studies, when myosin was heated and cooled to 5 °C, G' of K^+ treated myosin decreased in the order pH $11.0 > 11.5 \sim 12.0$, while G' of Na^+ treated myosin decreased in the order, pH $11.0 \sim 11.5 > 12.0$. High gel strength (G') at pH 11.0 could be due to denaturation of myosin at this pH. Denatured myosin may form stronger cross links with higher G' on heating compared to native myosin.

Acknowledgement

This work was supported by the Cooperative State Research, Education and Extension Service, US Department of Agriculture by Grant 2004-35503-14119 of the USDA NRI Competitive Grants Program.

References

Acton, J. C., Ziegler, G. R., & Burge, D. L. (1983). Functionality of muscle constituents in the processing of comminuted meat-products. *CRC Critical Reviews in Food Science and Nutrition, 18*(2), 99–121.

Arakawa, T., & Tokunaga, M. (2004). Electrostatic and hydrophobic interactions play a major role in the stability and refolding of halophilic proteins. *Protein & Peptide Letters, 11*(2), 125–132.

Asghar, A., Samejima, K., & Yasui, T. (1985). Functionality of muscle proteins in gelation mechanisms of structured meat-products. *CRC Critical Reviews in Food Science and Nutrition, 22*(1), 27–106.

Cacace, M. G., Landau, E. M., & Ramsden, J. J. (1997). The Hofmeister series: Salt and solvent effects on interfacial phenomena. *Quarterly Reviews of Biophysics, 30*(3), 241–277.

Connell, J. J. (1960). Studies on the proteins of fish skeletal muscle. 7. Denaturation and aggregation of cod myosin. *Biochemical Journal, 75*, 530–538.

Cross, R. A., Bardsley, R. G., Ledward, D. A., Small, J. V., & Sobieszek, A. (1984). Conformational stability of the myosin rod. *European Journal of Biochemistry, 145*(2), 305–310.

Ebel, C., Faou, P., Kernel, B., & Zaccai, G. (1999). Relative role of anions and cations in the stabilization of halophilic malate dehydrogenase. *Biochemistry, 38*(28), 9039–9047.

Gornall, A. G., Bardawill, C. J., & David, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *Journal of Biological Chemistry, 177*(2), 751–766.

Goto, Y., Calciano, L. J., & Fink, A. L. (1990). Acid-induced folding of proteins. *Proceedings of the National Academy of Sciences of the United States of America, 87*(2), 573–577.

Goto, Y., & Fink, A. L. (1989). Conformational states of beta-lactamase – molten-globule states at acidic and alkaline pH with high salt. *Biochemistry, 28*(3), 945–952.

Hochachka, P. W., & Somero, G. N. (1984). *Biochemical adaptation*. Princeton, NJ: Princeton University Press.

Hultin, H. O., & Kelleher, S. D. (2000). *High efficiency alkaline protein extraction*. US Patent 6, 136, 959.

Ingadottir, B. (2004). *The use of acid and alkali-aided protein solubilization and precipitation methods to produce functional protein ingredients from Tilapia*. Gainesville: Department of Food Science and Human Nutrition, University of Florida, 105.

Katoh, N., Nozaki, H., Komatsu, K., & Arai, K. I. (1979). New method for evaluation of the quality of frozen surimi from Alaska pollack. Relationship between myofibrillar ATPase activity and kamaboko forming ability of frozen surimi. *Bulletin of the Japanese Society of Scientific Fisheries, 45*(8), 1027–1032.

Kim, Y. S., Park, J. W., & Choi, Y. J. (2003). New approaches for the effective recovery of fish proteins and their physicochemical characteristics. *Fisheries Science, 69*(6), 1231–1239.

King, L., & Lehrer, S. S. (1989). Thermal unfolding of myosin rod and light-meromyosin – circular-dichroism and tryptophan fluorescence studies. *Biochemistry, 28*(8), 3498–3502.

Konno, K., Yamanodera, K., & Kiuchi, H. (1997). Solubilization of fish muscle myosin by sorbitol. *Journal of Food Science, 62*(5), 980–984.

Kristinsson, H. G. (2001). Evaluation of different methods to isolate cod (Gadus morhua) muscle myosin. *Journal of Food Biochemistry, 25*(3), 249–256.

Kristinsson, H. G., & Liang, Y. (2006). Effect of pH-shift processing and surimi processing on atlantic croaker (Micropogonias undulates) muscle proteins. *Journal of Food Science, 71*(5), C304–C312.

Kristinsson, H. G., Theodore, A. E., Demir, N., & Ingadottir, B. (2005). A comparative study between acid- and alkali-aided processing and surimi processing for the recovery of proteins from channel catfish muscle. *Journal of Food Science, 70*(4), C298–C306.

Kunz, W., Henle, J., & Ninham, B. W. (2004). 'Zur Lehre von der Wirkung der Salze' (about the science of the effect of salts): Franz Hofmeister's historical papers. *Current Opinion in Colloid and Interface Science, 9*(1–2), 19–37.

Li, S. J., & King, A. J. (1996). Lipid oxidation and myosin denaturation in dark chicken meat. *Journal of Agricultural and Food Chemistry, 44*(10), 3080–3084.

Lobley, A., Whitmore, L., & Wallace, B. A. (2002). DICHROWEB: An interactive website for the analysis of protein secondary structure from circular dichroism spectra. *Bioinformatics, 18*(1), 211–212.

MacLean, D. S., Qian, Q. S., & Middaugh, C. R. (2002). Stabilization of proteins by low molecular weight multi-ions. *Journal of Pharmaceutical Sciences, 91*(10), 2220–2229.

Melander, W., & Horvath, C. (1977). Salt effects on hydrophobic interactions in precipitation and chromatography of proteins – interpretation of lyotropic series. *Archives of Biochemistry and Biophysics, 183*(1), 200–215.

Mulvihill, D. M., & Kinsella, J. E. (1988). Gelation of beta-lactoglobulin – effects of sodium-chloride and calcium-chloride on the rheological and structural-properties of gels. *Journal of Food Science, 53*(1), 231–236.

Ooizumi, T., Hashimoto, K., Ogura, J., & Arai, K. (1981). Quantitative aspect for protective effect of sugar and sugar alcohol against denaturation of fish myofibrils. *Bulletin of the Japanese Society of Scientific Fisheries, 47*(7), 901–908.

Phillips, L. G., Whitehead, D. M., & Kinsella, J. E. (1994). *Structure-function properties of food proteins*. San Diego: Academic Press.

Rayment, I., Rypniewski, W. R., Schmidtbase, K., Smith, R., Tomchick, D. R., Benning, M. M., et al. (1993). Three-dimensional structure of myosin subfragment-1 – a molecular motor. *Science, 261*(5117), 50–58.

Salman Muzammil, Y. K. S. T. (2000). Anion-induced stabilization of human serum albumin prevents the formation of intermediate during urea denaturation. *Proteins: Structure, Function, and Genetics, 40*(1), 29–38.

Samejima, K., Ishioroshi, M., & Yasui, T. (1981). Relative roles of the head and tail portions of the molecule in heat-induced gelation of myosin. *Journal of Food Science, 46*(5), 1412–1418.

Sharp, A., & Offer, G. (1992). The mechanism of formation of gels from myosin molecules. *Journal of the Science of Food and Agriculture, 58*(1), 63–73.

Togashi, M., Kakinuma, M., Nakaya, M., Ooi, T., & Watabe, S. (2002). Differential scanning calorimetry and circular dichroism spectrometry of walleye pollack myosin and light meromyosin. *Journal of Agricultural and Food Chemistry, 50*(17), 4803–4811.

Visessanguan, W., & An, H. (2000). Effects of proteolysis and mechanism of gel weakening in heat-induced gelation of fish myosin. *Journal of Agricultural and Food Chemistry, 48*(4), 1024–1032.

Weber, G., & Farris, F. J. (1979). Synthesis and spectral properties of a hydrophobic fluorescent-probe – 6-propionyl-2-(dimethylamino) naphthalene. *Biochemistry, 18*(14), 3075–3078.

Xiong, Y. L. L. (1994). Myofibrillar protein from different muscle-fiber types – implications of biochemical and functional-properties in meat processing. *Critical Reviews in Food Science and Nutrition, 34*(3), 293–320.

Yamasaki, M., Yano, H., & Aoki, K. (1991). Differential scanning calorimetric studies on bovine serum albumin: II. Effects of neutral salts and urea. *International Journal of Biological Macromolecules, 13*(6), 322–328.

Zayas, J. F. (1997). *Functionality of proteins in food*. New York: Springer.