JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 4241–4249 4241
DOI:10.1021/jf903219u

Structural Changes and Dynamic Rheological Properties of Sarcoplasmic Proteins Subjected to pH-Shift Method

Panchaporn Tadpitchayangkoon,[†] Jae W. Park,[§] Steven G. Mayer,[#] and Jirawat Yongsawatdigul*,[†]

[†]School of Food Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand, [§]Oregon State University Seafood Laboratory, 2001 Marine Drive #253, Astoria, Oregon 97103, and [#]Chemistry Department, University of Portland, 5000 North Willamette Boulevard, Portland, Oregon 97203

Structural changes and dynamic rheological properties of sarcoplasmic proteins from striped catfish (*Pangasius hypophthalmus*) treated by various pH-shift processes were investigated. Isoelectric precipitation of acid-extracted sarcoplasmic proteins led to the lowest solubility in water. Sarcoplasmic proteins were unfolded after extremely acidic and alkaline extraction, exposing tryptophan and aliphatic residues. The α-helical structure was converted to β-sheet following acidic extraction, whereas alkaline treatment did not disturb the α-helical structure of sarcoplasmic proteins. Disulfide formation, hydrogen bonding via tyrosine residues, and hydrophobic interactions occurred under extreme pH extraction. Acidic extraction induced denaturation and aggregation of sarcoplasmic proteins to a greater extent than did alkaline treatment. Hydrophobic interactions via aliphatic and aromatic residues were formed during isoelectric precipitation. Sarcoplasmic proteins were partially refolded after isoelectric precipitation followed by neutralization. Sarcoplasmic proteins prepared from an alkaline pH-shift process readily aggregated to form a gel at 45.10 °C, whereas higher thermal denaturation temperatures (>80 °C) and gel points (~78 °C) were observed in acid-treated sarcoplasmic proteins. The pH condition used for extraction, precipitation, and neutralization greatly affected structural changes of sarcoplasmic proteins, leading to different thermal and dynamic rheological properties.

KEYWORDS: Sarcoplasmic proteins; striped catfish; FT-Raman spectroscopy; pH-shifting; dynamic rheological properties

INTRODUCTION

The pH-shift process is defined as the process in which muscle proteins are extracted at either acidic or alkaline pH to obtain the maximum solubility followed by the recovery of extracted proteins at their isoelectric point (*1*, *2*). Most myofibrillar and sarcoplasmic proteins are recovered by the pH-shift process. Several studies have indicated the improvement of gel texture using the pH-shift process (*3*−*6*), whereas others reported lower gel qualities compared to the conventional washing process (*7*, *8*). In general, alkaline extraction has been reported to result in superior textural properties of fish protein isolate as compared with acid extraction (*3*, *4*, *6*). Unlike thermally induced gelation of muscle proteins, gelation of fish protein isolate is not clearly understood.

Conformational changes of myofibrillar proteins by a pH-shift process have been widely studied. A pH-shift process will induce a globular head of myosin heavy chain to expose hydrophobic clusters and sulfhydryl groups, thereby rendering conversion to a molten globule (*9*). Partial unfolding of myosin and myofibrillar proteins induced by acid and alkali solutions was followed by refolding after isoelectric precipitation. This promoted the exposure of surface hydrophobicity, surface interfacial activity, and sulfhydryl groups that, in turn, led to an improvement of gelation and emulsification properties (*3*, *10*−*12*). In contrast to myofibrillar proteins, structural changes and gelation properties of sarcoplasmic proteins subjected to a pH-shift process have not yet been extensively investigated.

Denaturation and/or aggregation of muscle proteins, particularly myosin, subjected to a pH-shifting treatment have been investigated (*9*). However, the effect of the pH-shift method on conformational changes of another important group of fish muscle proteins, namely, sarcoplasmic proteins, has not been systematically determined. Several techniques are available for protein structure elucidation. Circular dichroism is typically applied to investigate the secondary and tertiary structures of protein (*9*, *13*, *14*). However, this application is limited to only the diluted protein solution, not to the solid state. Whereas fluorescence spectroscopy and probe spectrofluorometry can be used to monitor conformational changes of various proteins based on the environments of tyrosine and tryptophan or hydrophobic residues (*9*, *11*, *13*, *14*), information regarding secondary structural changes is impossible to obtain from this technique. Raman spectroscopy is a powerful technique for protein structure determination and

*Corresponding author (telephone 66-44-224359; fax 66-44-224150; e-mail Jirawat@sut.ac.th).

Published on Web 03/17/2010

offers several advantages that overcome the shortcomings mentioned above. Raman spectroscopy is used to characterize the backbone conformation of protein, thus giving information on the relative properties of different types of secondary structures in polypeptides or proteins in both liquid or solid form (*15*). It can also be used to determine the microenvironment of the side chains, including disulfide bond conformation, as well as aliphatic and aromatic side chains (*16*). Understanding the effect of the pH-shift process at the molecular level would reveal the role of sarcoplasmic proteins in the gelation of fish protein isolate. Therefore, our objective was to investigate conformational and structural changes of sarcoplasmic proteins using Fourier-transform (FT) Raman spectroscopy. In addition, the dynamic rheological and thermal behaviors of sarcoplasmic proteins were studied as affected by pH treatments.

MATERIALS AND METHODS

Sarcoplasmic Protein Preparation. Striped catfish (*Pangasius hypophthalmus*) with a size of 1−1.5 kg were purchased from a local market in Nakhon Ratchasima, Thailand, approximately 5 h after catch. Fish were transported to the Suranaree University of Technology laboratory in a polystyrene box filled with ice within 30 min. Immediately upon arrival, fish were eviscerated, deskinned, and minced using a meat grinder (model 8-22, Marblehead, OH). The fish mince was homogenized with cold deionized (DI) water at a ratio of 1:3 (w/v) using an IKA homogenizer (IKA works Asia, Bhd, Malaysia) set at speed 3 for 3 min. The homogenate (pH 6.4 ± 0.2) was centrifuged at 10000*g* and 4 °C for 20 min. The supernatant as a sarcoplasmic protein fraction with protein concentration of 10 ± 2 mg/mL was analyzed using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) gel and lyophilized (model Lyovac GT2-S, GEA Lyophil GmbH, Huerth, Germany). The lyophilized sarcoplasmic protein powder was vacuum-packed and kept at −80 °C until used within 6 months.

The lyophilized sarcoplasmic proteins with protein content of ∼912 mg/g were subjected to various pH treatments as used in the pH-shift process. According to our previous study, striped catfish mince showed the highest solubility at pH 3 and 11 and the lowest solubility at pH 5.5. Therefore, pH values of 3, 5.5, and 11 were selected for this study. The pH-adjusting step was divided into three conditions as follows:

First, 1 g of lyophilized sarcoplasmic proteins was mixed with 20 mL of cold DI water (pH 6.3 ± 0.2) and then adjusted to pH 3.0, 5.5, 7.0, and 11.0, respectively, using either 2 N NaOH or 2 N HCl solutions. The final pH value after each pH adjustment step was within ±0.2. The final volume of each sample was brought to 25 mL, and the mixture was stirred at 4 °C for 30 min (*17*); this was referred to as "proteins subjected to extraction pH" (EP).

Second, 1 g of lyophilized sarcoplasmic proteins was mixed with 20 mL of cold DI water. The pH of the mixture was adjusted to pH 3.0 or 11.0 using either 2 N HCl or 2 N NaOH solution, respectively, and then stirred at 4 °C for 30 min. The pH of the mixture was then adjusted to pH 5.5. The final volume was brought to 25 mL, and the mixture was stirred at 4 °C for 30 min (*17*); this was referred to as "isoelectrically precipitated proteins" (IP).

Third, 1 g of lyophilized sarcoplasmic proteins was mixed with 20 mL of cold DI water. The pH of the mixture was adjusted to pH 3.0 or 11.0 using either 2 N HCl or 2 N NaOH solution, respectively, and then stirred at 4 °C for 30 min. The pH of the mixtures was adjusted to pH 5.5, and then the mixture was stirred at 4 °C for 30 min, followed by adjustment of the pH to 7. The final volume was brought to 25 mL, and the mixture was stirred at 4 °C for 30 min; this was referred to as "neutralized proteins" (NP).

The experimental treatments are shown in **Figure 1**. The experiment was carried out in triplicate. The EP, IP, and NP samples obtained from various pH treatments in the form of a protein suspension were evaluated for protein solubility and total sulfhydryl (SH) content. Microdifferential scanning calorimetry (micro-DSC) and oscillatory dynamic properties of these samples were also investigated. Some portions of each sample were lyophilized (Freezone12L; Labconco Corp., Kansas, MO), manually ground, and kept at −80 °C for Raman spectroscopy measurement within 3 days.

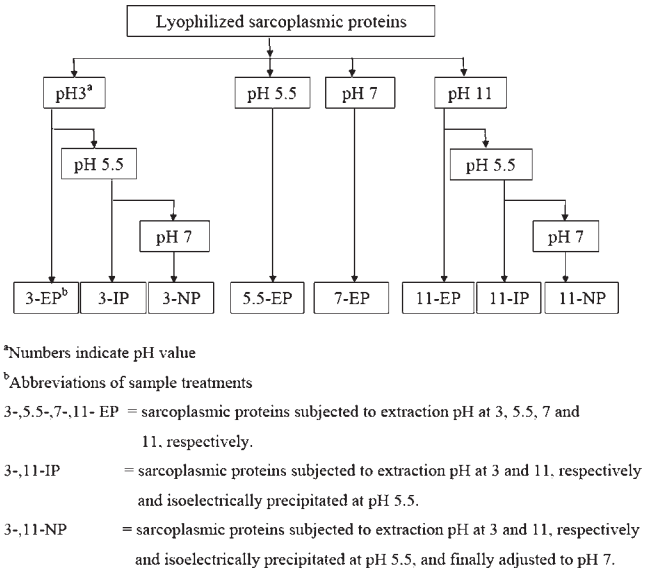

Figure 1. Flowchart of sample preparation.

Protein Solubility. All samples obtained from various pH treatments were centrifuged at 10000*g* and 4 °C for 20 min. The protein content of the supernatant was determined using the Lowry method (*18*) with bovine serum albumin (BSA) as a standard. The protein solubility was expressed as milligrams per milliliter and percentage of total protein of the lyophilized powder. Each sample was analyzed in duplicate.

Total Sulfhydryl Content. The total SH content of all samples was determined according to the method of Yongsawatdigul and Park (*4*). The sample (1 mL) was homogenized in 9 mL of a solubilizing buffer (0.2 M Tris-HCl, 2% SDS, 10 mM ethylenediaminetetraacetic acid, 8 M urea, pH 7.0). The homogenates were heated at 100 °C for 5 min and centrifuged at 10000*g* for 15 min (Eppendorf model 4515C; Westbury, NY). To a 1 mL aliquot of the supernatant was added 0.01 mL of Ellman's reagent (10 mM 5,5′-dinitriobis[2-nitrobenzoic acid]). The mixture was incubated at 40 °C for 25 min. The absorbance at 412 nm was measured to calculate the total SH content using the extinction coefficient of 13600 M^{-1} cm^{-1}. Each sample was analyzed in duplicate.

Raman Spectral Analysis. Approximately 1.8 g of lyophilized samples was placed in a 2 mL screw-top vial with cap (Supelco, Bellefonte, PA) and measured using a FT-Raman spectrometer (NXR FT-Raman module, Thermo Scientific Inc., Waltham, MA) at room temperature with the use of the dynamically aligned Vecta-Plus interferometer installed in the FT-IR bench (Nicolet 6700, Thermo Scientific Inc.). Raman spectral data were collected with an InGaAs detector, and the fundamental of a Nd:YLF laser (1064 nm) was used as the source. A 632.8 nm HeNe laser was used to calibrate the interferometer. The spectral resolution was 4.0 cm^{-1} from 98.2842 to 4001.5630 cm^{-1}. The spectra were an average of 64 scans and were smoothed. The recorded spectrum was analyzed using OMNIC Professional software version 7.3. The presented spectra and intensity were the average of triplicate measurements. The intensity of individual bands was normalized using the peak near 1004 cm^{-1} as an internal standard, which was reported to be insensitive to the microenvironment (*19*). Assignments of the bands in the spectra to protein vibrational modes were made according to published literature (*15*, *19*−*22*).

Micro-Differential Scanning Calorimetry. Micro-DSC studies were performed using a Setaram micro-DSC III (Setaram Co., Lyon, France). The instrument was calibrated for temperature accuracy using naphthalene. Samples were accurately weighed to approximately 500 mg in a stainless steel vessel. In the reference vessel, DI water was added. Samples were scanned at 1 °C/min over the range of 10−100 °C. At least two duplicate samples with reproducible thermograms were analyzed for peak temperature (T_{max}) and enthalpy of denaturation, and the average values are presented.

Oscillatory Dynamic Properties. Oscillatory dynamic properties of the pH-treated sarcoplasmic proteins were measured as a function of temperature using a CS-50 rheometer (Bohlin Instruments, Inc., Cranbury, NY). A coaxial bob and cup having a 25 mm diameter bob (C25 DIN

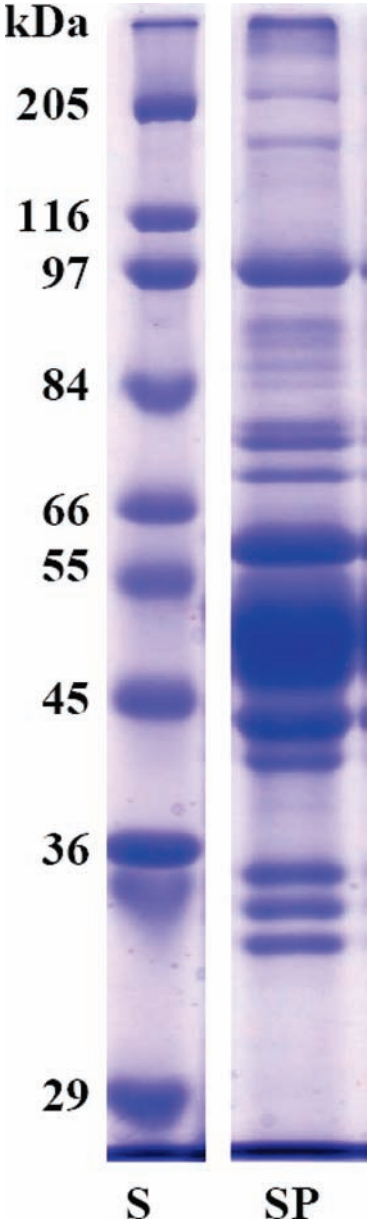

Figure 2. SDS-PAGE pattern (12.5% acrylamide) of lyophilized sarcoplasmic protein. S and SP denote standard molecular weight and sarcoplasmic proteins, respectively.

53019) was used. Shear stress of 0.02−0.05 Pa with a frequency of 0.1 Hz was found to be in the linear viscoelastic range of the evaluated samples. The samples were heated at 1 °C/min over the range of 10−90 °C. A plastic cover with a moistened sponge inside was used to prevent sample drying during heating. Elastic modulus (G') and phase angle (degree) of at least two duplicate samples were averaged and are presented.

Statistical Analysis. The significance of the difference among samples from triplicate experiments was analyzed by ANOVA and Duncan's multiple-range test (DMRT) (*23*). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 13.0 for Windows, SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Protein Solubility. Sarcoplasmic proteins of striped catfish contained various proteins with major molecular weight (MW) ranging from 36 to 97 kDa (**Figure 2**). Myofibrillar proteins, particularly myosin (MW = 205 kDa), can be considered as insignificant, implying that mostly sarcoplasmic proteins are extracted by the condition (mince to water ratio of 1:3) applied in this study.

Table 1. Protein Solubility and Total Sulfhydryl Content of Sarcoplasmic Proteins Treated at Various pH Values[a]

pH treatment[b]	protein solubility[c] (mg/mL)	total sulfhydryl content (mol/10^5 mg of protein)
3-EP	18.95 ± 0.83 a (51.94)	27.10 ± 0.48 d
3-IP	2.60 ± 0.41 d (7.12)	31.41 ± 0.17 c
3-NP	3.20 ± 0.56 d (8.77)	23.13 ± 0.46 e
5.5-EP	14.65 ± 0.9 b (40.15)	37.37 ± 0.74 b
7-EP	19.02 ± 0.13 a (52.13)	40.09 ± 1.39 a
11-EP	21.25 ± 0.43 a (58.25)	11.24 ± 0.02 g
11-IP	6.57 ± 0.34 c (18)	19.68 ± 0.17 f
11-NP	7.91 ± 0.32 c (21.68)	27.65 ± 1.21 d

[a] Means with different letters in the same column are significantly different ($P<0.05$). [b] Abbreviations of pH treatments are the same as **Figure 1**. [c] Number in parentheses indicates the percentage of soluble protein as compared to total protein of lyophilized sarcoplasmic proteins.

The solubility of lyophilized sarcoplasmic proteins after various pH treatments is shown in **Table 1**. The protein content of sarcoplasmic proteins treated at pH 7 was approximately 19.02 mg/mL, indicating only 52% of total sarcoplasmic proteins were soluble. However, sarcoplasmic proteins freshly extracted from Atlantic mackerel exhibited high solubility (approximately 78%) after the pH had been adjusted to 5.5 (*24*). The relatively low solubility observed in our study could have arisen from conformational changes of proteins induced by lyophilization. The solubility of sarcoplasmic proteins decreased when the pH was adjusted to pH 5.5 (5.5-EP). The solubility of lyophilized sarcoplasmic proteins was not affected by extremely acidic (3-EP) or alkaline (11-EP) extraction.

Isoelectric precipitation of the acid- and alkali-extracted samples (3- and 11-IP) resulted in a marked decrease in protein solubility that was much lower than that of 5.5-EP, despite the fact that they had the same pH of 5.5 ($P < 0.05$). This result indicated that shifting the pH from the extreme condition (3 → 5.5, 11 → 5.5) led to extensive conformation rearrangement, reflected in a marked decrease in solubility of sarcoplasmic protein. Acid treatment induced thiol oxidation and decreased surface hydrophobicity of sarcoplasmic proteins to a greater extent than did alkaline treatment (*25*). These might eventually lead to more protein aggregation and a decreased solubility as found in 3-IP. Because the solubility of 3-IP was lower than that of 11-IP, it could be presumed that protein underwent denaturation under acidic extraction to a greater extent than under alkaline extraction. Our findings on the effect of acid pH-shift treatment were in agreement with previous studies. The lower recovery of sarcoplasmic proteins was observed with treatment at pH 2.0 or 3.0 and then shifted to pH 5.5 as compared to the alkaline extraction (*17*, *26*). The solubility of trout hemoglobin, which was unfolded at pH 2.5−3.0 and readjusted to pH 5.5, was also lower than when unfolded at alkaline pH (*26*). Protein solubility remained low after neutralization (3- and 11-NP) and lower than that of 7-EP ($P < 0.05$). This implied that neutralization did not fully refold protein conformations back to their native state (7-EP).

It should be pointed out that the ionic strength of each sample was different due to the difference in the amounts of HCl and NaOH added. The estimated ionic strengths based on the concentrations of Na^+ and Cl^- of various treatments of EP, IP, and NP were 20−60, 52−62, and 130−140 mM, respectively. It was obvious that an increase in ionic strength did not increase protein solubility in this case. The effect of ionic strength on solubility also seemed to be negligible as the 3-NP sample exhibited higher solubility than the 11-NP sample ($P < 0.05$, **Table 1**), despite their comparable ionic strengths (130−140 mM). Our results demonstrated that the solubility of sarcoplasmic proteins was greatly

affected by the pH-shift process and that the acid pH-shift treatment resulted in lower solubility than did the alkaline counterpart.

Total SH Content. Total SH content of sarcoplasmic proteins varied with pH treatments (**Table 1**). The total SH content of 7-EP was highest and decreased when the pH was adjusted to 3.0, 5.5, and 11.0 ($P < 0.05$), indicating that thiol oxidation of sarcoplasmic proteins occurred at acidic and alkaline extraction. This result was in agreement with Thawornchinsombut and Park (*27*), who reported lower total SH content of Pacific whiting mince at pH 4.0 and 10.0 as compared to pH 7.0. The conformational changes of sarcoplasmic proteins subjected to either acidic or alkaline extraction resulted in the exposure of SH groups and led to disulfide bond formation.

Isoelectric precipitation and neutralization affected total SH content of sarcoplasmic proteins in different manners. Under acid extraction, total SH content after isoelectric precipitation (3-IP) was greater than that of the 3-EP ($P < 0.05$), indicating the exposure of SH groups upon isoelectric precipitation. Neutralization of isoelectric precipitated proteins (3-IP) to pH 7.0 appeared to induce protein−protein interactions through disulfide linkages. In the alkaline-extracted samples, total SH content increased after isoelectric precipitation (11-IP) and neutralization (11-NP) ($P < 0.05$). Our results indicated that reduction of disulfide bonds to sulfhydryl groups occurred in alkali-extracted sarcoplasmic proteins during isoelectric precipitation and neutralization. The extent of disulfide formation was presumably greater in the acidic pH-shift process than in the alkaline counterpart.

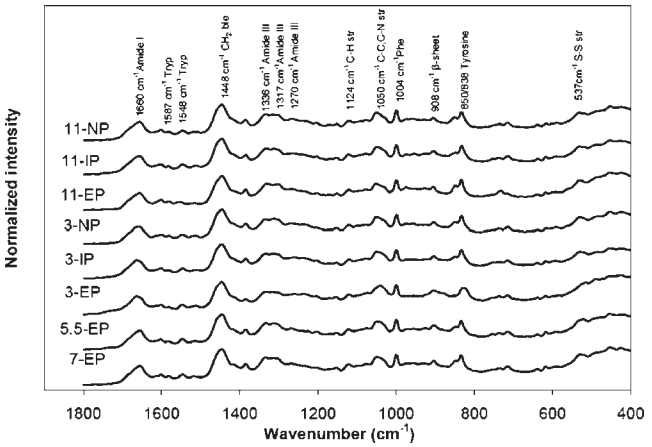

Figure 3. Raman spectra (400−1800 cm^{-1}) of sarcoplasmic proteins treated at various pH values. Abbreviations of pH treatments are the same as in **Figure 1**.

Raman Spectroscopy. Raman spectra and tentative assignment of Raman bands of sarcoplasmic proteins treated at various pH values are shown in **Figure 3** and **Table 2**, respectively. Amide I consists of amide carbonyl C═O stretching, with smaller contributions of C—N stretching and N—H bending. The amide III bands involve significant C—N stretching, N—H bending, and C—C stretching (*16*, *26*, *28*). Generally, an amide I band consists of overlapped bands at 1650−1660, 1667−1673, and 1662−1668 cm^{-1} corresponding to α-helix, β-sheet, and random coil structure, respectively. The α-helical, β-sheet, and random coil contents are also indicated by an amide III band near 1260−1350, 1230−1240, and 1245 cm^{-1}, respectively (*15*, *16*, *19*, *29*−*31*). The amide III′ band at 935 cm^{-1} is also attributed to the α-helical structure (*15*).

A predominant α-helical structure was found in sarcoplasmic proteins at pH 7.0 (**Table 2**; **Figure 4**). When the protein was adjusted to either pH 5.5 (5.5-EP) or pH 11 (11-EP), the amide I band was not affected (**Figure 4**), suggesting that the overall α-helical structure of sarcoplasmic proteins did not undergo significant changes at pH 11. Initially, sarcoplasmic proteins did not show major β-sheet structure at pH 7.0 (**Table 2**; **Figure 4**). As the pH was adjusted to pH 3 (3-EP), the amide I band shifted to 1668 cm^{-1} (**Figure 4**), indicating an increase in β-sheet structure. This was in agreement with Clark et al. (*32*), who reported that β-sheet content typically increased concomitant with a decrease in α-helix content. The amide I at 1660 cm^{-1} indicated that the isoelectric precipitation had less effect on the α-helical structure of sarcoplasmic proteins than did the extraction process (**Table 2**; **Figure 4**). The amide I and III bands of sarcoplasmic proteins treated at pH 11 (11-EP) were similar to those of 7-EP. These results indicate that alkaline extraction (pH 11) did not disrupt the α-helical structure of sarcoplasmic proteins.

Isoelectric precipitation of the acid-extracted sarcoplasmic proteins (3-IP) induced significant changes in the shift of amide I (**Figure 4**) and Raman intensities of amide III at 1239 cm^{-1} (**Table 2**), indicating a drastic change in secondary structures

Table 2. Normalized Intensity at Selected Regions of Raman Spectra of Sarcoplasmic Proteins Treated at Various pH Values

		normalized intensity[a]							
peak assignment[b]	wavenumber (cm^{-1})	3-EP	3-IP	3-NP	5.5-EP	7-EP	11-EP	11-IP	11-NP
S−S stretching	537	1.081 a (518)	0.953 c	0.931 d	1.039 a (525)	0.980 b	0.987 b	0.940 cd	0.956 c
ratio of tyrosyl doublet (R*tyr*)	838/850	nd	0.817 ab	0.835 ab	0.832 ab	0.850 a	0.789 b	0.810 ab	0.855 a
amide III′	908	0.847	0.831	0.806	0.854	0.831	0.806	0.837	0.804
	935	0.688 (942)	0.735 (923)	0.732 (927)	0.76 (927)	0.763	0.776	0.771	0.753
amide III (β-sheet) (1230−1240 cm^{-1})	1239	0.733	0.783	0.734	0.68 (1232)	nd	nd	0.741	nd
amide III (α-helix) (1260−1350 cm^{-1})	1270	0.738 (1263)	0.789 (1263)	0.763 (1274)	0.755 (1274)	0.752	0.744	0.781 (1266)	0.755
	1317	0.811	0.906	0.96	0.906	0.919	0.888 (1305)	0.907	0.885
	1336	0.812	0.902	0.886	0.908	0.929	0.903	0.911	0.894
CH_2 bending	1448	1.083 b	1.173 a	1.163 a	1.166 a	1.163 a	1.116 b	1.183 a	1.166 a
Trp	1548	0.440 b	0.477 a	0.448 ab	0.449 ab	0.430 b	0.377 c	0.451 ab	0.425 b
Trp	1587	0.369 c	0.426 a	0.413 ab	0.392 abc	0.396 abc	0.392 abc	0.409 ab	0.388 bc
amide I (1650−1668 cm^{-1})	1660	0.679 (1668)	0.715 (1668)	0.675 (1664)	0.698	0.689	0.634	0.696	0.684
CH stretching (shoulder)	2933	1.776 bc	1.696 c	1.776 bc	1.996 a	2.006 a	1.803 bc	1.846 bc	1.886 ab
═CH stretching	3056	0.367 bc	0.347 c	0.367 bc	0.403 a	0.387 a	0.360 bc	0.379 ab	0.381 ab

[a] Means with different letters in the same row are significantly different ($P < 0.05$). Number in parentheses refers to wavenumber if altered. nd, not detected. [b] Abbreviations of pH treatments are the same as **Figure 1**.

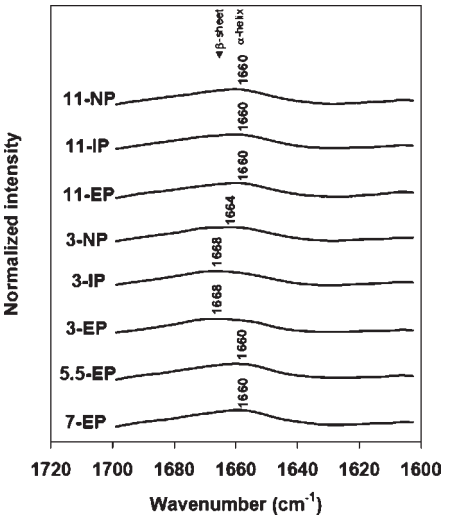

Figure 4. Raman spectra (1600−1700 cm^{-1}) of sarcoplasmic proteins treated at various pH values. Abbreviations of pH treatments are the same as in **Figure 1**.

upon isolectric precipitation. Isoelectric precipitation also induced an overall increase in β-sheet structure in the alkali-extracted sarcoplasmic proteins (11-IP, **Table 2**).

When 3-IP was readjusted to pH 7.0 (3-NP), the amide I band was shifted to 1664 cm^{-1}, suggesting that the β-content decreased concomitantly with an increase in random coil after neutralization. It should be noted that neutralization of alkali-extracted sarcoplasmic protein (11-NP) drastically reduced the intensity of the amide III band at 1230−1240 cm^{-1} assigned to β-sheet structure (**Table 2**), whereas α-helical structure was not affected (**Figure 4**). On the basis of the Raman spectra at amide I and III, structural changes of 11-NP were similar to those of 7-EP. Therefore, an alkaline pH-shift process appeared to minimally affect the overall secondary structure of sarcoplasmic proteins, whereas an acidic pH-shift treatment reduced overall α-helical structure, resulting in an increase of β-sheet and random coil structure.

The intensity of Raman bands near 935 and 908 cm^{-1}, resulting from the C−C stretching vibration, will also indicate changes of α-helices and β-sheets, respectively (*21*). The shifting of the 935 cm^{-1} band of 3-IP and 3-NP resulted from the loss of α-helical structure (**Table 2**). An increase in intensity of the 908 cm^{-1} band in 3-EP (**Table 2**) confirmed that an acidic pH-shift process induced changes of the α-helical structure to β-sheet.

It was presumed that acid treatment mainly interrupted a rearrangement of hydrogen bonds, resulting in β-sheet formation. Aggregation of protein molecules has been shown to involve the formation of intermolecular antiparallel β-sheet structure (*32*, *33*). A decrease in the α-helical structure of 3-IP and 3-NP occurred to a greater extent than that of 11-IP and 11-NP, respectively. This result suggested that an acidic extraction induced greater structural changes and denaturation than an alkaline process. Aggregation of the antiparallel β-sheet structure was likely to be a reason why acid-extracted proteins exhibited lower protein solubility (**Table 1**).

Raman stretching at 537 cm^{-1}, corresponding to S−S stretching vibration of the disulfide bonds, was observed in 7-EP (**Table 2**). The vibration at 537 cm^{-1} assigned to be the trans−gauche−trans (t-g-t) conformation in the disulfide bridge disappeared in 3-EP with the new Raman band at 518 cm^{-1}, indicating the gauche−gauche−trans (g-g-t) conformation. Conformational changes of disulfide bridges were induced by acidic extraction. The lower shifting of frequency from 537 to 525 cm^{-1} was also identified in 5.5-EP. The t-g-t conformation was recovered in 3-IP and 3-NP, suggesting that disulfide conformation was restored after isoelectric precipitation and neutralization. The alkaline extraction did not affect the microenvironment of disulfide bridges, but isoelectric precipitation and neutralization of alkali-extracted sarcoplasmic proteins appeared to modify conformation of disulfide bridges (**Table 2**). Irreversible denaturation ruptured S−S bonds with the decrease in the S−S stretching intensity at 504 cm^{-1} (*16*). Thus, a pH-shift treatment independent of pH applied for protein extraction appeared to have an effect on disulfide bonds of sarcoplasmic proteins. Total SH content also implied that oxidation of sulfhydryl groups to disulfide bonds was promoted to a greater extent in an acidic pH-shift process (3-NP, **Table 1**).

The ratio of the tyrosyl doublet (R_{Tyr}) at 850 and 838 cm^{-1}, which is an indicator of hydrogen bonding by tyrosine phenoxyls (*34*), has been used to determine whether the tyrosine residues in proteins are exposed or buried. An R_{Tyr} between 0.9 and 1.45 indicates the exposure of tyrosine residues that could interact with water molecules as a hydrogen bond donor and acceptor (*19*, *20*). An R_{Tyr} between 0.7 and 1 reflects buried tyrosine groups, and an R_{Tyr} lower than 0.3 indicates strong hydrogen bond acceptors interacted with a negative acceptor (*19*, *20*). On the basis of these values, tyrosine residues of 7-EP were buried (**Table 2**). The Raman band at 850 cm^{-1} was not found in the 3-EP sample. Extraction at extremely acidic pH resulted in the vibration of the para-substituted benzene ring of the tyrosine residues, leading to hydrogen bond formation of tyrosine phenolic groups (*15*). The tyrosine residues of 11-EP samples were more buried in a hydrophobic environment than 7-EP as evidenced by a decrease of R_{Tyr} of the former ($P < 0.05$). A decrease of R_{Tyr} of α-lactalbumin indicated an increase in the role of phenolic hydroxyl groups as strong hydrogen bond donors (*35*). Extreme pH values also led to a decrease in the tyrosyl doublet band intensity of oat globulin, thus indicating that the tyrosine residues were buried to a much greater degree (*29*). The R_{Tyr} of sarcoplasmic proteins extracted at acidic or alkaline pH increased after the pH had been shifted to 5.5 (3- and 11-IP) and neutralized to 7.0 (3- and 11-NP), indicating that isoelectric precipitation and neutralization induced changes in the microenvironment of tyrosine residues. The high value of R_{Tyr} (> 1) was also reported in alkali-treated rockfish protein isolate stored at pH 5.5 and 7.0 (*5*). It seems that the microenvironment of tyrosine residues of sarcoplasmic proteins subjected to either acidic or alkaline pH-shift treatment was in the same state as 7-EP. Thus, it could be postulated that the pH-shift process does not have a significant effect on the tyrosine residue burying regardless of the extraction pH applied.

The vibration at 1448 cm^{-1} was assigned to the CH and CH_2 bending vibrations, indicating changes of the environment around aliphatic side chains. Adjusting the pH to 5.5 (5.5-EP) did not affect the environments of aliphatic side chains as compared to pH 7 ($P > 0.05$, **Table 2**). A decrease in the intensity of the band at 1448 cm^{-1} was observed at acidic (3-EP) and alkaline (11-EP) extraction ($P < 0.05$), indicating the exposure of aliphatic residues upon the extreme pH extraction. When the pH was adjusted to 5.5 (3- and 11-IP) and subsequently to 7 (3- and 11-NP), the aliphatic residues became buried, resulting in an environment similar to that of 7-EP. Therefore, refolding of proteins could be induced by isoelectric precipitation and neutralization. An increase in the intensity of the Raman vibrational band near 1450 cm^{-1} indicated hydrophobic interactions of proteins, which was observed during frozen storage of hake fillets (*21*). The change in CH and CH_2 bending vibrations was

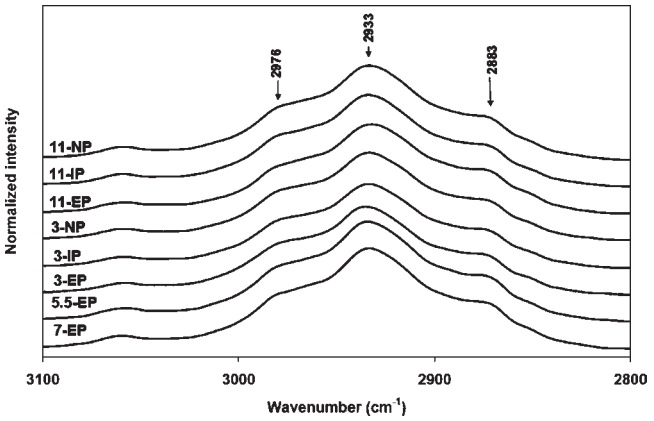

Figure 5. Raman spectra (2933 and 3060 cm^{-1}) of sarcoplasmic proteins treated at various pH values. Abbreviations of pH treatments are the same as in **Figure 1**.

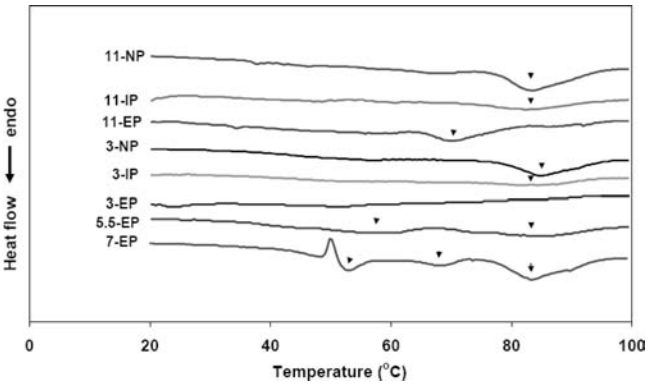

Figure 6. Micro-DSC thermograms of sarcoplasmic proteins treated at various pH values. The arrows indicate the endothermic peak. Abbreviations of pH treatments are the same as in **Figure 1**.

also observed for cod myosin after frozen storage (*36*) and for whey proteins with interaction of lysozyme (*37*).

Raman bands at 1548 and 1587 cm^{-1} derived from indole ring vibrations indicate the degree of burying of tryptophan residues (*19*). Alkali extraction resulted in the exposure of tryptophan residues as the intensity of these bands of 11-EP was lower than those of 7-EP ($P < 0.05$, **Table 2**). In contrast, acid extraction did not affect the degree to which the tryptophan residues were buried. An increase in the intensity of tryptophan residue vibrations was observed after isoelectric precipitation (3- and 11-IP) and neutralization (3- and 11-NP), reflecting the degree to which the tryptophan was buried after proteins were isoelectrically precipitated and subsequently neutralized. It should be stated that the degree of burying of tryptophan and aliphatic residues after pH-shift treatments was similar to that of 7-EP ($P > 0.05$). Sarcoplasmic proteins subjected to either acidic or alkaline pH-shift treatment could undergo refolding to achieve a degree of burying of hydrophobic residues similar to that of the native (7-EP).

Hydrophobic amino acids of sarcoplasmic proteins subjected to various pH treatments exhibited −C—H stretching vibrational bands in the 2700−3200 cm^{-1} region. Bands near 2874−2897 cm^{-1} have been assigned to CH_3 symmetric stretching and R_3C-H stretching bands, whereas =C—H stretching bands of aromatic amino acids can be found around 3061−3068 cm^{-1} (*14*, *33*). Aromatic and aliphatic amino acids as well as charged amino acids (proline, threonine, and histidine) show −C—H stretching bands near 2935−2955 cm^{-1} (*38*). It has been suggested that the exposure of hydrophobic residues of protein would lead to an increase in intensity of these bands (*21*). The 7-EP sample exhibited a major C—H stretching band at 2933 cm^{-1} with a shoulder around 2883 and 2976 cm^{-1} (**Figure 5**). The intensities of the −C—H stretching band at 2933 and 3056 cm^{-1} between 7-EP and 5.5-EP were comparable (**Table 2**). Thus, adjusting the pH from 7 to 5.5 did not affect the environment around aliphatic and aromatic amino acids. A decrease in the intensity of the Raman band was observed in 3-EP and 11-EP, indicating that extreme pH induced hydrophobic interactions among sarcoplasmic proteins. Isoelectric precipitation and neutralization did not change the microenvironment of hydrophobic residues of the acid-extracted sarcoplasmic protein (**Table 2**). However, more recovered hydrophobic groups were observed in 11-NP than in 3-NP, implying that 3-NP aggregated through hydrophobic interactions to a greater extent than 11-NP. In addition, isoelectric precipitation and neutralization resulted in spectral changes of =C—H stretching bands of aromatic amino acids with a decrease in intensity when compared to the native sample of 7-EP. These results confirmed that hydrophobic interactions of sarcoplasmic proteins occurred upon pH-shift treatment with a higher degree of such interactions under acidic extraction than the alkaline counterpart. Aggregation of sarcoplasmic proteins upon pH-shift treatment might explain why the solubility of 3-NP and 11-NP was much lower than that of 7-EP (**Table 1**).

On the basis of Raman spectroscopic data, fish sarcoplasmic proteins were unfolded after extremely acidic and alkaline extraction, exposing tryptophan and aliphatic residues. Acidic extraction had a greater effect on disrupting the α-helical structure, whereas alkaline extraction did not affect the secondary structure of sarcoplasmic proteins. Hydrophobic interactions of aromatic side chains were initiated during extreme pH (3 and 11) extraction. Disulfide formation, hydrogen bonding via tyrosine residues, and hydrophobic interactions also took place under both acid and alkali pH extractions. Sarcoplasmic proteins subjected to acidic extraction underwent denaturation and aggregation via hydrophobic interactions to a greater extent than those extracted at alkaline condition. Interactions of aliphatic and aromatic hydrophobic groups, such as tryptophan and tyrosine residues, were formed after isoelectric precipitation. Acid-extracted sarcoplasmic proteins underwent aggregation upon isoelectric precipitation to a greater extent than did alkali-extracted proteins. Some sarcoplasmic proteins were partially refolded after subsequent isoelectric precipitation and neutralization. More exposure of tyrosine residues and hydrophobic groups to the environment was detected when proteins were neutralized from alkaline pH. However, alkaline extraction did not alter the peptide backbone conformation.

Micro-DSC. The thermal denaturation pattern of sarcoplasmic proteins varied with pH treatment (**Figure 6**). The 7-EP sample exhibited both major exothermic peaks at 50.66 °C, indicating the aggregation of sarcoplasmic proteins, and two endothermic peaks at 69.38 and 88.70 °C. An exothermic peak around 37−38 °C and an endothermic peak at 74.2 °C were also observed for rockfish sarcoplasmic proteins (*17*). Sarcoplasmic proteins of striped catfish and rockfish exhibited a similar pattern of exothermic and endothermic peaks with different onset temperatures. Higher onset temperatures were observed in sarcoplasmic proteins of striped catfish, indicating a higher thermal stability of these proteins. Proteins extracted from striped catfish aquacultured in the tropical area are expected to have higher thermal stability than those obtained from rockfish that inhabit a colder environment. A similar finding was also reported in Pacific whiting, which showed a lower thermal transition temperature than threadfin beam (*39*). Transition temperatures of 3-EP were not detected, suggesting the complete unfolding of proteins during acidic

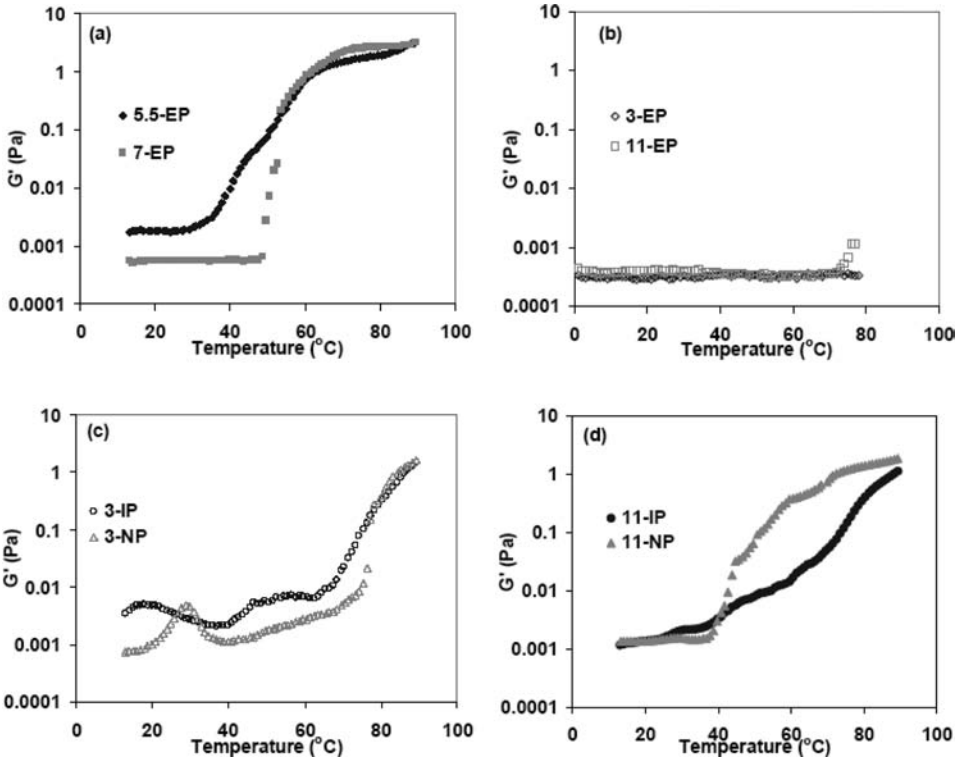

Figure 7. Changes in storage modulus (G') of sarcoplasmic proteins treated at various pH values. Abbreviations of pH treatments are the same as in **Figure 1**.

extraction, which corresponded well to a loss of α-helical structure and a marked decrease in the intensity of several Raman bands (**Table 2**). Despite the acid-induced denaturation of 3-EP, its solubility (47%) was comparable to that of 7-EP, presumably, the native state of saroplasmic proteins (**Table 1**). We speculate that some sarcoplasmic proteins would remain soluble even in the denatured state.

The exothermic peak was not observed in the 5.5-EP sample, indicating that the protein component(s) responsible for aggregation at pH 7 did not aggregate at pH 5.5. As the polypeptide backbone and environments of amino acid side chains of 5.5-EP were similar to those of 7-EP (**Table 2**), aggregation of such component(s) could be hampered by different charge characteristics between these two pH values. The maximum temperature (T_m) of the endothermic peak of 5.5-EP was shifted to lower temperatures of 53.51 and 83.16 °C as compared to the respective T_m of 69.77 and 84.48 °C of 7-EP. Structures of sarcoplasmic proteins at pH 5.5 could be partially unfolded, resulting in a lower denaturation temperature. It should be noted that any structural changes induced by pH 5.5 were not obvious from the Raman spectra. The 11-EP exhibited only an endothermic peak at 70.97 °C. The aggregation of some sarcoplasmic proteins did not take place at extremely alkaline pH due to the electrostatic repulsive force. These results indicated that some sarcoplasmic proteins were more stable under alkaline extraction than the acid counterpart, which was in agreement with the conformational changes deduced from the Raman spectra.

The T_m of sarcoplasmic proteins was recovered at >80 °C when the pH was adjusted for isoelectric precipitation and neutralization. This evidence suggests the refolding of sarcoplasmic proteins upon neutralization. Endothermic peaks of alkali-treated myosin were detected at ~37 and 45 °C (*40*). Pacific whiting surimi containing myoglobin at various concentrations exhibited four endothermic transition peaks at 30, 37, 45, and 60 °C (*41*). Adding sarcoplasmic proteins also increased the denaturation temperature of myosin and actin of pollock surimi (*17*). Our study indicated that the thermal stability of sarcoplasmic proteins increased after being subjected to isoelectric precipitation and neutralization and was also much higher than that of myosin. T_m values of 3-IP, 3-NP, 11-IP, and 11-NP were 86.32, 84.87, 82.94, and 83.72 °C with endothermic enthalpies of 0.058, 0.405, 0.073, and 0.163 J/g of sample, respectively. On the basis of enthalpy results, sarcoplasmic proteins refolded after isoelectric precipitation followed by neutralization. The thermal stability of 3-NP was likely to be higher than that of 11-NP as the endothermic enthalpy of the former was greater. Disulfide bonds and hydrophobic interactions obtained from Raman spectra and total SH content results could be mainly responsible for the higher thermal stability of 3-NP.

Oscillatory Dynamic Properties. Changes in the storage modulus (G') and phase angle of sarcoplasmic proteins varied with pH treatments (**Figure 7**). The 7-EP sample exhibited higher G' (**Figure 7**) and lower phase angle value (data not shown) at 90 °C than others. The onset temperature of G' increasing of 7-EP was at 48.75 °C (**Figure 7a**; **Table 3**), which was lower than the aggregation temperature obtained from DSC (50.66 °C, **Figure 6**). These results indicated that gel network formation of 7-EP started developing even before complete denaturation. Aggregation of 7-EP continuously occurred and reached the gel point defined as a temperature in which G' is equal to G'' at 52.15 °C (**Table 3**).

An increase in G' values was also observed at pH 5.5 (**Figure 7a**). The protein−protein interaction would be predominant in sarcoplasmic proteins with almost zero net charge, thereby allowing aggregation of 5.5-EP at a lower temperature (~30 °C) relative to that of 7-EP. The results are highly correlated to the lower T_m of 5.5-EP observed from DSC (**Figure 6**). On the other hand, sarcoplasmic proteins treated at pH 3.0 (3-EP) and 11.0 (11-EP) did not show gel network development upon heating (**Figure 7b**). Sarcoplasmic proteins treated at pH 3.0 (3-EP) underwent denaturation with significant changes in peptide backbone, hampering

Table 3. Gel Point of Sarcoplasmic Proteins Treated at Various pH Values[a]

pH treatment[b]	onset of G' rising (°C)	gel point ($G' = G''$, °C)	G' value at 90 °C (Pa)
3-EP	nd	nd	nd
3-IP	38.15 ± 0.77 b	77.28 ± 3.78 a	1.514 ± 0.067 bc
3-NP	38.60 ± 1.27 b	78.35 ± 1.34 a	1.638 ± 0.332 bc
5.5-EP	31.85 ± 0.07 d	44.55 ± 2.75 c	3.046 ± 0.030 a
7-EP	48.75 ± 0.07 a	52.15 ± 0.63 b	3.182 ± 0.126 a
11-EP	nd	nd	nd
11-IP	34.25 ± 0.63 c	53.55 ± 2.75 b	1.395 ± 0.229 c
11-NP	37.70 ± 0.00 b	45.10 ± 0.84 c	1.937 ± 0.064 b

[a] Means with different letters in the same column are significantly different ($P < 0.05$). nd, not detected. [b] Abbreviations of pH treatments are the same as in **Figure 1**.

gel network formation. Although structural changes were not detected in 11-EP, alkaline extraction would result in negatively charged proteins that limited the gel network formation.

Isoelectric precipitation and neutralization modified the viscoelastic properties of sarcoplasmic proteins (**Figure 7c**,**d**). Sarcoplasmic proteins precipitated from extremely alkaline pH (11-IP) exhibited a G' pattern similar to that of 5.5-EP, whereas a different G' pattern was observed for 3-IP. G' values of 3-IP continually increased after 63.35 °C (**Figure 7c**). On the other hand, the gel network of 11-IP started developing at 34.25 °C and gradually increased thereafter (**Figure 7d**).

The onset of G' rising and the gel point of 3-IP were higher than those of 11-IP and 5.5-EP ($P < 0.05$, **Table 3**). Sarcoplasmic proteins aggregated to a greater extent after being subjected to extreme pH extraction (3.0 and 11.0) followed by isoelectric precipitation because isoelectric adjustment induced hydrophobic interactions (**Table 2**). The 3-IP sample underwent extensive aggregation induced by pH treatment; therefore, it required higher energy to unfold before aggregation could take place. The DSC thermogram also revealed that 3-IP exhibited relatively higher T_m (**Figure 6**).

A difference of G' pattern between 3-NP and 11-NP was observed. G' values of 3-NP started to increase at 23.85 °C, with a peak at 29.85 °C, and then gradually decreased to reach a minimum at 38.70 °C and continuously increased afterward (**Figure 7c**). In contrast, G' of 11-NP sharply increased at 37.70 °C (**Figure 7d**). Although the G' pattern of 11-NP was similar to that of 7-EP (**Figure 7a**,**d**), the gel point of the former was lower (**Table 3**). Sarcoplasmic proteins treated by alkaline pH-shift treatment underwent refolding, resulting in a structure similar to that of the native (7-EP) deduced from the Raman spectra (**Table 2**). However, hydrophobic residues of 11-NP were more recovered than those of 3-NP (**Table 2**), and hydrophobic interactions of 11-NP occurred to a higher degree than for the native sample (7-EP) as indicated by the intensity of ═C—H stretching bands (**Table 2**). Therefore, 11-NP readily aggregated to form a network upon heating at a lower temperature than did 7-EP.

Sarcoplasmic proteins did not completely refold to their native state as a G' value at 90 °C for 3- and 11-NP was lower than that for 7-EP ($P < 0.05$, **Table 3**). On the basis of the structural changes evident in the Raman spectra, DSC thermograms, and G' patterns, it was presumed that sarcoplasmic proteins formed aggregates via hydrophobic interactions upon a pH-shifting process. The alkaline extraction process appeared to induce structural changes that enabled more elastic gel network development of sarcoplasmic proteins. Our results might explain, in part, why the alkaline pH-shift treatment resulted in higher textural properties compared to the acidic pH-shift process (*3*, *4*, *6*).

ACKNOWLEDGMENT

We thank Dr. Eunice C. Y. Li-Chan of the University of British Columbia, Vancouver, British Columbia, Canada, for providing critical comments on FT-Raman spectra.

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Received for review September 11, 2009. Revised manuscript received March 3, 2010. Accepted March 4, 2010. We are grateful for the financial support from the Commission of Higher Education, Ministry of Education, Thailand. S.G.M. acknowledges the support of a Major Research Instrumentation grant from the National Science Foundation (Award 0618973) and a grant from the American Chemical Society Petroleum Research Fund (Award 44487-B4).