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Raman Spectroscopy Determines Structural Changes Associated with Gelation Properties of Fish Proteins Recovered at Alkaline pH

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Structural changes of alkali-treated rockfish protein isolate (AKPI) during frozen storage were elucidated using a Raman spectrometer and scanning electron microscope (SEM). The results were compared to conventional surimi (CS). No significant textural difference was noted between AKPI stored at pH 5.5 and 7.0. The strongest texture was found for AKPI frozen with cryoprotectants and CS, while the weakest texture was observed in AKPI frozen without cryoprotectants. SEM revealed the most discontinuity in gels of AKPI with no cryoprotectants and a more aggregated microstructure after storage at pH 5.5 than at neutral pH. Raman spectral analysis demonstrated refolding of AKPI by pH readjustment to 7.0, although the refolded structure was not identical to that before the pH shift. CS showed higher α-helix content (~50%) than AKPI (~20−30%). Frozen storage induced a decrease and an increase in the α-helix content of CS and AKPI samples, respectively. AKPIs were slightly less stable than CS during frozen storage.

KEYWORDS: Alkali-treated protein isolate; frozen storage; texture properties; Raman spectroscopy; SEM

INTRODUCTION

Alternative methodologies to conventional surimi (CS) processing to produce a functional protein concentrate from small pelagic fish species and other surimi resources in the U.S. have been developed. In general, this novel procedure features a method for isolating edible protein from animal muscle by decreasing or increasing the pH of the protein slurry to a level sufficient to solubilize more than 75% of the protein. Membrane lipids and other insoluble parts are removed by centrifugation. Protein is precipitated from the aqueous phase by adjusting to the isoelectric pH (*1*−*5*). Although the pH of this protein isolate is readjusted to neutrality, the proteins that have undergone pH-induced treatments may have alternative structures and stability while kept under frozen conditions.

During frozen storage, structural changes of surimi can occur, leading to protein denaturation and resulting in the loss of gel-forming ability. The stability of CS while storing under frozen conditions can be improved by inclusion of cryoprotectants (*6*). Park and others (*7*) reported that sucrose and sorbitol retard freeze denaturation of Alaska pollock surimi. However, it is yet unknown whether the cryoprotectant would be required or not for fish protein isolate and whether the protein structures are unfolded/refolded by altering pH. In addition, on the basis of the conventional protein chemistry, it has been believed that most proteins are more stable at their isoelectric point (pI) than at other pH values (*8*). Our previous study (*9*) to investigate changes of protein functionality, conformation, and stability as a function of various frozen storage conditions indicated that the protein isolate, obtained by the pH-shift method, substantially lost its gelation properties and critically required cryoprotectants to retard freeze denaturation and aggregation. Differential scanning calorimetry (DSC) thermograms showed a slight difference when proteins were stored at neutral pH and at the pI. However, there was no significant difference detected when analyzed by other methods, including total sulfhydryl content, surface hydrophobicity, and gel qualities by the punch test (*9*). More sensitive protein analysis methods are required to monitor the changes of the alkali-treated protein isolate (AKPI).

The structural changes of protein can be examined at the molecular level by several methods such as circular dichroism, fluorescence spectroscopy, nuclear magnetic resonance (NMR), infrared absorption, and Raman spectroscopy. Raman spectroscopy can be used advantageously to determine the molecular structure of food proteins in liquid or solid form without the need to extract a purified component. This technique yields information on the secondary-structure fractions, disulfide bond conformation, and/or the aromatic side-chain environment (*10*).

Because of the opaque or solid nature of many foods, changes in protein functionalities that cannot be differentiated by other methods are amenable to Raman spectroscopy. Proteins have

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been studied extensively using this technique. The reversible denaturation of bovine serum albumin solutions by heat, acid, and alkali were studied using the Raman spectroscopic technique (*11*). In 1997, Raman spectroscopy was used to study the in situ protein structure in raw, salted surimi, gel setting, and cooking (*12*). Careche and others (*13*) assessed the structural properties of aggregates formed during frozen storage of hake muscle using Raman spectroscopy and found that the protein backbone adopted a conformational structure rich in β-sheet content. Protein structural changes of hake muscle kept frozen at different temperatures (−10 and −30 °C) were monitored using a Raman spectrometer. An increase of β-sheet structure at the expense of the α-helix structure was revealed. Raman data also indicated protein denaturation through the exposure of aliphatic hydrophobic groups to the solvent (*14*).

The primary objective of this study was to investigate the stability of AKPI at various frozen storage conditions with or without cryoprotectants using the Raman technique. In addition, the gel microstructure, textural properties, and molecular structure of the AKPI were compared to CS.

MATERIALS AND METHODS

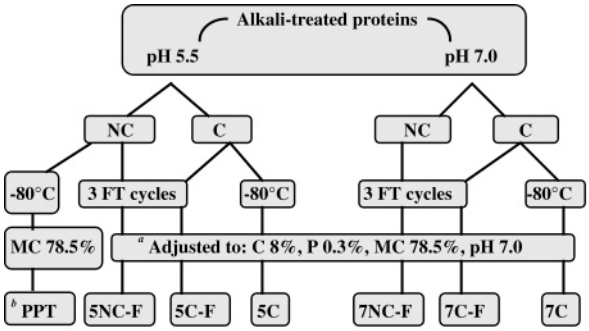
Materials. Fresh whole rockfish (*Sebastes flavidus*), 1−2 days old, 25−30 cm in length, were transferred on ice from Pacific Surimi (Warrenton, OR) to the OSU Seafood Lab within 30 min. Fish were then filleted and ground into mince. Mince was used as the raw material for AKPI.

Sample Preparations. For the conventional method, mince was washed 3 times at a 1:3 ratio (mince/water). Final washing was conducted using a 0.3% NaCl solution to facilitate the dewatering step. The homogenate was centrifuged at 6000*g* for 20 min at 4 °C for each washing step. Washed mince was then mixed with cryoprotectants (4% sucrose, 4% sorbitol, and 0.3% sodium tripolyphosphate). Surimi was adjusted to pH 7.0 and 78.5% moisture and then vacuum-packed and stored at −80 °C.

Alkali-treated rockfish proteins (pH 11) were prepared as detailed in Kim (*15*). Mince was homogenized with cold deionized (DI) water at a 1:9 ratio using a Power Gen 700 homogenizer (GLH 115, Fisher Scientific, Inc., Pittsburgh, PA) for 2 min at a speed level of 2. The pH of homogenates was adjusted using 1 and/or 2 N cold HCl and NaOH to pH 11.0. The pH measurements were conducted using a pH-meter (HI 9025 microcomputer pH-meter, Hanna Instruments, Inc., Woonsocket, RI) with a Spear Gel Combo pH probe (Corning Incorporated Life Sciences, Acton, MA). After pH adjustment, samples were centrifuged (Sorvall RC-5B, DuPont Co., Newtown, CT) at 8000*g* for 25 min at 4 °C to separate the insoluble parts. Soluble proteins were subsequently recovered at pH 5.5. Protein precipitates were then collected by centrifugation (4000*g* for 20 min at 4 °C).

The pH and cryoprotectant content of the experimental treatments are diagrammed in **Figure 1**. Two pH (5.5 or 7.0) treatments were applied to the recovered pellet, which were further subdivided into two sets of cryoprotectants: 0 and 8% (sucrose/sorbitol = 1:1), respectively. Samples with no freeze/thaw treatment were stored at −80 °C for 2 weeks before analysis. After 11 days, one set of each treatment was subjected to 3 freeze−thaw cycles at −18 °C (18 h) freezing and 4 °C (6 h) thawing, respectively, which were completed in 3 days. Before evaluation, the ratio of water/protein of the samples with and without cryoprotectants was controlled (**Table 1**). Prior to protein analysis or gel preparation, all samples were thawed at 5 °C for 30 min before adjusting to maintain equal pH (7.0), cryoprotectants, and moisture content (78.5%). The precipitate, pellets recovered at the pI after alkaline solubilization, was adjusted for equal moisture content (pH = 5.5) and subjected only to Raman analysis. Gels were prepared without salt except CS, which was chopped with 2% NaCl, and the process was conducted in a walk-in cold room (5−6 °C).

Texture Analysis. Paste (21.5 g) was stuffed into a plastic mold (30 mm i.d. and 25 mm in height), vacuum-packed in a plastic bag, and then cooked at 90 °C for 15 min. Cooked gels were cooled in iced water for 15 min and refrigerated overnight. The chilled gels were set at room temperature for 2 h before fracture analysis. Gels were subjected to the punch test using a texture analyzer (TA.XT.plus, Texture Technologies Corp., New York, NY). A spherical probe (5 mm in diameter) was used as a measuring tool, and the penetration speed was set at 1 mm/s. Breaking force (*g*) and deformation (in millimeters) required to fracture the surface of the gel were recorded.



[a] All treatments were adjusted to an equal cryoprotectant content and pH 7.0 before testing (C : cryoprotectants (sucrose + sorbitol = 1 + 1); P : 0.3 % sodium tripolyphosphate; MC : moisture content)

[b] Abbreviations of treatments:

PPT = protein precipitate (pH 5.5) was not added cryoprotectants;
5 = frozen storage at pH 5.5; 7 = frozen storage at pH 7.0;
NC = without cryoprotectants; C = with 8% cryoprotectants
F = with 3 freeze/thaw cycles (freezing at −18 ± 2 °C, 18 h; thawing at 4 ± 2 °C, 6 h)

Figure 1. Experimental design.

Table 1. Compositions of Experimental Treatments before Adjusting to Equal pH, Moisture, and Cryoprotectants

treatment	crude proteins (%)	moisture content (%)	sucrose + sorbitol =1:1 (%)	ratio of water/ protein	ratio of protein/ cryoprotectant
NC[a]	16.0	83.7	0	5.24	0
C[b]	14.7	77.0	8	5.24	1.84

[a] NC, 0% cryoprotectants (sucrose + sorbitol = 1 + 1). [b] C, 8% cryoprotectants (sucrose + sorbitol = 1 + 1).

The folding test is another indicator measuring gel cohesiveness. It was conducted by slowly folding a 3 mm thick slice of gel (diameter = 30 mm) in half lengthwise and then in half again while examining it for structural failure. A folding score from 1 to 5 was given based on the rating system of Kudo and others (*16*): 1 = broke under finger pressure; 2 = broke immediately when folded in half; 3 = broke gradually when folded in half; 4 = did not break when folded in half but broke when folded in quarter; and 5 = folded in quarter without breaking.

Scanning Electron Microscope (SEM). Sample preparation for SEM was as described by Feng (*17*) with a slight modification. Small samples of heated gels were cut ($5 \times 5 \times 5$ mm^3) with a razor blade and fixed with 2.0% glutaraldehyde in 0.1 M sodium phosphate buffer at pH 7.2 and ambient temperature for 1 h. Samples were then washed twice with 0.1 M sodium phosphate buffer at pH 7.2. Fixed samples were dehydrated through a series of ethanol solutions of increasing concentration (25, 50, 75, 95, and 100%, v/v) for 30 min each. Dehydrated samples were stored in 100% ethanol at 4 °C until dried. The samples were dried by the hexamethyldisilazane (HMDS) method (*18*). Dried samples were mounted on aluminum specimen stubs and coated with gold/polonium (60:40 wt %) using a Sputter Coater (Edwards High Vacuum S 150 B, West Sussex, U.K.). The samples were examined at 7500× using a SEM (AmRay 3300 FE, Bedford, MA) at an accelerated voltage of 10 kV.

Raman Spectroscopy. Raw frozen samples were packed in an ice box containing iced gel packs and transferred within 8 h to the Food

Science building at the University of British Columbia (Vancouver, Canada). Upon arrival, the samples were stored at −35 °C until tested.

Samples were thawed at 4 °C for 10 min before examining by a visible laser Raman spectrometer (JASCO model NR-1100, Japan Spectroscopic Co. Ltd., Tokyo, Japan). Samples were placed in a capillary tube (Nichiden-Rika Glass Co. Ltd., Tokyo, Japan) and held horizontally in the spectrometer, thermostated at 4.0 ± 0.1 °C using a JASCO temperature controller model RT-1C (Japan Spectroscopic Co. Ltd., Tokyo, Japan). The incident laser beam was vertical, i.e., perpendicular to the capillary axis. Raman spectral data were collected on the Raman spectrometer with 488 nm excitation from an argon ion laser (Coherent Innova 70C series, Coherent Laser Group, Santa Clara, CA), cooled with the Coherent Laser Pure heat exchanger system. The conditions used were as follows: incident laser power of 100 mW, slit height of 4 mm, spectral resolution of 5.0 cm^{-1} at 19 000 cm^{-1}, sampling speed of 120 cm^{-1} min^{-1} with data collected every 1 cm^{-1}. Frequency calibration of the instrument was performed daily using the 1050 cm^{-1} (±1 cm^{-1}) band of a 1 M KNO_3 standard solution. Optical alignment was also monitored by checking the intensity of this standard solution. To increase the signal-to-noise ratio, at least six scans of each sample were collected to obtain averaged spectral data. Triplicate samples of each treatment were scanned.

Spectra were smoothed, baseline corrected, and normalized against the phenylalanine band at 1007 cm^{-1} by GRAMS/32 Spectral Notebase version 4.14 level II (Galactic Industries Corp., Salem, NH). Estimation of the secondary-structure composition of the proteins based on the Raman spectra in the amide I region was carried out by least-squares analysis using the Raman Spectral Analysis Package (RSAP version 2.1) of Przybycien and Bailey (*19*). Assignments of peaks in the Raman spectra to specific vibrational modes of amino acid side chains or the polypeptide backbone were made according to published literature (*12*, *13*, *20*−*24*).

Statistical Analysis. At least two replicates were performed for texture analysis of all treatments. Data were analyzed using an analysis of variance (ANOVA) procedure. A general linear model was applied with further analysis using Tukey's test to determine differences ($p < 0.05$) among treatment means (SPSS for Windows, version 10.0, SPSS, Inc., Chicago, IL). For peak intensity from Raman data, standard deviations were calculated and were reported as *y* error bars.

RESULTS AND DISCUSSION

Texture Analysis. Regardless of pH during frozen storage, the textural properties of AKPIs stored at −80 °C with cryoprotectants (5C and 7C) exhibited superior quality followed by samples with cryoprotectants and FT cycling (5C−F and 7C−F) and samples without cryoprotectants with FT cycling (5NC−F and 7NC−F), respectively (**Figure 2**).

Unlike AKPI from rockfish, our previous study using Pacific whiting (PW) as a raw material demonstrated a detrimental decrease of gel elasticity when AKPI was treated with freeze/thaw cycles regardless of an inclusion of cryoprotectants (*9*). This was probably due to the fact that PW is a gadoid species, which tends to undergo more denaturation during frozen storage. Formaldehyde produced from the enzyme trimethylamineoxide (TMAO) in this species triggers protein denaturation and subsequent aggregation, resulting in changes in texture and functionalities (*20*, *25*, *26*). There was no significant difference of gel quality between the AKPI kept frozen at pH 7.0 and 5.5. Nevertheless, the textural qualities of 5NC−F seemed to show a slightly higher breaking force than 7NC−F. We actually observed a slightly poorer quality in 5NC−F, which represented a more brittle and discontinuous texture than 7NC−F. This observation was also supported by SEM analysis (**Figure 3**). In this study, the penetration test might not be sensitive enough when proteins showed poor elasticity because of aggregation. The results suggested that cryoprotectants are needed to preserve

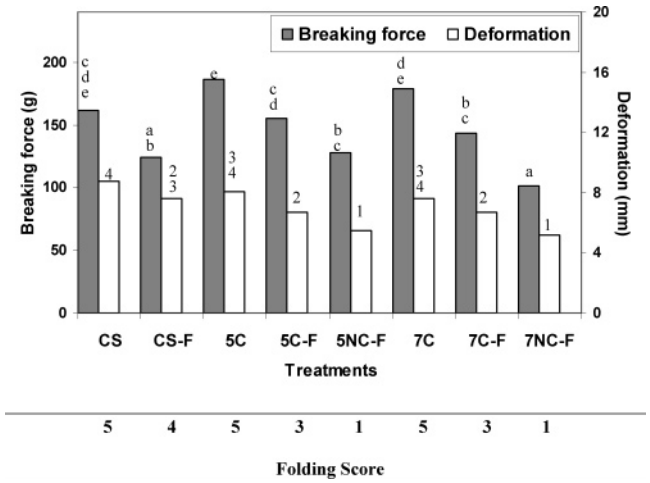


Figure 2. Texture properties of gels prepared from rockfish surimi and AKPIs at various storage conditions. Abbreviations for treatments are the same as in **Figure 1** and CS = conventional surimi. Different letters on each bar represent significant differences ($p < 0.05$).

AKPI during frozen storage. The gel strength of CS was slightly lower than AKPI; however, after being frozen/thawed, CS exhibited a slightly better gel quality regarding elasticity, as evidenced by the folding score.

SEM. Gel microstructures of CS and AKPI with 7C treatment showed a relatively smooth and continuous structure, while that with 5C treatment appeared discontinuous with several voids (**Figure 3**). This difference, however, could not be differentiated using the texture analyzer (**Figure 2**). When AKPI with added cryoprotectants were subjected to freeze−thaw cycles (the 5C−F treatment), they exhibited a more discontinuous and aggregated structure. When samples without cryoprotectants were treated with FT cycles, the gels were more aggregated, exhibiting less continuous structures with several big voids and interconnecting grooves. Samples stored at pH 5.5 also showed more discontinuity and were grainier than samples stored at neutrality. Thus, with regard to the effects of pH during frozen storage, the SEM images were in accordance with texture qualities. The greater the continuity of the gel microstructure, the better the gel quality obtained. These results strongly indicated that AKPI requires cryoprotectants to prevent protein aggregation when frozen and also suggested that protein quality could be better maintained when kept frozen at neutral pH.

Raman Spectroscopy: Secondary-Structure Estimation from the Amide I Band. Figure 4 shows the Raman spectra of all treatments in the 400−1800 cm^{-1} range. **Table 2** shows the assignment of major bands to amino acid side chains and peptide backbone vibrations. The most outstanding band, centered near 1655 cm^{-1} for CS, has been assigned to the amide I vibration mode, which mainly involves C═O stretching and, to a lesser degree, C−N stretching, C_α−C−N bending, and N−H in-plane bending of peptide groups. In general, the amide I band consists of overlapped band components falling in the 1658−1650, 1680−1665, and 1665−1660 cm^{-1}, which are attributable to α-helix, β-sheet, and random-coil structures, respectively (*27*, *28*).

For CS and CS−F, the amide I region was observed at 1657 and 1661 cm^{-1}, respectively (**Figure 4**). A similar band shift was observed upon freeze-induced denaturation of PW muscle and reported to indicate a decrease in α-helix structure and an increase in undefined or random-coil structure (*21*). In AKPI samples, the amide I band was located around 1661−1663 cm^{-1} for PPT, 5C, and 7C treatments, which indicates a random-coil

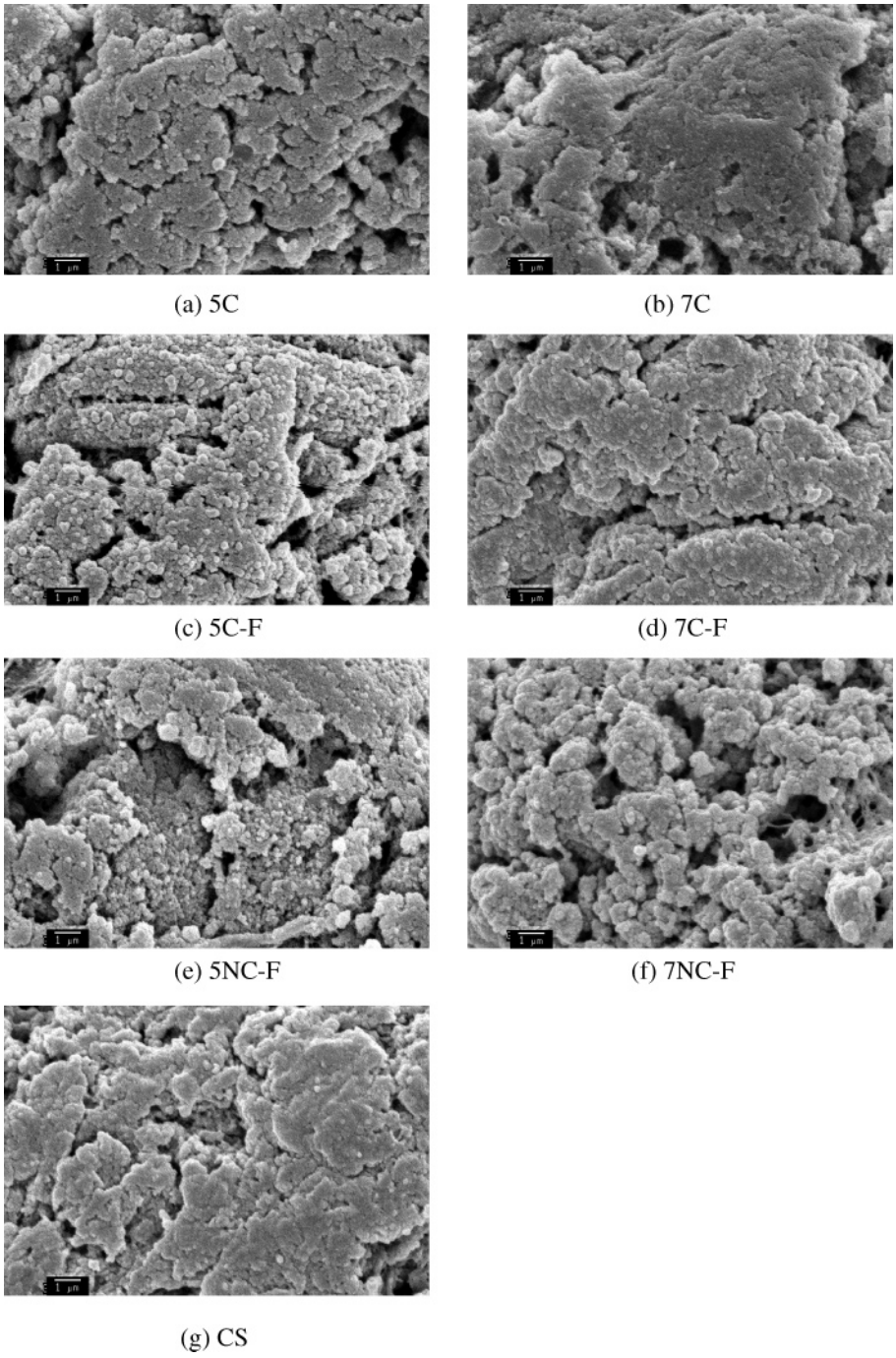


Figure 3. Microstructures of gels prepared from rockfish surimi and AKPIs at various storage conditions (magnification of 7500×, bar = 1 μm). All treatments were adjusted to the same cryoprotectant concentration and pH 7.0 before gel preparation. Abbreviations for treatments are the same as in **Figure 1** and CS = conventional surimi.

structure. The peak intensity (*I*) of this band was significantly lower in all AKPI treatments than CS. When pH 5.5 treatments with and without cryoprotectants were treated with FT cycling, the band shifted to a lower frequency (1659 cm^{-1} for 5C−F and 1655 cm^{-1} for 5NC−F), reflecting a recovery of the helical structure. However, no significant change was noted in pH 7.0 treatments.

Another way of looking at these Raman spectra is the use of the C−C stretching vibrations near 940, and 990 and 1239 cm^{-1}, which are characteristics of α-helix and β-sheet structures, respectively. Generally, the gradual loss of these structures leads to broadening and weakening in intensity (*27*−*29*). A marked decrease in I_{941} was evident in CS after FT cycling (0.716 ± 0.127 versus 0.567 ± 0.024). A significantly lower I_{941} was recorded in all AKPI treatments (0.388−0.535) when compared with that of CS.

More detailed evaluation of changes of the protein secondary structures was achieved by quantitative estimation using least-squares analysis of the amide I band (**Table 3**). The results demonstrated that CS predominantly contained α-helix structure (53%) and an approximately equal amount of β-sheet and random-coil structures (23 and 24%, respectively). PW surimi consisted of 44, 24, and 32% of α-helix, β-sheet, and random-coil structures, respectively (*12*). Freezing and thawing resulted in a substantial diminution of α-helix content and an elevation of β-sheet and random-coil contents. Other researchers have

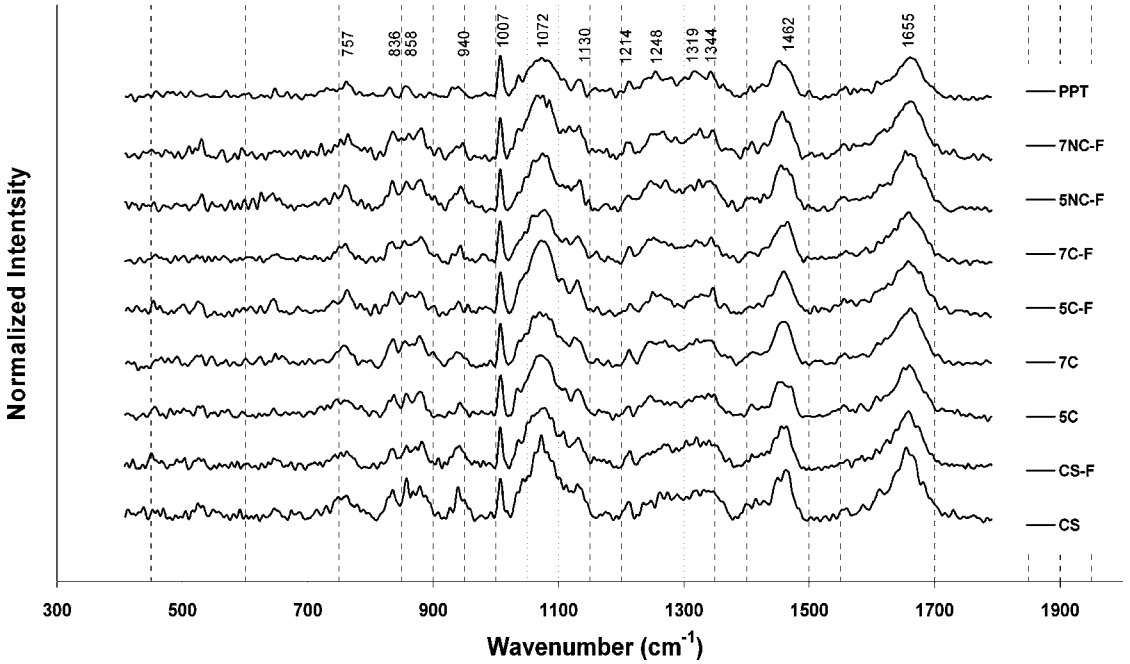


Figure 4. Raman spectra in the 400–1800 cm^{-1} region of rockfish surimi and AKPIs at various storage conditions. Abbreviations for the legend are the same as in **Figure 1** and CS = conventional surimi.

Table 2. Tentative Assignment of Some Bands in the Raman Spectrum of Rockfish Surimi

wavenumber (cm^{-1})	tentative assignment
524	cystine S–S stretch or aliphatic C–C–C deformation
757	tryptophan
836, 858	tyrosine
880	tryptophan
941	α helix
1007	phenylalanine
1072	backbone C–C, C–N stretch
1130	backbone C–N stretch
1214	tyrosine or phenylalanine
1248	amide III polypeptide backbone
1304	CH bending or amide III (α helix)
1320, 1339	tryptophan or aliphatic C–H bending
1400	C═O stretch of Asp, Glu, Coo^{-}
1462	CH_2 bending
1655	amide I polypeptide backbone
2938	aliphatic (alkyl) C–H stretching
3068	aromatic or unsaturated C–H stretching
3236	water O–H stretching

Table 3. Secondary Structure Fractions Estimated from the Amide I Band[a]

treatment[b]	total α helix	total β sheet	total random coil
CS	0.53	0.24	0.23
CS–F	0.15	0.49	0.35
PPT	0.23	0.47	0.30
5C	0.21	0.45	0.34
5C–F	0.42	0.35	0.23
5NC–F	0.47	0.29	0.23
7C	0.34	0.38	0.28
7C–F	0.46	0.33	0.22
7NC–F	0.36	0.43	0.21

[a] Average values from at least two duplicate analyses. [b] Abbreviations for treatments are the same as in **Figure 1** and CS = conventional surimi.

reported concurring results in hake fillet (*21*), ling cod actomyosin (*23*), and cod fillet (*26*).

Generally, gelation of CS is accomplished by first grinding surimi with salt to increase the solubility or the extractability of the myofibrillar proteins. The resulting paste is then cooked at high temperature (*6*). From the study in our lab by Kim (*15*) and the present case, AKPI, however, can form a gel without adding salt at pH 7.0. It is perhaps due to the reorganization of protein molecules as a result of pH adjustments. Thus, it is interesting to note that the protein structures, particularly the secondary fractions of CS and AKPI examined by Raman spectroscopy without salt addition, were discrepant.

A study using Raman spectroscopy with globular protein found that, when a gel is formed, there is a tendency for the β-sheet content to increase with a simultaneous decrease in α-helix content (*30*). Similar results were also discovered in heat-induced PW surimi gel (*12*). Boye and others (*31*) suggested that increasing pH from 3.0 to 9.0 as well as heating (26−97 °C) of β-lactoglobulin increased the formation of the β-sheet structure. Casal and others (*32*) found that the intermediate stages of alkaline and thermal denaturation were suggested to be similar, but the final stage of thermal denaturation is completely different from alkaline denaturation because protein unfolding is more affected by high pH than by heating as a result of repelling forces of the negatively charged functional groups. The lower α-helix and greater β-sheet contents of AKPI than CS may explain its ability to form gels at a suitable pH of neutrality (7C) without adding salt (**Table 3**). Interestingly, too much β-sheet structure, such as observed in the PPT or 5C samples, did not favor the ability to form a good gel. Furthermore, freezing of AKPI resulted in secondary-structure fractions more similar to CS than AKPI before freezing, but these changes did not correlate with good gelling ability. This suggests additional subtle structural differences upon freezing that are not reflected by merely estimating the fractions of the secondary structures.

The pellets recovered from alkali-treated proteins at the pI (PPT) and 5C (stored at pH 5.5 with cryoprotectants but adjusted to a final pH of 7.0) exhibited similar proportions of the secondary fractions, which contained only ∼50% of the α-helix content of CS and the highest proportion of β-sheet content. A

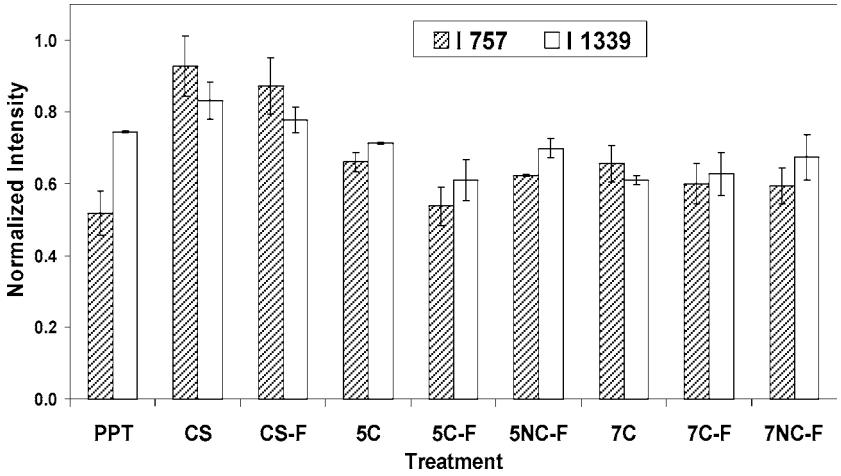


Figure 5. Raman intensity of the 757 and 1339 cm^{-1} of rockfish surimi and AKPIs at various storage conditions. Abbreviations for treatments are the same as in **Figure 1** and CS = conventional surimi.

decrease in α-helix content and an increase in β-sheet content are indicative of protein−protein interactions, which have also been reported for heat-denatured proteins (*33*) and freeze-denatured fish proteins (*21*, *23*).

After the denaturation process produced by frozen storage, one can obtain some protein aggregates that are not extractable by detergents that break hydrophobic interactions (*34*, *35*). Protein aggregates from other sources that were found to be insoluble in water as well as in the presence of sodium dodecyl sulfate (SDS) showed high proportions of β-sheet structures (*36*, *37*). These changes could be due to an unfolding of the helical structures (during frozen storage for CS and/or chemical treatment during the process for AKPI), followed by the formation of sheet structures, possibly through intermolecular interactions between exposed hydrophobic residues (*12*).

It was noteworthy that a contrasting trend to CS was observed when AKPIs were subjected to FT cycling. There was an increase in α-helix structure and slight decline of β-sheet and random-coil structures after FT cycling for AKPI at both storage pH. After FT cycles, samples without cryoprotectants stored at the pI (5NC−F) showed relatively similar composition to 5C−F (greater α-helix and less β-sheet contents), while those stored at pH 7.0 contained less α-helix but higher β-sheet structures. Similar increases in helical structure were reported after freezing of ling cod without cryoprotectants (*23*) and upon the aquacade concentration of cod myosin (*20*). It was suggested that aggregation of myosin heads and arrangement of the helical tails in a daisy wheel configuration (*38*) may lead to increased Raman signal intensity characteristic of helical structures and that these changes can be induced by the solute concentration, including that which occurs during freezing.

Changes of Tryptophan Residue Bands. Many Raman bands such as those at 760, 879, 1336, 1359, 1363, and 1557 cm^{-1} display information about the microenvironment of the tryptophan (Trp) residues. The intensity of these bands, particularly at 757, 1339, and 1557 cm^{-1}, slightly decreased for the CS sample treated with FT cycles (**Figure 5**), which perhaps indicated the exposure of Trp residues. Similar findings were reported by Careche and others (*21*) and Badii and Howell (*24*).

The I_{757} and I_{1339} of all AKPI treatments were significantly lower than CS; however, no significant change was noticed when FT and/or no cryoprotectant treatments were applied regardless of pH during storage (**Figure 5**). It should be noted that some overlap of the Trp band at 757 cm^{-1} with the band at 755 cm^{-1}, assigned to aliphatic residues, was reported for cod myosin. Similarly, the 1339 cm^{-1} band can also be assigned to aliphatic CH bending (*20*). Hence, changes of bands at 757 and 1339 cm^{-1} may have resulted from changes in the aliphatic side chains and CH bending, respectively, as well as Trp.

Tyrosine Doublet Bands. The intensity ratio I_{836}/I_{858} was determined for the tyrosine (Tyr) doublet at 836 and 858 cm^{-1}. It was suggested by Yu and others (*39*) that the ratio of the tyrosine ring vibrations at 850 and 830 cm^{-1} (R_{Tyr}) reflects "buried" and "exposed" tyrosine groups. $R_{Tyr} \geq 1$ in the myosin Raman spectrum was indicative of Tyr residues relatively exposed on the protein surface, which could interact with solvent water molecules as a hydrogen-bond donor and acceptor. If R_{Tyr} falls between 0.7 and 1.0, the Tyr residues may be considered buried. If the ratio is as low as 0.3, strong hydrogen bonding to a negative acceptor is indicated (*40*).

All treatments had relatively high R_{Tyr}, suggesting that the Tyr residues were exposed to the solvent in all cases. Nevertheless, some significant differences were observed. The precipitate from alkali-treated proteins had the highest intensity ratio (1.4) (**Figure 6**). A significant decrease was attained when PPT was adjusted to neutrality even after frozen storage at pH 5.5 for 2 weeks (5C and 7C), suggesting protein refolding. The intensity ratio of 5NC−F demonstrated a high value (1.3), thus representing the exposed Tyr residues. This may indicate that the proteins stored at pH 5.5 without the preservative effect of cryoprotectants could not be refolded even after altering the pH to neutral. In contrast, no difference in the intensity ratio among the AKPI samples stored at pH 7.0 (with or without cryoprotectants) was evident. These results corroborated the microstructures observed by SEM, in that the AKPI without cryoprotectants stored at the pI underwent greater detrimental protein structural changes than those kept frozen at pH 7.0.

Polypeptide Backbone Stretching at 1072 cm^{-1}. The Raman intensity of the CS at ∼1072 cm^{-1}, assigned to conformationally sensitive skeletal vibrations (mostly C−N and C−C stretch modes) (*41*), decreased after FT cycling, with a slight shift to a higher wavenumber at 1078 cm^{-1} (**Figure 7**). A dramatic decrease in the intensity of this band was observed in the PPT, which was partially recovered in AKPI treatments after pH adjustment.

Although published literature interpreting the significance of changes in this region of the Raman spectrum is limited, Raman bands near 1100 cm^{-1} have been reported to be useful as a

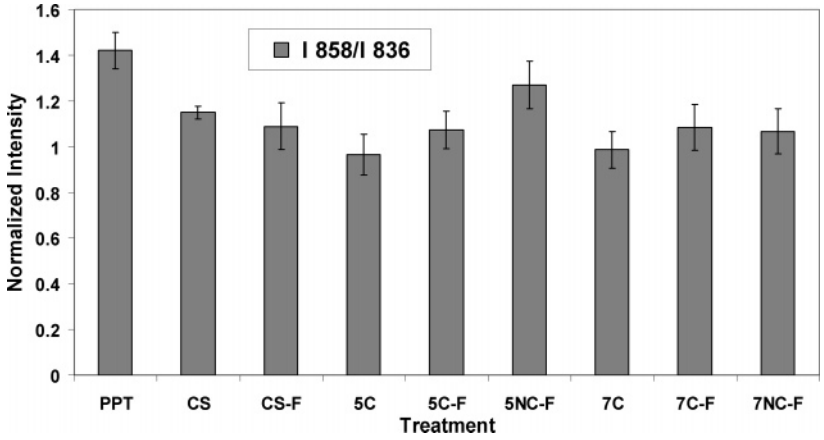


Figure 6. Raman intensity ratio of the tyrosine doublet of rockfish surimi and AKPIs at various storage conditions. Abbreviations for treatments are the same as in **Figure 1** and CS = conventional surimi.

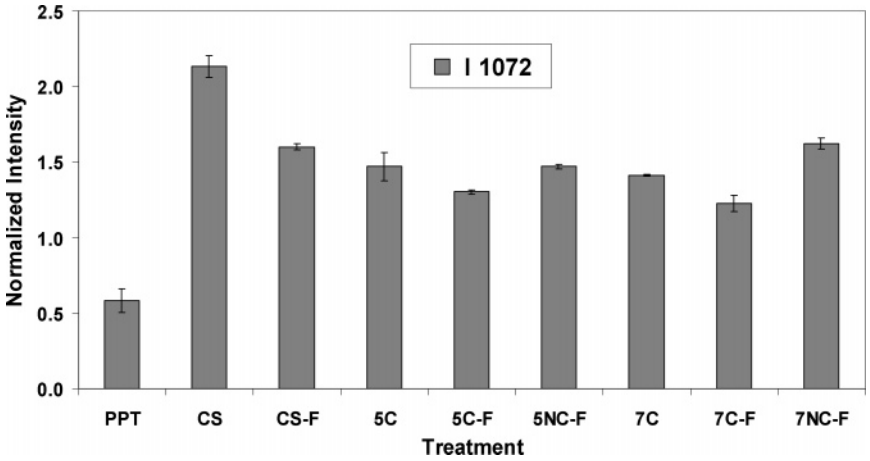


Figure 7. Raman intensity at 1072 cm^{-1} of rockfish surimi and AKPIs at various storage conditions. Abbreviations for treatments are the same as in **Figure 1** and CS = conventional surimi.

conformational change marker that broadens and loses intensity upon denaturation (*42*). Carew and others (*41*) reported that thermal denaturation of myosin from rabbit muscle leads to changes in the 1040−1120 cm^{-1} region, while Ogawa and others (*22*) reported a diminution of Raman intensity in the region of 1050−1100 cm^{-1} upon heating (setting at 40 °C) of rockfish actomyosin. Similarly, Caillé and others (*43*) indicated that a 1045 cm^{-1} band assigned to the C−N stretching vibration was sensitive to conformation, decreasing in intensity when the muscle fibers were denatured.

On the other hand, a pronounced increase in the peak intensity around 1060−1080 cm^{-1} was reported when the lysozyme solution was heated to form an opaque gel (*34*, *44*). A small increase in this region was observed in the Raman spectra of the gel formed by heating a solution of bovine serum albumin at pH 7 in the absence but not in the presence of κ-carrageenan (*45*), while no change was observed in gels formed by heating α-lactalbumin or β-lactoglobulin (*34*). Cod collagen showed a considerably lower peak intensity at 1096 cm^{-1}, which also is assigned to backbone stretching, after being stored at higher freezing temperatures; several studies have reported a reduction in collagen solubility because of frozen storage, particularly at a higher storage temperature (*46*).

These divergent reports in the literature suggest that the Raman band near 1100 cm^{-1} is sensitive to conformational changes of the polypeptide backbone that are associated with specific intermolecular interactions. In the present study, a predominantly low intensity of this band (I_{1072}) was detected for the PPT sample. This information probably suggested that a significant decrease in this band intensity is related to intermolecular interactions of protein molecules at the pI to form aggregates. The intensity of this band subsequently increased in the AKPI samples after cryoprotectant addition and/or pH adjustment to approximately the same extent as the CS−F sample. However, the intensity was not restored to that of CS without freezing/thawing, and no distinct trends were observed between AKPI samples treated under different storage conditions, suggesting that some of the conformational changes resulting from the alkali recovery process could not be reversed even upon readjustment of the pH to neutrality.

Aliphatic Bending Vibrations. Most C−H deformations appear in the region of 1400−1500 cm^{-1}, that is, at a wavenumber region roughly half of that of the C−H stretching vibrations (*41*). A band observed at 1462 cm^{-1} for the methylene (CH_2) bending in the CS sample showed a slight decline in its peak intensity (1.2−1.0) after FT cycling. A slight shift of the band toward a lower frequency with a lower intensity of its shoulder (∼1400−1440) for AKPI stored at the pI compared to those at pH 7.0 was illustrated (**Figure 4**). Ling cod natural actomyosin showed a band at 1460 and at 1446 cm^{-1} after freezing without cryoprotectants with a shoulder at 1464 cm^{-1} (*23*). Such a decrease has been observed for myosin isolated from cod, particularly after frozen storage in the presence of formaldehyde (*20*), and hake fillet (*21*). Changes of this band may have resulted from hydrophobic interactions of the aliphatic residues (*47*).

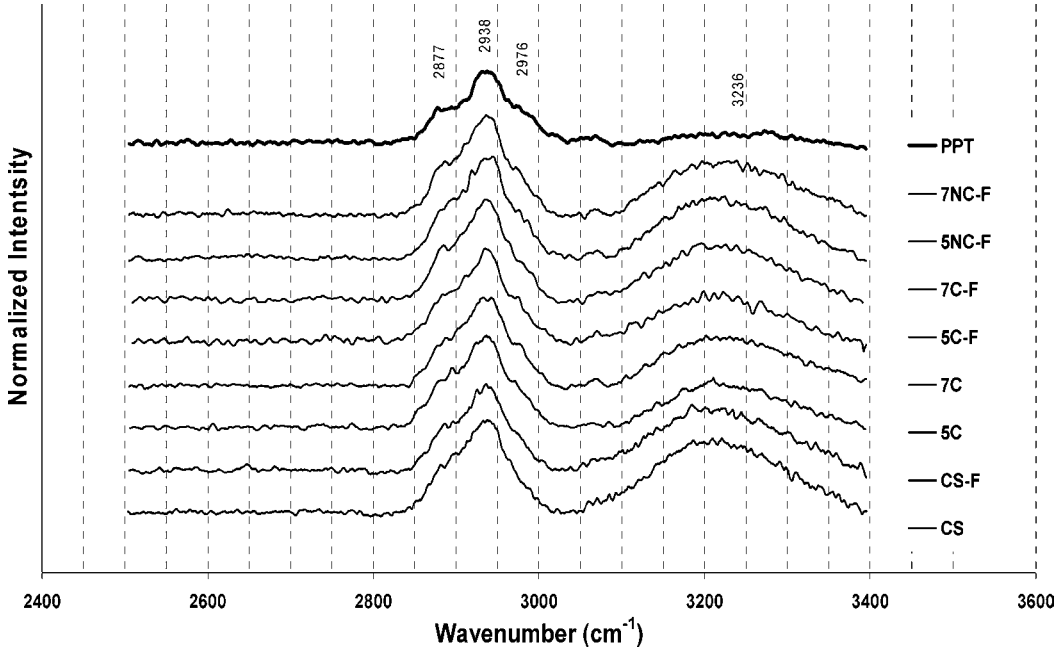


Figure 8. Raman spectra in the 2500–3400 cm^{-1} region of rockfish surimi and AKPIs at various storage conditions. Abbreviations for the legend are the same as in **Figure 1** and CS = conventional surimi.

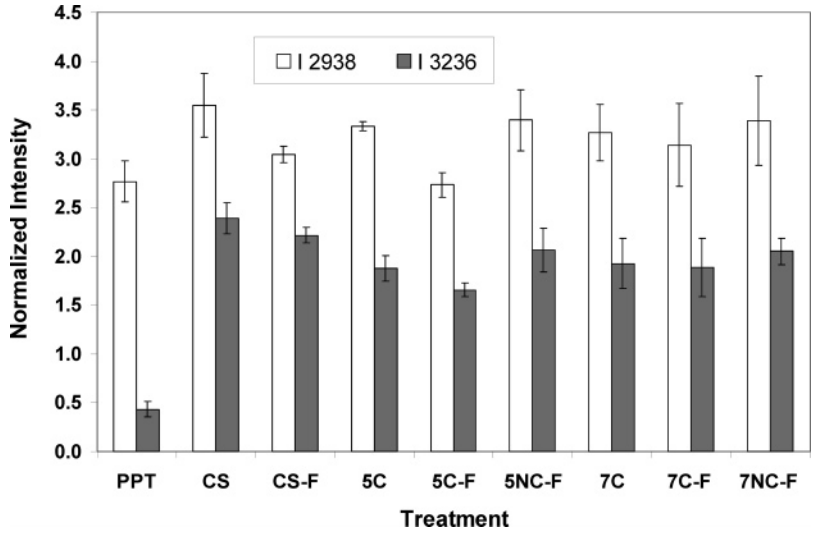


Figure 9. Raman intensity at 2938 and 3236 cm^{-1} (C–H and water O–H stretching, respectively) of rockfish surimi and AKPIs at various storage conditions. Abbreviations for treatments are the same as in **Figure 1** and CS = conventional surimi.

C−H Stretching Vibrations in the 2500−3400 cm^{-1} Region. Hydrophobic groups of amino acids, peptides, and proteins exhibit C−H stretching vibrational bands in the 2800−3100 cm^{-1} region. Bands found near 2874−2879 cm^{-1} are assigned to CH_3 symmetrical stretching and R_3C-H stretching bands of aliphatic amino acids, whereas the C−H stretching bands of aromatic amino acids can be found near 3061−3068 cm^{-1} (*48*). The CS sample showed two major bands at 3236 and 2938 cm^{-1} (with a shoulder at 2900 cm^{-1}). CS−F treatment demonstrated a slight decrease of I_{2938} with a more distinct shoulder around 2897 cm^{-1} and a small shoulder appearing at 2977 cm^{-1} (**Figure 8**). The shoulders at wavenumbers around 2903 and 2976 cm^{-1} were clearly distinguishable in the AKPI samples. The PPT treatment exhibited both shoulders with a substantially lower I_{2938} (2.8 compared to 3.5 and 3.0 for the CS and CS−F, respectively).

Sultanbawa and Li-Chan (*23*) and Badii and Howell (*24*) reported a similar trend in ling cod actomyosin and cod fillets, respectively, under various frozen storage conditions. When fish proteins were stored under harsher conditions (i.e., without a cryoprotectant, higher frozen storage temperature, and/or freeze−thaw treatment), the greater evidence of a peak shoulder of a band at 2938 cm^{-1} was illustrated.

The band at 3236 cm^{-1}, which reflects the OH stretch, indicated a lower intensity for all AKPI treatments and particularly the PPT in comparison to CS (**Figures 8** and **9**). This probably suggested that the AKPI underwent a higher rate of dehydration because of protein−protein interactions through aggregation mechanisms during the protein recovery process and/or frozen storage. Storage pH and cryoprotectants demonstrated a marked influence on this band. At the pI storage condition, protein molecules lost more water after being frozen/thawed. However, when cryoprotectants and pH of the 5NC−F were adjusted, rehydration could be observed. This observation was less prominent in samples stored at pH 7.0.

A similar finding was reported by Sultanbawa and Li-Chan (*23*). The OH stretch band intensity decreased for the frozen natural actomyosin and surimi without a cryoprotectant compared to the fresh samples. They proposed that such decreases may be related to dehydration as a result of the formation of ice crystals during frozen storage. Careche and others (*21*) reported a decrease in the intensity ratio of O−H/C−H bands, which correlated with the harshness of the condition of freezing or frozen storage of the hake fillets. This intensity ratio was suggested to be of use for monitoring quality change because of freezing and frozen storage. Nevertheless, our results did not clearly reveal this phenomenon. Only the PPT sample showed a significant decrease in the intensity of the OH band; no significant difference was discernible among other AKPI treatments (data not shown), which might be attributed to the adjustment of all of those samples in the present study to pH 7.0 and 78.5% moisture prior to analysis.

Texture qualities of AKPI decreased in the following descending order: samples with cryoprotectants, samples with cryoprotectants and subjected to FT cycles, and samples without cryoprotectants and subjected to FT cycles. No distinct difference of the gel texture was detected between AKPI stored at neutral pH and the pI. Nevertheless, a greater reduction in protein qualities was observed by SEM and Raman analysis when AKPI were kept frozen at pH 5.5 than at pH 7.0. When the storage condition became harsher, the microstructure of the gel became more discontinuous. The Raman spectral data showed that the protein structure in the pellet recovered from alkali-treated proteins could be partially refolded by adjusting to neutral pH. However, this reorganized structure was not identical to CS. There was a decrease in the α-helix content from 53 to 15% when the CS was frozen/thawed, while the β-sheet structure and random-coil content increased. In contrast, for the AKPI stored at pH 5.5 (5C), the α-helix fraction increased (21−42%), with a diminution of β-sheet and random-coil structures. The AKPI stored at neutrality (7C) contained a higher content of α-helix structure than the pI-stored samples but less than CS. Such changes of Raman bands led to the conclusion that several amino acid side chains and peptide backbones of AKPI samples were more exposed during frozen storage in comparison to CS. As a result, protein−protein interactions (aggregation) were induced. In addition, the mechanism of deterioration of AKPI under frozen storage seemed to take place differently compared to CS. Cryoprotectants played an important role in the stabilization of the unfolded/refolded AKPI. When the protein isolate was kept frozen at the pI, the decrease of protein functionalities and the structural changes were more intense. The results suggest that AKPI is slightly less stable than CS under frozen storage conditions.

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