Structural Changes in Natural Actomyosin and Surimi from Ling Cod (*Ophiodon elongatus*) during Frozen Storage in the Absence or Presence of Cryoprotectants

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Surimi and natural actomyosin (NAM) from ling cod (*Ophiodon elongatus*) were subjected to frozen storage in the absence or presence of cryoprotectants (sorbitol, sucrose, lactitol, and Litesse, either individually or in combination). Effects of frozen storage were studied for NAM frozen at −10 °C for 10 days and for surimi after eight freeze−thaw cycles. A commercial blend cryoprotectant (4% sucrose and 4% sorbitol), individual cryoprotectants at 8%, and optimal blends at 4, 5.5, 6, and 8%, were effective in maintaining the gel strength of surimi and NAM gels. Surimi or NAM frozen in the absence of cryoprotectants or with only 4% individual cryoprotectants, showed increased percent α-helical content by Raman analysis. Increased disulfide content was also observed in the treatment without cryoprotectants by the Raman SS stretching band and by chemical determination. Tyrosine residues were in a buried environment before and after freezing for all treatments, and surface hydrophobicity measured by 1-anilinonaphthalene-8-sulfonate decreased after frozen storage in the absence of cryoprotectants.

Keywords: *Frozen storage; cryoprotectants; surimi; natural actomyosin; Ophiodon elongatus; gel; hydrophobic interaction; disulfide linkages; α-helix*

INTRODUCTION

Surimi is the Japanese term for minced fish from which most of the water-soluble components, including sarcoplasmic proteins, have been removed by leaching with potable water, and the remaining myofibrillar fraction is blended with cryoprotectants prior to freezing (*1*). The inclusion of cryoprotectants is required to ensure long-term stability, as deterioration of the functional and textural properties of fish muscle proteins during frozen storage in the absence of cryoprotection is well documented (*2*−*5*). A commercial cryoprotectant blend consisting of 4% sucrose, 4% sorbitol, and 0.2−0.3% polyphosphates has been used successfully to confer stability to surimi proteins during frozen storage. However, this blend has been reported to be too sweet (*6*), and blends consisting of sucrose and sorbitol with polyols such as lactitol and Litesse (which have reduced sweetness and caloric value) have been proposed as alternative cryoprotectant blends (*7*, *8*).

Several theories have been proposed with regard to the mechanism of cryoprotection (*9*−*11*). Gel-forming ability is an important determinant of surimi quality (*12*), and it may be lost by the denaturation and aggregation of fish proteins that occur in the absence of cryoprotection (*13*). However, few studies have been reported on the structural properties of fish proteins in situ, i.e., in the surimi or gel.

The structural changes occurring during frozen storage, leading to frozen-storage-induced myosin aggregation and consequent loss of gelling capacity of fish muscle systems, have been investigated primarily by using model systems of myosin or actomyosin (*14*, *15*). The globular heads of myosin are responsible for its enzymatic (ATPase) activity, which is sensitive to changes in the configuration of the molecule around the enzymic site (*5*). Many studies on fish have established that there is a significant loss in ATPase activity following frozen storage (*1*, *3*, *10*, *16*). However, loss of ATPase activity is not necessarily synonymous with frozen-storage-induced aggregation of myosin (*17*). Sulfhydryl (SH) groups are considered to be the most reactive functional group in proteins, being easily oxidized to disulfide (SS) groups. There has been great interest in knowing whether this form of covalent bonding occurs during frozen storage of fish (*5*). Recently, Ramírez et al. (*17*) suggested that frozen storage of myosin in suspension results in aggregation involving side-to-side interactions of the rod with low formation of disulfide bonds; on the other hand, when myosin is solubilized prior to frozen storage, mainly head-to-head interactions with a higher formation of disulfide bonds are implicated in the aggregations. Extrinsic fluorescence probes such as 1-anilinonaphthalene-8-sulfonic acid (ANS) and *cis*-parinaric acid (CPA) have been widely used to study surface hydrophobicity in fish proteins (*18*, *19*). Mackie (*5*) reported that an increase in fluorescence is an indication of denaturation of a protein, with the exposure at the surface of hydrophobic groups that are normally buried in the interior of the molecule.

Raman spectroscopy is useful to study a food system as it is, in the solid or liquid state, and without the need to extract a purified component. Examples of such studies are reported by Li-Chan (*20*). Proteins have been

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Table 1. Cryoprotectant Treatments Used in This Study

	cryoprotectant composition, % (w/w)				
treatment no.[a]	sucrose	sorbitol	Litesse	lactitol	total
1	**4.0**	0.0	0.0	0.0	4.0
2	0.0	**4.0**	0.0	0.0	4.0
3	0.0	0.0	**4.0**	0.0	4.0
4	0.0	0.0	0.0	**4.0**	4.0
5	**8.0**	0.0	0.0	0.0	8.0
6	0.0	**8.0**	0.0	0.0	8.0
7	0.0	0.0	**8.0**	0.0	8.0
8	0.0	0.0	0.0	**8.0**	8.0
9	**4.0**	**4.0**	0.0	0.0	8.0
10	**2.0**	**2.0**	**2.0**	**2.0**	8.0
11	**1.0**	**1.0**	**1.0**	**1.0**	4.0
12	**2.0**	**2.0**	0.0	**1.5**	5.5
13	**2.0**	**2.0**	**1.0**	**1.0**	6.0
14	0.0	0.0	0.0	0.0	0.0

[a] Treatments 1−4, individual cryoprotectants at 4%; treatments 5−8, individual cryoprotectants at 8%; treatment 9, commercial blend of cryoprotectants; treatments 10−13, optimal blends with final concentrations of 4, 5.5, 6, and 8% formulated in a previous study (*8*); treatment 14, no cryoprotectant (control).

studied extensively using this technique. Information about the polypeptide secondary structure (α-helix, β sheet, and random coil) can be obtained from the amide I and amide III vibrational modes. Characterization of cysteine SH groups and cystine SS bonds, and the exposure to environments of aromatic side chains of tyrosine (Tyr) and tryptophan (Trp) can also be determined from the Raman spectra.

Careche and Li-Chan (*18*) used Raman spectroscopy to show that the presence of formaldehyde decreased α-helical content from 95% to 60% in a cod myosin model system. The amide I region of the Raman spectrum indicated that the predominantly α-helical structure of raw Pacific whiting surimi changed to higher antiparallel β sheet and lower α-helix contents in the cooked kamaboko gel (*21*). Ogawa et al. (*22*) reported that the gel setting phenomenon of fish actomyosins requires *gauche*−*gauche*−*trans* conformation of disulfide bonds as well as a slow unfolding of the α-helix. To our knowledge, no reports have been published on applying Raman spectroscopy to investigate the effects of cryoprotectants on surimi protein structure during frozen storage. This technique has a unique advantage in being applicable to analyze changes occurring as a function of frozen storage using either model systems of protein extracts or in situ using the surimi itself.

The objective of this research was, therefore, to investigate the effects of frozen storage on the properties of the proteins in ling cod, when individual polyols or blends of polyols are used as cryoprotectants. Structural changes in the proteins in a natural actomyosin (NAM) system and a surimi system were studied using Raman spectroscopy, as well as by conventional measurements of surface hydrophobicity, sulfhydryl and disulfide group content, and gel properties.

MATERIALS AND METHODS

Materials. Ling cod (*Ophiodon elongatus)* and ingredients for preparing natural actomyosin (NAM) and surimi were obtained as described by Sultabawa and Li-Chan (*7*).

Preparation and Freeze−Thaw Treatment of Surimi. Surimi was prepared from 6.5 kg of filleted fish after removal of dark muscle, according to Sultanbawa and Li-Chan (*7*). The dewatered mince was divided into 300-g portions, to which were added one of the 14 cryoprotectant combinations given in Table 1, as well as 0.15% (w/w) sodium pyrophosphate and 0.15% (w/w) of pentasodium tripolyphosphate. The order of preparation of the surimi formulations was chosen randomly.

Each treatment was divided into 125-g portions, and each portion was placed in a vacuum pouch and then vacuum sealed. One set of these pouches was placed in the freezer at −20 ± 2 °C and subjected to 8 freeze−thaw cycles. Each freeze−thaw cycle comprised freezing at −20 ± 2 °C for 18 h and thawing at 4 ± 2 °C for 6 h. The other set of pouches was analyzed without freezing.

Preparation and Frozen Storage Treatment of NAM. Natural actomyosin was extracted from ling cod fillets in a walk-in cold room at 4 ± 2 °C according to Ogawa et al. (*23*) with modifications as follows. The dark muscle of the filleted fish was removed, and 2.5 kg of the remaining (white) muscle was weighed out and divided into 250-g portions. Each portion was homogenized in a Waring commercial blender for 1 min with 1.5 L of 0.05 M NaCl in 20 mM Tris-maleate buffer at pH 7.0. The combined homogenates were centrifuged at 4500*g* for 20 min at 4 °C. After the supernatant was decanted off, the pellets were combined and again divided into 250-g portions and homogenized in the Waring blender for 1 min with 1.5 L of 0.6 M NaCl in 20 mM Tris-maleate buffer, pH 7.0. The homogenates were combined, filtered through a double layer of cotton gauze, then diluted by adding 30 L of 20 mM Tris/maleate buffer at pH 6.9 and kept overnight. The aqueous layer on the surface of the tank was decanted and the residue at the bottom was pelleted out by centrifuging at 4500*g* for 20 min at 4 °C. The pellets collected were then concentrated by centrifuging at 27000*g* for 60 min at 4 °C. The pellet from this final centrifugation step is hereinafter referred to as "natural actomyosin" (NAM).

The extracted NAM was divided into 50-g portions, to which were added one of the 14 combinations of cryoprotectants given in Table 1, as well as 0.15% (w/w) sodium pyrophosphate and 0.15% (w/w) of pentasodium tripolyphosphate. One set of the 14 treatments was kept at −10 °C for 10 days, and the other set was analyzed without any freezing treatment.

Determination of Protein Content in Surimi and NAM. A 2-g portion of surimi or NAM from each treatment, brought to a final volume of 30 mL with 0.6 M NaCl in 40 mM Tris/maleate buffer at pH 7.0, was sonicated for 1 min with a Tekmar sonic disruptor (Tekmar Co; Cincinnati, OH) using a microtip probe, duty cycle of 50, output control 5, and pulse setting of 30. The samples were placed in ice during sonication. Protein contents of the sonicated samples of surimi and actomyosin were determined according to the Biuret method, using reagents and the microplate procedure provided by Sigma (Sigma Diagnostics, St. Louis, MO). Bovine serum albumin (BSA) (A-4503, Sigma, Chemical Co., St. Louis, MO) was used as the standard. Measurements were conducted in triplicate wells on a single microplate. These sonicated samples of surimi and NAM were also used to determine hydrophobicity, SH, and SS contents.

Determination of Moisture in Surimi. Moisture in surimi was determined according to the official methods of the AOAC (*24*). A 5-g aliquot of surimi was dried in a vacuum oven (< 50 mm Hg) at 75−80 °C for ca. 18 h, to a constant weight. Moisture determination was carried out in triplicate on all 14 treatments before freezing.

Gel Properties of Surimi and NAM. Surimi (115 g) from each treatment was adjusted to 83% moisture after thawing overnight at 4 ± 2 °C. The surimi was then mixed in a Kitchen Aid model K5A mixer for 1 min. Salt was added at 3% (w/w) and mixed for another 2 min, after which 2% (w/w) whey and 5% (w/w) wheat starch were added and mixed for a further 3 min. The paste was filled into plastic molds of 4-cm length and 3.1-cm diameter. The molds were wrapped in plastic films and placed in vacuum pouches and vacuum-sealed. The gels were formed by setting at 40 °C for 30 min and cooking for 15 min, based on the methods reported by Reppond et al. (*25*) and Park et al. (*26*). Gels were kept in the cold room at 4 ± 2 °C for at least 24 h and equilibrated to room temperature prior to analysis. Gel strength was measured for five replicates of each treatment by the standard puncture test according to Lanier (*27*) and Nielsen and Pigott (*28*) using a TA.XT2 texture

analyzer (Texture Corp., Scarsdale, NY/Stable Microsystems, Godalmin, Surrey, UK). Gels were removed from the molds and cut into 2.5-cm long sections. A plunger of 5-mm diameter resting on the cylindrical gel samples and moving at a speed of 0.5 mm/sec was used. Gel strength was expressed as force to rupture (g) multiplied by the deformation distance (cm).

NAM gels were prepared according to Ogawa et al. (*23*) with modifications. A 10-g portion of NAM from each treatment was mixed separately with a mortar and pestle for 1 min; NaCl (3% w/w) was added, and the protein content was adjusted to 10% (w/w) with distilled water, then the material was mixed for 2 min. The resulting paste was placed in a polyethylene cone and extruded into 2-mL siliconized microcentrifuge tubes of 10-mm diameter. Tubes were deaerated by centrifugation at 10000*g* for 30 s in a benchtop centrifuge, placed in vacuum pouches, and vacuum-sealed. Gels were allowed to set at 40 °C for 30 min and cooked at 90 °C for 30 min in a thermostated water bath. Gels were cooled on ice, then kept at 4 ± 2 °C for at least 24 h, and brought to room temperature before analyses. Gels were removed from the microcentrifuge tubes and cut into 1-cm long sections. Gel strength was measured as described above for surimi using a plunger of 3-mm diameter and a test speed of 0.5 mm/sec.

Sulfhydryl (SH) Groups and Disulfide (SS) Bonds in NAM. The contents of total and reactive SH groups before and after freezing of NAM were determined colorimetrically with Ellman's reagent (*29*) by modification of the methods of Hardman (*30*) and Beveridge et al. (*31*). Samples were diluted in 85 mM Tris/100 mM glycine/4 mM EDTA buffer in the absence or presence of 2.5 M guanidine thiocyanate for measurement of reactive or total SH groups, respectively. Measurements were conducted in duplicate for the unfrozen samples and in triplicate for the samples stored at −10 °C.

The SS content was determined using 2-nitro-5-thiosulfobenzoate (NTSB) by minor modification of the methods of Thannhauser et al. (*32*) and Damodaran (*33*). NAM solution (200 μL; 4 mg/mL) was mixed with 1.5 mL of NTSB assay solution and the mixture was incubated in the dark for 25 min. The absorbance was then measured at 412 nm, as described for the SH content. The reagent blank contained 1.5 mL of NTSB assay solution mixed with 200 μL of distilled water.

Hydrophobicity of NAM. Protein surface hydrophobicity was determined using the ANS fluorescent probe method as described by Careche and Li-Chan (*18*). Standardization of net relative fluorescent intensity (RFI) values was based on measuring the RFI for ANS (10 μL) in methanol (10 mL) and normalizing to a standard value of 50. Surface hydrophobicity was expressed as the initial slope of the plot of standardized net RFI values vs percentage protein concentration, computed by least squares regression analysis.

Raman Spectral Analysis of Surimi and NAM. Raman spectra were recorded at 4.0 ± 0.1 °C on a JASCO model NR-1100 laser Raman spectrophotometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan) with excitation at 488 nm supplied by an argon ion laser with Spectra Physics model 168B power supply (Spectra Physics, Mountain View, CA), according to Careche and Li-Chan (*18*). Spectral files were baseline-corrected, smoothed, and normalized using Grams 386 (Galactic Industries Corporation, Salem, NH). The spectra were baseline-corrected using the Array basic program's multiple point baseline-correction function, and smoothed using the maximum entropy smoothing (ESM) of the Razor analysis program; the selected peak shape was Gaussian. The spectral data were also normalized to the intensity of a band at 1004 ± 2 cm^{-1} which was reported to arise from vibrational motions of phenylalanine residues and to be insensitive to microenvironment (*34*). In addition, the relative proportions of secondary fractions were estimated using the Raman spectral analysis package (RSAP) of Przybycien and Bailey (*35*), which is based on the algorithm of Williams (*36*) for least-squares analysis for the amide I band.

Statistical Analyses. Analysis of variance (ANOVA), Tukey's, and Fisher's comparison tests were carried out using Minitab for Windows Release 12Xtra (Minitab Inc., State College, PA).

Table 2. Properties of Gels Formed from Natural Actomyosin before Freezing and after Frozen Storage for 10 days at −10 °C in the Absence or Presence of Cryoprotectants; Treatments Are Listed in Order of Increasing Gel Strength after Frozen Storage

	gel strength, g·cm[a]	
treatment	before freezing	after frozen storage
no cryoprotectants added	23 ± 2^{d}	10 ± 1^{a}
4% lactitol	16 ± 1^{a}	11 ± 1^{a}
4% sorbitol	19 ± 1^{bc}	14 ± 1^{b}
4% sucrose	16 ± 2^{a}	14 ± 1^{b}
4% litesse	19 ± 0^{bc}	14 ± 1^{b}
8% litesse	20 ± 1^{c}	17 ± 1^{c}
8% sucrose	17 ± 1^{ab}	19 ± 1^{c}
8% lactitol	24 ± 2^{d}	22 ± 1^{d}
optimal blend 3	20 ± 1^{c}	22 ± 1^{d}
optimal blend 4	23 ± 1^{d}	23 ± 1^{d}
commercial blend	23 ± 2^{d}	23 ± 1^{d}
8% sorbitol	20 ± 1^{c}	24 ± 1^{d}
optimal blend 1	23 ± 1^{d}	24 ± 1^{d}
optimal blend 2	23 ± 1^{d}	24 ± 1^{d}

[a] Given values are averages ± standard deviations of 8 replicate measurements. The compositions of the commercial blend and optimal blends 1, 2, 3, and 4 are given in Table 1 and correspond to treatments 9, 10, 11, 12, and 13, respectively. Values bearing different superscript letters within each column are significantly different ($P < 0.05$).

RESULTS AND DISCUSSION

Moisture and Protein Content of Natural Actomyosin and Surimi. Moisture content of the surimi before addition of cryoprotectants was 82.6%, which is similar to the 83.4% reported by Sych et al. (*37*). The crude protein contents for the control sample (no cryoprotectant) and the sample treated with the commercial blend of 4% sucrose and 4% sorbitol were 16.84 and 14.77%, respectively, which are similar to the values of 16.6 and 14.5%, respectively, reported by Sych et al. (*37*) for cod (*Gadus morhua*). The moisture content of all surimi treatments was adjusted to 83% moisture prior to gel formation and evaluation. The protein concentration of NAM samples was adjusted to 10% prior to gelation and evaluation.

Gel Properties of Natural Actomyosin and Surimi. The properties of gels prepared from NAM and surimi with or without cryoprotectant treatments, before and after frozen storage, are shown in Tables 2 and 3, respectively.

The highest gel strength of unfrozen NAM was observed for treatments containing 8% lactitol, 8% sorbitol, commercial blend or any of the optimal blends, and was maintained after frozen storage (Table 2). Similarly, the highest gel strength values obtained before freezing of surimi were observed for the treatments containing commercial blend, optimal blends 1 and 2, and 4% or 8% sucrose, and the gelling property was generally maintained after 8 freeze−thaw cycles in the presence of these cryoprotectants. In contrast, the NAM and surimi frozen without cryoprotectants formed only very weak gels − the strength of gels formed from NAM after 10 days at −10 °C was 10 g·cm compared to 23 g·cm before freezing, and the gel strength from surimi after 8 freeze−thaw cycles was 59 g·cm, compared to 457 g·cm before freezing.

The gel strength for freeze−thawed surimi and frozen NAM samples with no cryoprotectants was generally lower than that of those treated with cryoprotectants. In addition, gel strength of samples with individual cryoprotectants added at the 4% level were generally

Table 3. Properties of Gels Formed from Surimi Before and After 8 Freeze−Thaw Cycles in the Absence or Presence of Cryoprotectants; Treatments Are Listed in Order of Increasing Gel Strength after Freeze−Thaw Treatment

	gel strength, g·cm[a]	
treatment	before freezing	after freeze−thaw
no cryoprotectants added	457 ± 27^{bd}	59 ± 3^{a}
4% sorbitol	419 ± 24^{ab}	326 ± 21^{b}
4% sucrose	524 ± 12^{deg}	360 ± 24^{bc}
4% Litesse	457 ± 21^{bd}	377 ± 25^{cd}
4% lactitol	500 ± 26^{def}	404 ± 20^{cd}
optimal blend 4	469 ± 21^{cd}	409 ± 12^{d}
8% Litesse	386 ± 29^{a}	449 ± 28^{ed}
optimal blend 1	571 ± 20^{g}	454 ± 24^{e}
optimal blend 2	557 ± 26^{g}	478 ± 8^{e}
8% sucrose	524 ± 18^{fg}	524 ± 23^{f}
8% sorbitol	441 ± 15^{b}	549 ± 22^{fg}
commercial blend	525 ± 29^{cefg}	577 ± 22^{g}
8% lactitol	457 ± 23^{bd}	627 ± 20^{h}
optimal blend 3	446 ± 14^{bc}	649 ± 12^{h}

[a] Given values are averages ± standard deviations of 5 replicate measurements. The compositions of the commercial blend and optimal blends 1, 2, 3, and 4 are given in Table 1 and correspond to treatments 9, 10, 11, 12, and 13, respectively. Values bearing different superscript letters within each column are significantly different ($P < 0.05$).

significantly lower ($P < 0.05$) than those samples frozen with 8% individual cryoprotectants, the commercial blend, or with optimal blends containing 8, 6, 5.5, and 4% cryoprotectant. The exceptions were 4% Litesse and 4% lactitol in surimi, which were not significantly different ($P > 0.05$) from the 6% optimal blend (optimal blend 4) or 8% Litesse. The 5.5% optimal blend (optimal blend 3) and the 8% lactitol treatment gave significantly higher gel strengths than the commercial blend in the freeze−thawed surimi. Sych et al. (*38*) reported that the gel properties of cod surimi made with 8% Polydextrose, Palatinit, and lactitol together, or 4% lactitol alone, were comparable to that of surimi prepared with commercial blend, whereas Auh et al. (*39*) reported that an 8% solution of highly concentrated branched oligosaccharides showed cryoprotective effects similar to those of 8% sucrose or the commercial blend applied to actomyosin from Alaska pollock.

It should be noted that, in this study, gels from NAM treatments were compared on an equivalent protein (10%) basis, whereas surimi treatments were formulated on an equivalent moisture (83%) basis. After freeze−thaw treatment, surimi samples with higher protein concentration (no or 4% cryoprotectant addition) resulted in lower gel strength than those samples with lower protein concentration (8% cryoprotectant or blends). Because gel strength would usually be expected to increase with protein concentration, the observed differences in gel strength between surimi treatments are suggested to arise from changes in protein structure rather than changes in concentration.

Sufhydryl Groups and Disulfide Bonds in Natural Actomyosin. The reactive and total SH content and SS groups of NAM were measured before (data not shown) and after freezing (Table 4).

Unfrozen NAM contained ~4 reactive SH groups, 0.5−1 buried SH group, and 1 SS linkage per 10^5 g protein. The content of SH equivalents (SH + 2SS) in ling cod NAM was thus ~6−7 mol/10^5 g protein (30−35 SH residues/5 × 10^5 g protein), which is comparable to values reported by Suzuki (*1*). Reactive SH was not significantly different ($P > 0.05$) between any of the

Table 4. Reactive SH, Total SH, and SS Contents for Natural Actomyosin after 10 days at −10 °C in the Absence or Presence of Different Cryoprotectant Treatments

	moles/10^5 g of protein[a]			
treatment	reactive SH	total SH	SS	SH + SS
4% sucrose	2.69^{b}	3.90^{e}	1.21^{b}	5.11^{ac}
4% sorbitol	2.88^{b}	4.05^{c}	1.02^{bc}	5.07^{ac}
4% Litesse	2.19^{c}	4.15^{b}	0.96^{bcd}	5.11^{ac}
4% lactitol	3.61^{a}	4.17^{b}	0.91^{bcd}	5.08^{ac}
8% sucrose	2.53^{b}	4.02^{cd}	1.11^{bc}	5.13^{ac}
8% sorbitol	2.96^{b}	4.32^{a}	1.12^{bc}	5.44^{a}
8% Litesse	3.02^{a}	3.96^{d}	1.15^{bc}	5.11^{ac}
8% lactitol	3.18^{a}	4.18^{b}	0.66^{d}	4.84^{bc}
commercial blend	2.35^{b}	4.39^{a}	0.98^{bcd}	5.37^{a}
optimal blend 1	2.13^{c}	4.09^{c}	0.93^{bcd}	5.02^{ac}
optimal blend 2	3.36^{a}	3.79^{f}	1.21^{b}	5.00^{ac}
optimal blend 3	3.13^{a}	3.68^{g}	1.16^{bc}	4.84^{bc}
optimal blend 4	1.86^{cd}	3.97^{d}	0.95^{bcd}	4.92^{bc}
no cryoprotectants	1.73^{cd}	2.57^{h}	1.75^{a}	4.32^{d}

[a] Given values are averages of duplicate measurements determined by Ellman's reagent (for reactive SH and total SH content) or 2-nitro-5-thiosulfobenzoate (for SH + SS content). The SS content was calculated as the difference between (SH + SS) and total SH content. The compositions of the commercial blend and optimal blends 1, 2, 3, and 4 are given in Table 1 and correspond to treatments 9, 10, 11, 12 and 13, respectively. Values bearing different superscript letters within each column are significantly different ($P < 0.05$).

treatments for unfrozen NAM; the contents of total SH, SH + SS, and SS groups were significantly different ($P < 0.05$) between treatments but did not show a clear trend.

When NAM containing commercial blend was frozen (Table 4), the reactive SH groups decreased to 2 SH and the number of buried SH groups increased to 2, while the disulfide bonds remained at 1. The other treatments with cryoprotectants showed a similar trend after frozen storage. In contrast, for the NAM frozen without cryoprotectant, the number of reactive SH decreased from 4 to 2 and the number of SS bonds increased from 1 to 2, while the number of buried SH groups remained at 1. These changes could result from SH oxidation or SH−SS interchange reactions.

Reactive SH, total SH, SS, and SH + SS contents were all significantly different ($P < 0.05$) between different treatments for the frozen NAM. Treatments with 4% and 8% lactitol, 8% Litesse, and optimal blends 2 and 3 produced significantly higher ($P < 0.05$) reactive SH than the other treatments. Optimal blends 1 and 4, 4% Litesse, and the no-cryoprotectant control had significantly lower ($P < 0.05$) reactive SH than other treatments. The no-cryoprotectant control revealed significantly lower ($P < 0.05$) total SH and SH + SS than all other treatments, and significantly higher ($P < 0.05$) SS content than all other treatments. Studies done by Lim and Haard (*40*) on Greenland halibut, by LeBlanc and LeBlanc (*19*) on cod proteins, and by Ramírez et al. (*17*) on myosin from *Tilapia nilotica*, also provided evidence for the loss of SH groups and for formation of SS bonds during frozen storage. However, the extent of involvement of SH groups and disulfide bonds in frozen-storage-induced aggregation was suggested to depend on the initial physicochemical state of myosin, with a higher involvement being indicated for myosin when frozen in solubilized form than when in suspension (*17*).

From the data in our study on NAM, it was evident that the reactive SH groups decreased and the number

Table 5. Surface Hydrophobicity Values ($\%^{-1}$) for Unfrozen and Frozen (−10 °C, 10 days) Natural Actomyosin in the Absence or Presence of Different Cryoprotectant Treatments

	S_o ($\%^{-1}$)[a]	
treatment	unfrozen NAM	frozen NAM
4% sucrose	268[a]	181[bc]
4% sorbitol	190[b]	183[bc]
4% Litesse	304[a]	328[a]
4% lactitol	270[a]	219[b]
8% sucrose	271[a]	197[b]
8% sorbitol	203[b]	159[bcd]
8% Litesse	190[b]	190[bc]
8% lactitol	220[a]	217[b]
commercial blend[b]	209 (20)[b]	136 (8)[cd]
optimal blend 1	226[a]	184[bc]
optimal blend 2[b]	204 (14)[b]	196 (13)[b]
optimal blend 3	208[b]	187[bc]
optimal blend 4	211[b]	173[bcd]
no cryoprotectants added[b]	204 (15)[b]	124 (8)[d]

[a] The compositions of the commercial blend and optimal blends 1, 2, 3, and 4 are given in Table 1 and correspond to treatments 9, 10, 11, 12, and 13, respectively. The surface hydrophobicity (S_o) values were calculated as the initial slope of the plot of standardized net relative fluorescence intensity values vs % protein concentration. Values bearing different superscript letters within each column are significantly different ($P < 0.05$). [b] Surface hydrophobicity for duplicate trials of the commercial blend, optimal blend 2, and the control treatment with no cryoprotectants was measured in triplicate; the S_o values shown for these treatments are averages of triplicate measurements from the two trials, with standard deviation values given in parentheses.

of hidden SH groups increased during frozen storage. This indicates that some aggregation may have occurred. The number of total SH groups (∼4) and SS bonds (∼1) remained the same after freezing for the treatments with cryoprotectants, whereas total SH decreased and SS increased in the treatment without cryoprotectants. Hence, it is possible to hypothesize that SS bond formation is a secondary process of aggregation during freeze denaturation in the absence of cryoprotectants.

Hydrophobicity of Natural Actomyosin. The surface hydrophobicity was decreased after freezing of NAM for most treatments, with the exceptions of the samples treated with 4% and 8% Litesse (Table 5). The largest difference was observed after freezing the treatment without cryoprotectants. For the unfrozen NAM, although treatments could be classified into two significantly different ($P < 0.05$) groups on the basis of hydrophobicity values, there was no clear trend in relationship to the composition of the cryoprotectants. For frozen NAM, treatments without cryoprotectants and the commercial blend revealed significantly lower ($P < 0.05$) surface hydrophobicity values than samples treated with 4% Litesse, 4% lactitol, 8% sucrose, 8% lactitol, and optimal blend 2. Surface hydrophobicity of frozen NAM when Litesse was used as a cryoprotectant at the 4% level was significantly higher ($P < 0.05$) than all other treatments.

A number of studies have reported that the freezing process and frozen storage increased the hydrophobicity of myofibrillar proteins (*41*−*43*). Two recent studies by Careche and Li-Chan (*18*) on cod myosin and Careche et al. (*44*) on hake actomyosin reported a decrease in surface hydrophobicity of the proteins in the presence of formaldehyde during freezing and frozen storage. A possible explanation for the decrease in surface hydrophobicity after frozen storage could be the high concentration of NAM (100 mg/mL) in this study, which could have produced aggregates held by strong hydrophobic interactions that were not disrupted by sonication or dilution.

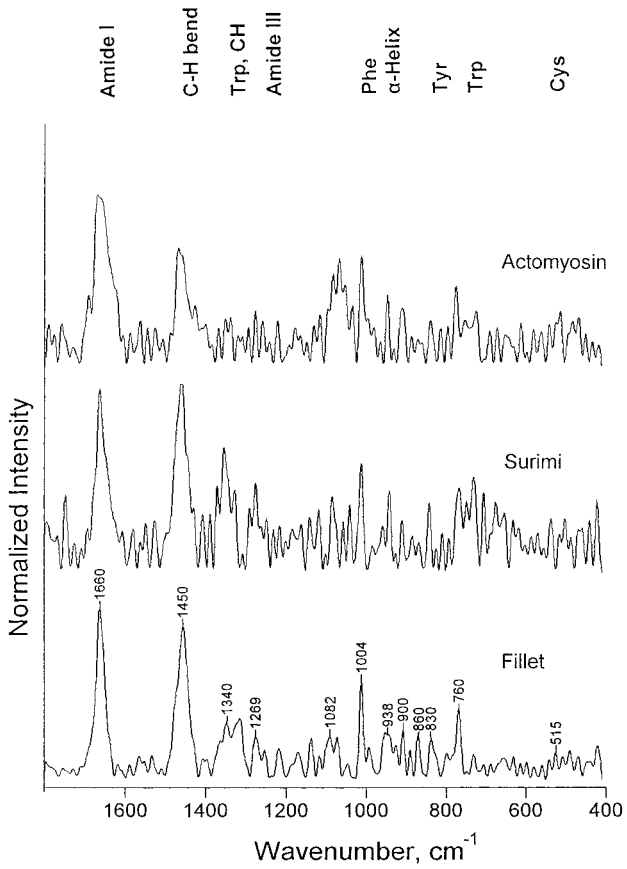

Figure 1. Raman spectra in the 400−1800 cm^{-1} wavenumber region of ling cod natural actomyosin, surimi, and fillet.

Raman Spectral Results for Natural Actomyosin and Surimi. Raman spectra of ling cod NAM and surimi before and after freezing, with and without cryoprotectants, were compared. The tentative assignments of predominant bands were made on the basis of previous studies on cod myosin (*18*).

Figure 1 reveals the Raman spectra in the 400−1800 cm^{-1} wavenumber region of the NAM, surimi, and fillet from ling cod, and Figures 2 and 3 show the spectra of NAM and surimi, respectively, as the unfrozen or frozen samples, and without cryoprotectants or with the commercial blend. Similar spectra were obtained for the other cryoprotectant treatments.

In this study, freezing, as well as treatments of NAM and surimi with cryoprotectants, resulted in changes in the amide III region and in the band near 934−944 cm^{-1} assigned to the α-helical structure, indicating secondary structural changes in the polypeptide backbone. Examination of the amide III region between 1230 and 1320 cm^{-1} for unfrozen NAM or surimi revealed typical α-helical bands at 1269 and 1302 cm^{-1}. After frozen storage or freeze−thaw treatment, these bands were observed at 1276 and 1304 cm^{-1}. Careche and Li-Chan (*18*) reported bands at 1258, 1295, and 1307 cm^{-1} for α-helical structures in cod myosin without freezing. Bouraoui et al. (*21*) reported bands at 1252 and 1304 cm^{-1} in raw surimi from Pacific whiting.

Similarly, the amide I region near 1650−1680 cm^{-1} (*45*) revealed changes for NAM and surimi with and without cryoprotectants. Before freezing, the spectrum

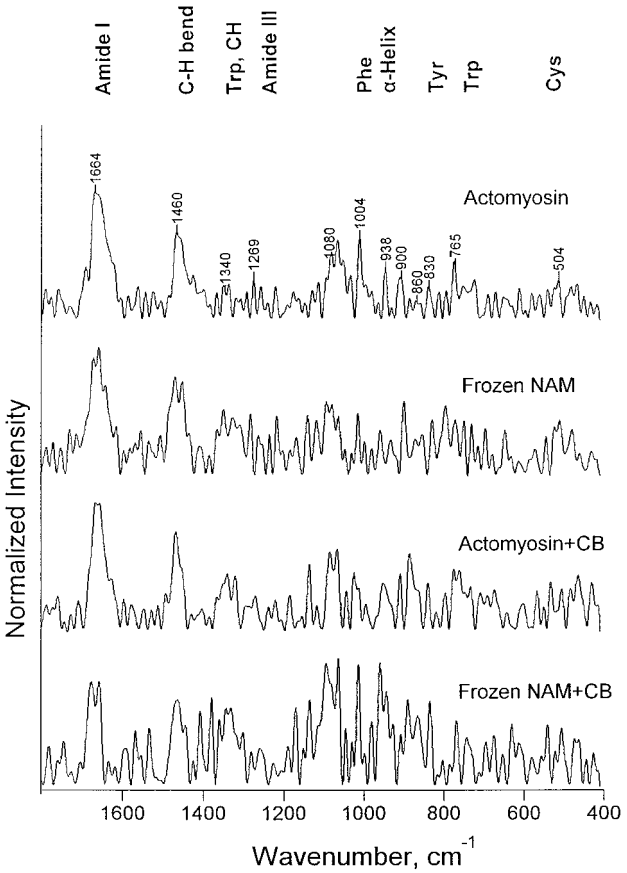

Figure 2. Raman spectra in the 400−1800 cm^{-1} wavenumber region of natural actomyosin (NAM), unfrozen or after frozen storage at −10 °C for 10 days, and in the absence or presence of the commercial blend (CB) of cryoprotectants (4% sucrose and 4% sorbitol).

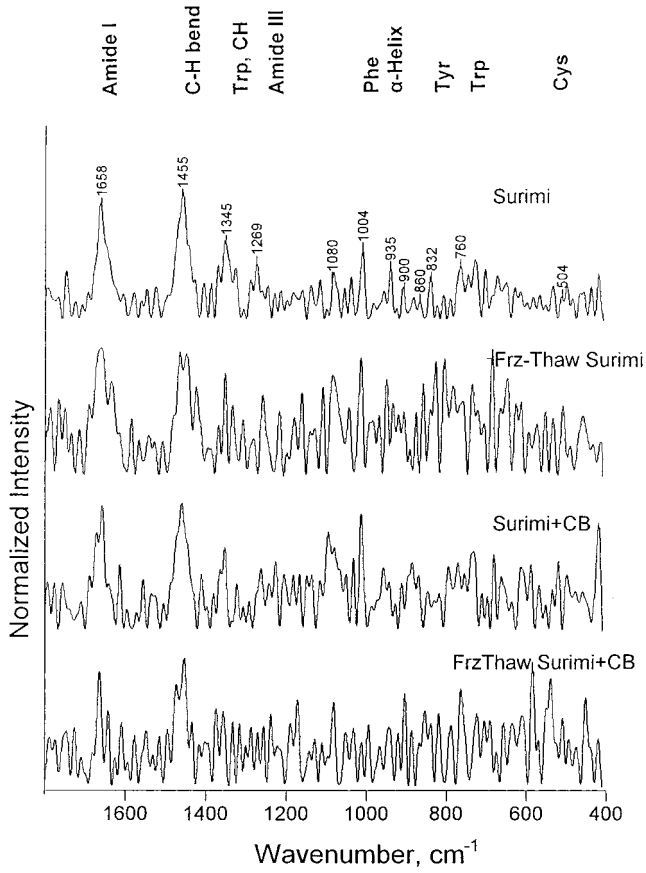

Figure 3. Raman spectra in the 400−1800 cm^{-1} wavenumber region of surimi, unfrozen or after 8 freeze−thaw cycles, and in the absence or presence of the commercial blend (CB) of cryoprotectants (4% sucrose and 4% sorbitol).

of NAM without cryoprotectants had a band at 1664 cm^{-1} in the amide I region; after freezing, this band was observed at 1655 cm^{-1} with a shoulder at 1668 cm^{-1}. In the NAM containing the commercial blend the band was observed at 1655 cm^{-1} with a shoulder at 1662 cm^{-1} before freezing. After freezing, this band was observed at 1654 cm^{-1} with a shoulder at 1673 cm^{-1}.

More detailed analysis of the amide I band was done using the method of Williams (*36*), computed by the RSAP program of Przybycien and Bailey (*35*). Because of the helical nature of myosin, and the relative enrichment of myosin obtained by processing ling cod fillet into surimi and natural actomyosin, higher α-helical contents were observed in the surimi (40%) and NAM (50%) than in the fillet (30%) (Table 6). Using circular dichroism measurements of model actomyosin preparations diluted to a protein concentration of 0.2 mg/mL, Ogawa et al. (*23*) reported α-helical content between 43 and 50% for carp, yellowtail, amberjack, sea bream, big eye tuna, stone flounder, sea bass, and greenling. The 50% content of α-helix observed for ling cod NAM in the present study is in agreement with these literature results.

Freezing resulted in an increase in the percentage of α-helix compared to that observed before freezing, from 50 to 70% for actomyosin frozen without cryoprotectant, and from 40 to 60% for surimi frozen without cryoprotectant (Table 6). Although these increases were not statistically significant at $P < 0.05$, the percent α-helix contents of frozen NAM and surimi with no cryoprotectant were significantly higher ($P < 0.05$) than those of frozen treatments with the commercial blend. It is interesting to note that the percent α helix decreased after freezing for both NAM and surimi treatments with commercial blend, as well as for NAM with 8% optimal blend or 8% lactitol; these were the treatments that showed good gel strength. Ogawa et al. (*22*) reported that gel formation of fish actomyosins requires a slow unfolding of the α-helix, and that the dissociation of α-helix is closely associated with the low-temperature gelling phenomenon (setting) of actomyosin. Setting is an important process for surimi-making because of enhancement of the gel strength of kamaboko.

Careche and Li-Chan (*18*) reported an α-helical content of 95% for an unfrozen myosin preparation concentrated using aquacide, but the helical content dropped to about 70% when the myosin preparation was concentrated by centrifugation. This indicates that as the water was removed the percentage of α-helix increased. In the present study, the NAM and surimi treatments without cryoprotectants lost more water due to freeze denaturation and also showed an increase in α-helical content. A similar trend was observed for treatments with 4% cryoprotectants. From electron microscope studies, Yamamoto (*46*) reported that monomeric myosin from rabbit skeletal muscle formed by the dissociation of filamentous myosin at high ionic strengths (0.5 M KCl, pH 6.0) tended to aggregate by intra- and intermolecular aggregation of myosin heads. At 40 °C, oligomers were observed in which the heads were tightly packed and no individual heads were distinguishable. The helical coiled tails were arranged in a "daisy wheel"

Table 6. Effects of Freezing and Cryoprotectants on Changes in Secondary Structure Content Estimated from the Raman Amide I Band of Ling Cod Natural Actomyosin (Frozen at −10 °C for 10 days) and Surimi (after 8 Freeze−Thaw Cycles)

	total α-helix (%)		total β-sheet (%)		total random coil (%)	
sample[a]	unfrozen	frozen	unfrozen	frozen	unfrozen	frozen
actomyosin						
no cryoprotectant	50^{ab}	70^{b}	15^{ab}	20^{ab}	35^{ab}	10^{a}
commercial blend	50^{ab}	35^{a}	30^{bc}	30^{bc}	20^{ab}	35^{ab}
8% sucrose	30^{a}	35^{a}	20^{ab}	20^{ab}	50^{b}	45^{b}
4% sucrose	n.d.[b]	75^{b}	n.d.	15^{a}	n.d.	10^{a}
8% sorbitol	30^{a}	n.d.	20^{ab}	n.d.	50^{b}	n.d.
4% sorbitol	n.d.	50^{ab}	n.d.	15^{a}	n.d.	35^{ab}
8% lactitol	70^{b}	50^{ab}	10^{a}	30^{bc}	20^{ab}	20^{ab}
4% lactitol	n.d.	60^{b}	n.d.	10^{a}	n.d.	30^{ab}
8% optimal blend	70^{b}	50^{ab}	10^{a}	30^{bc}	20^{ab}	20^{ab}
4% optimal blend	n.d.	60^{b}	n.d.	20^{ab}	n.d.	20^{ab}
surimi						
no cryoprotectant	40^{ab}	60^{b}	30^{bc}	20^{ab}	30^{ab}	20^{ab}
commercial blend	40^{ab}	20^{a}	10^{a}	40^{c}	50^{b}	40^{ab}
fillet	30^{a}	n.d.	20^{ab}	n.d.	50^{b}	n.d.

[a] The secondary structure content was estimated by the Raman spectral analysis package using spectral data averaged over 6 scans. Duplicate data sets were measured for each frozen sample, except for the surimi with commercial blend, fillet, and actomyosin samples with 8% sorbitol, 8% optimal blend, and 4% optimal blend. Secondary structure values (% helix, sheet, or random coil) bearing different superscript letters are significantly different ($P < 0.05$) [b] n.d. = not determined because of low S/N ratio.

configuration. Ramirez et al. (*17*) also suggested head-to-head interactions with a strong participation of disulfide bonds during frozen-storage-induced aggregation of myosin in 0.6 M KCl solution. The apparent increase in α-helical content after freezing of NAM or surimi without cryoprotectants, observed in the present study by Raman analysis, could be due to the aggregation of myosin heads due to changes in ionic strength caused by dehydration as a result of formation of ice crystals during frozen storage.

For NAM without cryoprotectants and before freezing, a band was observed at 1460 cm^{-1} for the CH_2 bending region and after freezing at 1446 cm^{-1} with a shoulder at 1464 cm^{-1}. For NAM with the commercial blend, the band was at 1462 cm^{-1} before freezing and at 1458 cm^{-1} after freezing. Careche and Li-Chan (*18*) reported that changes in aliphatic residues, particularly after frozen storage in the presence of formaldehyde were shown by both the CH stretching (broad band near 2940 cm^{-1}) and CH bending (band near 1450 cm^{-1}) vibrations.

The intensity ratio of the tyrosine doublet at 860 and 830 cm^{-1} for NAM and surimi varied between 0.3 and 1.0 for the various frozen and unfrozen treatments (data not shown). According to Tu (*45*), if this ratio is between 0.7 and 1.0, the tyrosine residues may be considered as buried. If the ratio is as low as 0.3, strong H-bonding to a negative acceptor is indicated. The ratio of the tyrosine doublet for all the treatments studied, whether with or without cryoprotectants, and before or after freezing, suggested tyrosine residues in a buried environment. This observation is in agreement with the low surface hydrophobicity values measured by the fluorescence probe method in this study (Table 5). A significant increase in the tyrosine doublet ratio from 0.4 to 0.8 was observed after freeze−thaw treatment of surimi in the absence of cryoprotectants, suggesting changes in the microenvironment of tyrosine residues.

The disulfide bond stretching region as described by Tu (*45*) can be at 510, 525, and 540 cm^{-1}. The observed wavenumbers ranged from 504 to 523 cm^{-1} for ling cod NAM and surimi with various treatments. The treatments without cryoprotectants revealed an increase in intensity of the S−S stretching band after freezing:

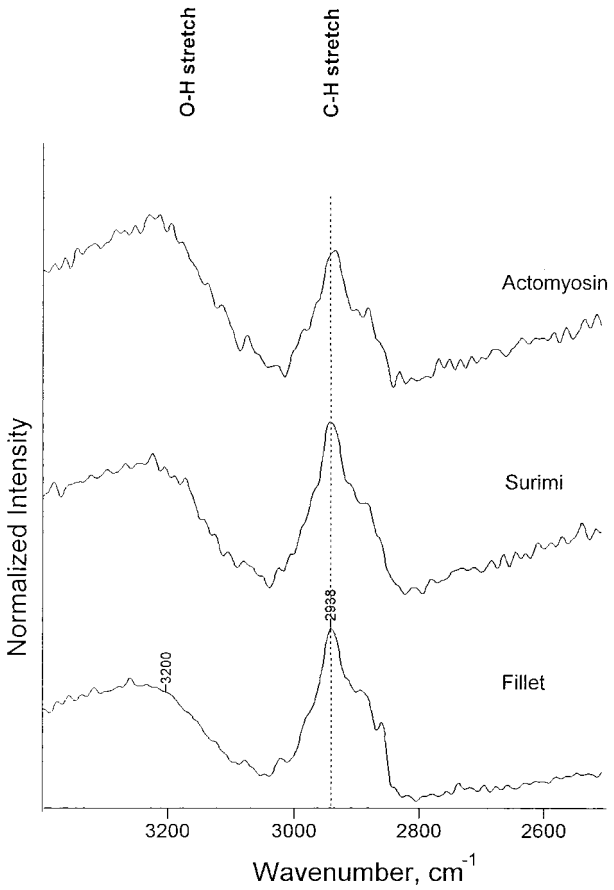

Figure 4. Raman spectra in the 2500−3400 cm^{-1} wavenumber region of ling cod natural actomyosin, surimi, and fillet.

from 0.50 to 0.99 for NAM and from 0.36 to 0.54 for surimi. These results are consistent with the increase in SS content determined by chemical analysis using Ellman's reagent and NTSB (Table 4). This increase was not observed for treatments with cryoprotectants.

Figure 4 shows the CH stretching region (2500−3400 cm^{-1}) of the Raman spectra for NAM, surimi, and fillet, and Figures 5 and 6 are the corresponding spectra for unfrozen and frozen NAM and surimi with and without

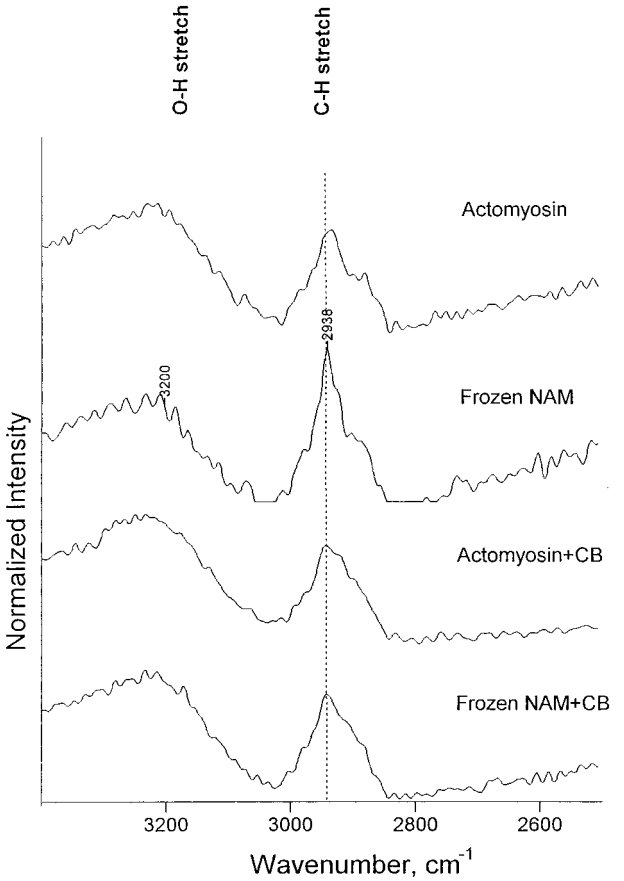

Figure 5. Raman spectra in the 2500−3400 cm^{-1} wavenumber region of natural actomyosin (NAM), unfrozen or after frozen storage at −10 °C for 10 days, and in the absence or presence of the commercial blend (CB) of cryoprotectants (4% sucrose and 4% sorbitol).

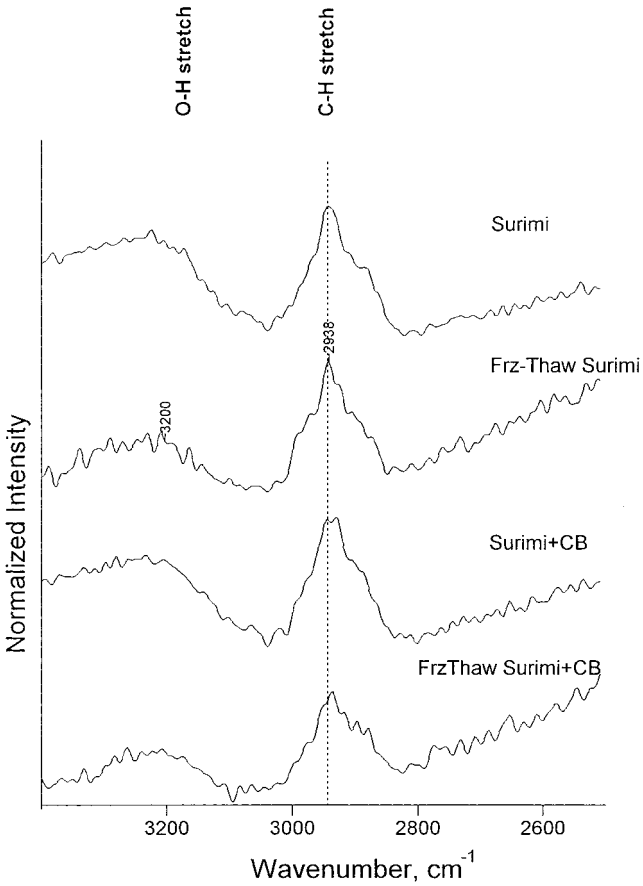

Figure 6. Raman spectra in the 2500−3400 cm^{-1} wavenumber region of surimi, unfrozen or after 8 freeze−thaw cycles, and in the absence or presence of the commercial blend (CB) of cryoprotectants (4% sucrose and 4% sorbitol).

cryoprotectants. A shift in the location of the peak intensity from 2931 to 2938 cm^{-1} was observed when comparing the spectra of NAM to that of surimi or fillet (Figure 4), or unfrozen to frozen NAM (Figure 5). A similar shift to a higher wavenumber was observed by Careche and Li-Chan (*18*) from 2933 cm^{-1} for unfrozen cod myosin to 2933 with a shoulder at 2945 cm^{-1} after freezing. The maximum peak intensity was observed near 2938 cm^{-1} for both unfrozen and frozen NAM samples containing the commercial blend (Figure 5) and for the surimi samples (Figure 6). For treatments with the other blends of cryoprotectants used in the present study, the shifts occurred to both higher and lower wavenumbers. Published data are scarce for the CH stretching band in protein spectra (*47*). Arteaga (*48*) reported a slight shift to higher wavenumbers for the CH band at 2940 cm^{-1} when urea was added to aqueous buffered solutions. Those results suggest partial unfolding and increased exposure of the aliphatic residues to an aqueous environment. Arteaga (*48*) also suggested that the height-to-width ratio of the CH stretching band may be related to protein hydrophobicity and functional properties such as emulsification. Interestingly, the presence of the commercial blend of cryoprotectants in both surimi and NAM prevented the narrowing of the broad CH stretching band that was observed upon freezing in the absence of cryoprotectants.

The OH stretch at 3200 cm^{-1} indicated decreases in intensity for the frozen natural actomyosin and surimi without cryoprotectant in comparison to the unfrozen samples. Decreases were also observed in frozen surimi with commercial blend and frozen NAM with 4% Litesse. Such decreases may be related to dehydration as a result of formation of ice crystals during frozen storage. Careche et al. (*49*) reported a decrease in the intensity ratio of νO-H/νC-H bands, which was correlated with harshness of the condition of freezing or frozen storage of fillets of hake (*Merluccius merluccius L.*). Changes in the O−H stretching band of the FT-Raman spectra of the frozen fillets were consistent with different water losses expected due to ice formation and crystal growth pattern under the freezing conditions studied, and the ratio was suggested to be of use for determining quality loss due to freezing and frozen storage. As shown in Figure 5, the presence of the cryoprotectant blend in frozen NAM prevented the decrease of the OH stretching band intensity.

CONCLUSIONS

A reduction in gelling capacity was observed in ling cod natural actomyosin (NAM) after frozen storage and surimi after freeze−thaw cycling in the absence of cryoprotectants. Addition of individual cryoprotectants at the 8% level, optimal blends at 4, 5.5, 6, and 8%, or a commercial blend consisting of 4% sucrose and 4% sorbitol, effectively prevented the frozen-storage-induced loss of gelling capacity in both NAM and surimi.

Both the colorimetric reaction with Ellman's reagent and Raman spectral analysis indicated that SS bonds were formed during frozen storage of NAM in the absence of cryoprotectants. Raman spectral analysis indicated that the tyrosine residues were in a buried

environment before and after freezing for all treatments. The decrease in reactive SH groups and in surface hydrophobicity in NAM and surimi after freezing or freeze−thaw abuse suggest that aggregation of NAM caused hydrophobic and SH sites to be buried during freeze denaturation. The Raman spectral data showed an increase in the percent α-helical content in NAM and surimi after frozen storage in the absence of cryoprotectants or with only 4% individual cryoprotectants. The OH stretching region also decreased in intensity in both surimi and NAM without cryoprotectants, in surimi with the commercial blend, and in NAM with 4% Litesse. A possible explanation is the formation of ice crystals which resulted in dehydration of the protein and aggregation of the myosin heads.

Significant differences in gel strength were observed between the control sample (no cryoprotectants) and samples treated with various cryoprotectants even before the NAM and surimi samples were frozen. Differences were also observed in the structural properties of NAM as indicated by measurements of SH and SS contents and surface hydrophobicity. Furthermore, the band shifts for the amide I, amide III, CH_2 bending vibrations, and CH stretching regions of the Raman spectra also indicated slight differences between the control and various cryoprotectant treatments even when the NAM and surimi samples were unfrozen. These results suggest that secondary and other structural changes induced by addition of the cryoprotectants to the NAM and surimi *before* freezing may play an important role in stabilization of the fish proteins against the deteriorative changes that are usually observed during frozen storage in the absence of cryoprotection.

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