Structural Changes of Hake (*Merluccius merluccius* L.) Fillets: Effects of Freezing and Frozen Storage

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Structural changes in hake (*Merluccius merluccius* L.) fillets as affected by freezing method and frozen storage temperature have been studied through Raman spectroscopy and related to changes in texture and functionality. Changes in protein secondary structure were observed due to storage temperature, accompanied by changes in apparent viscosity and shear resistance. Samples at −10 °C showed greater structural alteration than at −30 °C in terms of increase of β-sheets at the expense of α-helices. An increase of unordered protein structure was found only in samples stored at −10 °C. Exposure of buried tryptophan residues was observed at both storage temperatures. The decrease of the δCH_2 band upon storage suggested an increase of hydrophobic interactions of aliphatic residues. Except for liquid air frozen fillets, all samples showed a decrease of the $\nu O-H/\nu C-H$ band ratio compared to the fresh ones, this decrease being higher the harsher the conditions.

Keywords: *Freezing; frozen storage; Raman spectroscopy; hake; protein structure; functionality; texture*

INTRODUCTION

Freezing and frozen storage is an excellent method of preserving the organoleptic attributes and protein functionality of fish flesh during prolonged periods of time. Depending on intrinsic factors such as species and season and technological factors such as handling practices previous to freezing, freezing rate, temperature of storage, or presence of protective barriers against oxidation, the practical storage life of frozen fish may vary substantially. Therefore, the quality of fish found on sale is not always good, due to reasons ranging from unsuitable raw material to bad handling practices or storage conditions. This is also a problem for processing industries who have to purchase fish stocks of irregular quality, which may deteriorate at different rates during processing and retail sale. Although good handling and storage practices are broadly known, sometimes, due to technological or economical factors, they cannot be completely followed. For lean species of high commercial value such as hake, the end of practical storage life is reflected as a fibrous, dry product which becomes tough and which has lost important functional properties such as protein extractability in salt solutions or viscosity. Understanding of the underlying mechanisms involved in the deterioration of fish flesh and the interactions among them would lead one to find parameters to establish fish quality and also help predict practical storage life for each stock, with subsequent economic advantage for the fisheries sector and consumers.

Myofibrillar proteins, which are the main contributors imparting textural attributes and functional properties to muscle foods, are the ones most affected during cold storage. It is accepted that they suffer denaturation and/or aggregation due to factors such as partial dehydration due to freezing of water and the associated concentration of solutes in the tissue, formation of formaldehyde in certain species such as hake, or interactions of proteins with lipids or their oxidation products (Sikorski et al., 1976; Matsumoto, 1979, 1980; Shenouda, 1980; Haard, 1990; Sikorski and Kolakowska, 1994), but the way these factors lead to structural changes of proteins causing denaturation and aggregation remains to be established. Work has been done on the type of interactions responsible for aggregation of myofibrillar proteins, and attention has been drawn to the importance of hydrophobic interactions and disulfide bridges (Connell, 1975; Dingle et al., 1977; Gill et al., 1979; Laird et al., 1980; Lim and Haard, 1984; Rehbein and Karl, 1985; Owusu-Ansah and Hultin, 1986; Tejada et al., 1996; Careche et al., 1998). Muscle proteins become gradually inextractable in salt solutions and, depending on species, conditions, and time of storage, these proteins will be extracted to more or less extent in sodium dodecyl sulfate (SDS) or SDS plus mercaptoethanol, and eventually, a residue nonextractable in these solutions can be obtained (Tejada et al., 1996; Careche et al., 1998). This unextracted residue has a structure resembling the sarcomere, which tends to become more pronounced the longer the storage time (Tejada et al., 1996). Proteins most involved in the aggregation are myosin and actin, the relative proportion of them being dependent on species and storage conditions (Tejada et al., 1996; Careche et al., 1998; Del Mazo et al., 1999).

Although it is accepted that protein denaturation of myofibrillar proteins occurs during frozen storage, little direct evidence on the structural changes occurring in muscle proteins during freezing and frozen storage is found other than loss of protein function or functionality. It has been proposed that Raman spectroscopy can be a useful tool to probe protein structure in solid and liquid food systems (Li-Chan et al., 1994). Circular

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dichroism, on the other hand, is, in principle, another powerful physical method for the study of protein structure (Chen et al., 1974; Compton and Johnson, 1986), but this technique is not suitable for direct spectroscopic measurements of samples that are in the solid state. Fluorescence spectroscopy is another analytical tool for studying proteins, but this method is incapable of determining peptide backbone structure and is also limited to the study of the environments of tyrosine and tryptophan side chains (Mantulin and Pownall, 1986; Szöllösi et al., 1987). By contrast, Raman spectroscopy is a more suitable and direct technique that overcomes most of the above objections and can be used for solid samples and aqueous solutions, providing information on the peptide backbone structure, the environment of some side chains such as those of tyrosine and tryptophan, and the local conformations of disulfide bonds and methionine residues (Frushour and Koenig, 1975; Tu, 1982). In isolated myosin from cod, Raman spectroscopy has shown that structural changes occur upon formaldehyde addition and frozen storage, involving loss of α-helical content (Careche and Li-Chan, 1997). Changes in vibrational modes assigned to aliphatic residues suggested involvement of hydrophobic interactions after formaldehyde addition or frozen storage.

The primary aim of the present work was the study by Raman spectroscopy of the structural changes in hake muscle proteins as affected by freezing and frozen storage temperature and their relation with functionality and texture.

MATERIALS AND METHODS

Fish Source. Different lots of hake (*Merluccius merluccius* L.) were obtained between June 1997 and June 1998. The average length and weight of the fish were 43 ± 4 cm and 800 ± 173 g, respectively. They were transported in ice to the Instituto del Frío. The fish in post rigor condition were headed, gutted, filleted, and washed with iced water to remove blood, etc. One lot was frozen in a blast freezer (Frigoscandia, Aga Frigoscandia, Freezer Division, Helsingborg, Sweden), until the thermal center reached −40 °C (within 1.30 h), and the resulting frozen fillets were vacuum packed in Cryobac BB-1 bags and stored at −10 and −30 °C for 10 months until the analyses were performed. These were referred to as HTS (high-temperature storage) and LTS (low-temperature storage), respectively. In a second lot, half of the fish was analyzed fresh and the other half was frozen and vacuum packed in the above conditions and stored at −80 °C until analysis (within 1 week). These samples were referred to as FR (fresh) and BF (blast frozen samples). A third lot was also divided into two: half of it was analyzed fresh (FR), and the other half was frozen in liquid air (−180 °C) and named LA (liquid air frozen samples). A fourth lot was purchased and analyzed fresh only (FR). For analysis, frozen fish were thawed in a chill room at 4 °C overnight.

Apparent Viscosity. This was determined in a homogenate of thawed muscle in 50 mM phosphate buffer, pH 7.0, containing 5% NaCl, according to the method of Borderías et al. (1985), as modified by Barroso et al. (1998). Measurements were made at 12 rpm with an RV3 spindle, using a Brookfield model DV-III rotary viscometer (Stoughton, MA) and the Rheocalc V. 1.2 software system. Measurements were carried out at least in triplicate, and results were expressed in centipoises (cP).

Shear Resistance. Frozen samples were previously thawed at 4 °C, and fillets were cut in 1 × 1 × 3 cm portions. Determinations were performed on the raw portions using an Instron model 4501 Universal Testing Machine (Instron Corp., Canton, MA) and Instron Series IX software (Automated Materials Testing System V. 5) fitted with a Kramer shear cell developed by Kramer et al. (1951) with a head that exerted a maximum force of 5 kN at a speed of 100 mm/min, according to the method of Borderías et al. (1983) as modified by Barroso et al. (1998). A minimum of six replicates was performed per assay.

Raman Spectroscopy. Small muscle portions from different parts of a fillet (previous manual removal of connective tissue with tweezers) were taken and gently transferred with a rod into a glass tube (5 cm height and 5 mm i.d., Wilmad Glass Co., Inc., Buena, NJ) to fill ∼1 cm length. The tube contained ∼30 μL of water in the bottom to prevent desiccation. Filling of the tube was carried out making sure the sample did not touch the water. The sample-containing tube, previously sealed, was placed into a thermostated device at the 15−20 °C temperature range for Raman analysis.

FT-Raman spectra were recorded on a Bruker RFS 100/S FT-spectrometer. An Nd:YAG laser, which emits at a wavelength of 1064 nm, was used as the excitation source. The scattered radiation was collected at 180° to the source, and typical spectra were recorded at 4 cm^{-1} resolution with 2000 scans with 300 mW of laser power at the sample. Frequency-dependent scattering that occurs in the Raman spectra recorded with this spectrometer was corrected by multiplying point by point with $(\nu_{laser}/\nu)^4$. Frequencies cited are accurate to ±0.3 cm^{-1}. To get consistent, reproducible results, Raman analyses with different pieces of muscle from the same fish were performed. For that, three samples were taken in three different tubes, and the above 2000 scans were recorded for each of the samples of the same fish, thus producing 6000 scans in total but randomizing possible differences in composition. Additionally, this ensured samples were not denatured during the Raman measurements. Signals obtained were fed to a personal computer for storage, display, plotting, and processing, and the manipulation and evaluation of the spectra were carried out using OPUS 2.2 (Bruker, Karlsruhe, Germany) and SpectraCalc (Galactic Industries Corp., Salem, NH) software.

Assignment of the visible bands to vibrational modes of peptide backbone or amino acid side chains was carried out through comparison to Raman spectra of model polypeptides or monographs of Raman spectra of proteins (Frushour and Koenig, 1975; Tu, 1982; Krimm and Bandekar, 1986). Protein secondary structures were determined as percentages of α, β, turn, and random coil conformations (Alix et al., 1988). With this aim, the water spectrum was previously subtracted from the spectra by following the same criteria as those described in other literature works (Williams and Dunker, 1981; Williams, 1983; Alix et al., 1988). The Phe ν-ring band located near 1003 cm^{-1} was used as internal standard for normalization of the spectra, as it has been reported to be insensitive to the microenvironment (Lippert et al., 1981; Harada et al., 1982).

Statistical Analyses. For apparent viscosity and shear resistance, one-factor analyses of variance (ANOVA) were performed to detect differences among conditions: fresh (FR), liquid air freezing (LA), blast freezing (BF), and low- and high-temperature storage (LTS and HTS, respectively). The Bonferroni test was used for paired comparison. For secondary structure comparisons and other characteristic Raman peak intensity bands, ANOVAs were performed to see the main effects: fresh (FR), freezing (groupings LA and BF), and frozen storage (groupings LTS and HTS). When needed, for finding minimun differences, *t* tests were performed between pairs of conditions, and in the cases of lack of replicates nonparametric tests were also run. The software used was SPSS (SPSS Inc., Chicago, IL).

RESULTS

Apparent Viscosity and Texture. Table 1 shows the apparent viscosity and Kramer shear resistance values for hake muscle. There were no significant changes with effect of freezing, but during frozen storage for 10 months this functional property decreased very abruptly for the samples stored at the highest temper-

Table 1. Apparent Viscosity (η_{app}) and Shear Resistance Values (Kramer) in Hake Muscle (Mean ± SEM)[a]

conditions	η_{app}	Kramer
FR	$8750^{ab} \pm 1313$ (8)	$8.8^{ab} \pm 1.70$ (12)
LA	$8793^{ab} \pm 53$ (3)	$6.6^{b} \pm 0.83$ (6)
BF	$10140^{b} \pm 978$ (8)	$11.4^{a} \pm 2.08$ (13)
LTS	$7880^{a} \pm 149$ (3)	$12.7^{a} \pm 1.16$ (6)
HTS	$1406^{c} \pm 133$ (3)	$30.8^{c} \pm 7.42$ (6)

[a] FR, fresh muscle; LA, BF, liquid air and blast frozen samples; LTS, HTS, low- and high-temperature frozen storage. Different letters in the same column indicate significant differences ($P < 0.05$). Numbers in parentheses are the numbers of samples.

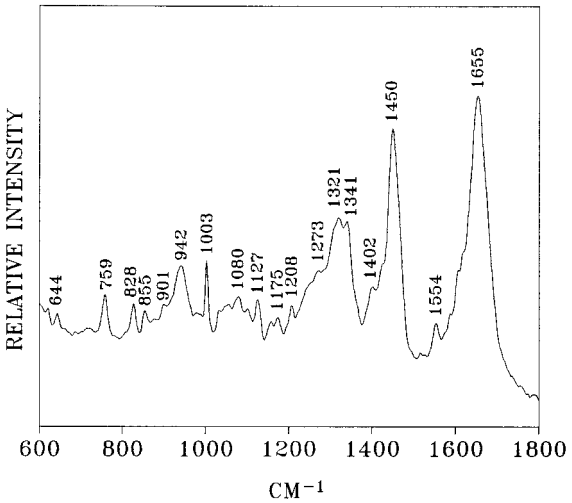

Figure 1. Raman spectrum of a sample of fresh muscle from hake.

ature. Apparent viscosity of samples stored at −30 °C also decreased significantly when they were compared only with the fresh samples belonging to the same lot (results not shown). Variability found in the initial values for this functional property (due to fish to fish variation, seasonal variations, etc.) leads to the fact that the comparison of an average of values from different individuals may mask changes due to frozen storage as those found at −30 °C in this work. There were no differences in the protein contents of the homogenate measured in apparent viscosity for both temperatures (37.9 ± 1.8 and 34.8 ± 0.3 mg/g at −10 and −30 °C, respectively), and therefore other events must occur that lead to such a big loss of this functional property at −10 °C.

Kramer shear resistance values (Table 1) showed a significant increase only for samples stored for 10 months at −10 °C, these values being ∼3 times higher than those found with the other conditions.

Secondary Structure. Figure 1 shows the Raman spectrum of a fresh hake fillet in the 1800−600 cm^{-1} range. The most prominent band, centered near 1655 cm^{-1} (Table 2), has been assigned unambiguously to the amide I vibrational mode (Krimm and Bandekar, 1986), which involves mainly C═O stretching and, to lesser degrees, C−N stretching, C_α−C−N bending, and N−H in-plane bending of peptide groups. Most of the work on protein Raman and infrared spectroscopy points out that there is a correlation between the frequencies of the amide I band and the amount of the types of protein backbone conformation (Tu, 1982; Alix et al., 1988). This is because the nature of the amide I band makes it sensitive to changes in the hydrogen bonding scheme involving the peptide linkages. Thus, generally speaking, the amide I band consists of overlapped band components falling in the 1658−1650, 1680−1665, and 1665−1660 cm^{-1} ranges, which are attributable to α-helices, β-sheets, and random coil structures, respectively (Frushour and Koenig, 1975; Tu, 1982).

Table 2. Assignment of Some Raman Bands of Hake Muscle[a]

freq, cm^{-1}	assignment	freq, cm^{-1}	assignment
1655 vs	amide I, H_2O	1159 vw	
1618 sh	Trp, Phe, Tyr ν-ring	1127 w	νCN (protein backbone)
1606 sh	Trp, Phe, Tyr ν-ring	1102 vw	νCN, νCC (protein backbone)
1587 sh	Trp ν-ring	1080 w	νCN, νCC (protein backbone)
1554 w	Trp ν-ring	1057 w	
1450 s	$\delta_{as}CH_3$, δCH_2, δCH	1032 w	Phe δ-ring
1425 sh	Asp, Glu, Lys	1003 m	Phe ν-ring
1402 w	$\nu_s COO^-$ (Asp, Glu)	942 m	νCC (α-helix)
1341 m	δCH	901 vw	νCC
1321 m	δCH	855 w	Tyr ν-ring
1309 sh	amide III (α-helix)	828 w	Tyr ν-ring
1273 m	amide III (α-helix)	759 m	Trp
1250 sh	amide III (β-sheets, random coil)	644 w	Tyr
1208 w	Tyr, Phe	622 w	Phe
1175 w	Tyr		

[a] Abbreviations: vs, very strong; s, strong; m, medium; w, weak; vw very weak; sh, shoulder; ν, stretching; δ, bending.

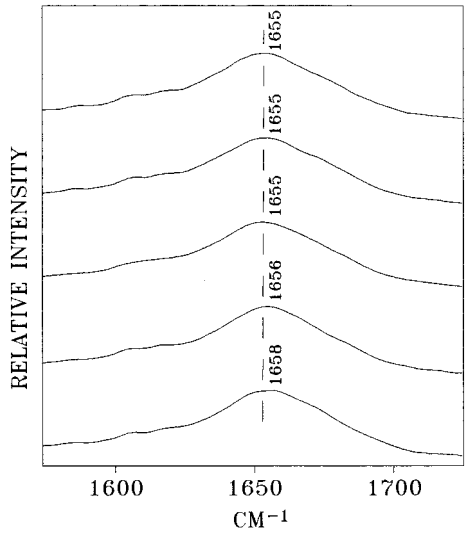

Figure 2. Raman spectra in the amide I region of hake muscle: (from top to bottom) fresh samples, blast frozen samples, liquid air frozen samples, low-temperature frozen stored samples, and high-temperature frozen stored samples.

Comparison of the amide I region of hake samples reveals slight shifting of the band maximum toward higher frequencies in going from fresh to frozen stored hake (Figure 2), with more marked changes after storage at −10 °C. On the basis of the above structurally characteristic frequency ranges, a reasonable explanation of this spectral change may be a decrease in α-helical structure. This is supported, on the one hand, by the intensity decrease of the νC−C band generated by α-helices, as described later and, on the other hand, by quantitative estimates of protein secondary structures included in Table 3 and the statistical analyses in Tables 4 and 5. The amide I spectral profile can provide quantitative information on protein backbone secondary structure. However, because hake muscle contains various proteins, the data of Table 3 are given

Table 3. Protein Secondary Structures (Percent) in Hake Muscle As Determined from Raman Amide I Spectra (Mean ± SEM)[a]

conditions	α-helices	β-sheets	turns	unordered
FR	63.1 ± 1.3 (5)	10.5 ± 1.3	15.5 ± 0.8	10.9 ± 0.4
LA	64.0 (1)	9.2	15.8	11.0
BF	61.9 ± 2.1 (3)	10.7 ± 1.5	16.4 ± 0.5	11.0 ± 0.2
LTS	58.7 ± 0.3 (2)	14.0 ± 0.0	16.2 ± 0.0	11.1 ± 0.3
HTS	43.8 ± 1.3 (2)	18.4 ± 1.63	16.8 ± 0.3	21.0 ± 0.0

[a] Abbreviations as in Table 1.

Table 4. One-Way Analysis of Variance of the Percentages of Protein Secondary Structure As Affected by Freezing and Frozen Storage[a]

conditions	α-helices	β-sheets	turns	unordered
fresh (FR)	a (5)	a	a	a
frozen (BF + LA)	a (4)	a	a	a
frozen stored (LTS + HTS)	b (4)	b	a	a

[a] Different letters in the same column indicate significant differences ($P < 0.05$). Abbreviations as in Table 1.

Table 5. *t* Tests for Pairs of Conditions of the Percentages of Protein Secondary Structure[a]

pairs of conditions	α-helices	β-sheets	turns	unordered
FR/BF	NS	NS	NS	NS
FR/LTS	*	*	NS	NS
FR/HTS	**	*	NS	***
BF/LTS	NS	NS	NS	NS
BF/HTS	**	*	NS	***
HTS/LTS	*	NS	NS	*

[a] NS, not significant; ***, $P \leq 0.05$; **, $P \leq 0.01$; *, $P \leq 0.001$. Abbreviations as in Table 1.

as secondary structure average percentages. The amide I spectral profiles of hake muscle frozen either with liquid air or with a blast freezer were not significantly different from those of fresh hake (Table 4). When pairs of conditions were analyzed (Table 5), the most significant differences were found for frozen storage at −10 °C and to lesser extent for frozen storage at −30 °C. In addition, Table 3 shows also that a decrease in α-helices was accompanied by an increase in β-sheets, the turn percentages remaining practically with the same values. Spectral changes resulting from frozen storage at −10 °C also involved an apparent increase in random coil structure.

The amide II vibration, expected in the 1560−1510 cm^{-1} range, involves mainly N−H in-plane bending and C−N stretching of the trans peptide group (Krimm and Bandekar, 1986). Because of the small change in polarizability associated with amide II, a distinct Raman amide II band usually cannot be detected in proteins. This vibrational mode, however, appears with medium-strong intensity in the infrared spectrum. Infrared measurements (results not shown) for hake films prepared from muscle stored at −30 °C revealed that the amide II band shifted to lower frequencies upon frozen storage, which can also be expected in terms of a decrease in α-helical structure (Zundel et al., 1984).

The use of the amide III band to answer the question of whether the above-mentioned decrease in α-helical content is due to an increase in the amount of β-structure or random coiled polypeptide arrangement is complicated and requires a very careful analysis of the amide III band components, which is still underway using proteins and polypeptides as models. This is because the β-structure and random coil bands overlap so that the intensity in the amide III region between

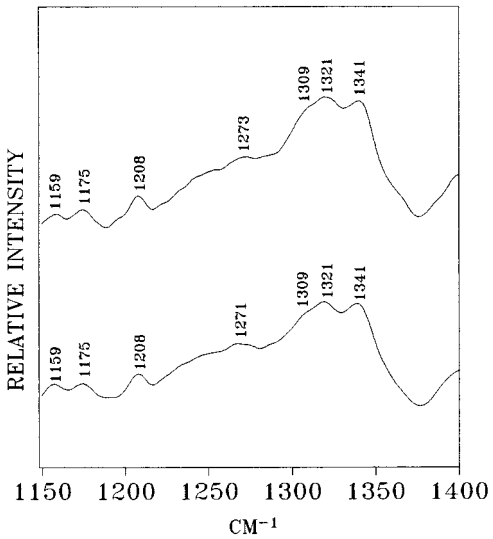

Figure 3. Raman spectra in the amide III region of hake muscle: fresh (top) and high-temperature frozen stored samples (bottom).

1250 and 1240 cm^{-1} is due to contributions from both random coil and β-structure (Yu et al., 1973; Lippert et al., 1976; Krimm and Bandekar, 1986). The amide III mode involves N−H in-plane bending and C−N stretching as well as contributions from C_α−C stretching and C═O in-plane bending. It is assigned here to the 1309 and 1273 cm^{-1} bands by comparison with the Raman spectrum of myosin (Careche and Li-Chan, 1997), which is a major protein in hake muscle. The band at 1309 cm^{-1} is present in hake muscle spectra as a shoulder (Figure 3) and is produced by the myosin tail region, but due to overlapping, this band appears as a not very distinct band to be easily used for protein structural analysis. One can observe, nevertheless, that the 1309 cm^{-1} shoulder is less pronounced for hake frozen stored at −10 °C when compared with fresh hake specimens. Although the remaining amide III region includes several strongly overlapped band components, a relative intensity increase in the 1240−1225 cm^{-1} interval for the frozen stored specimens is seen when compared with the fresh ones. This is due to the relative increase of β-sheets, which is in agreement with the quantitative results obtained from the amide I band region.

Another way of looking at these Raman data is the use of the C−C stretching vibrations near 940 and 900 cm^{-1}, which are characteristic of α-helices and β-sheets, respectively. These bands are known from work on model polypeptides and proteins to be sensitive to the conformation of the polypeptide backbone. Generally, gradual loss of these structures leads to broadening and weakening in intensity (Frushour and Koenig, 1975; Barret et al., 1978; Tu, 1982). In going from α- to β-structures of hake proteins there is a decrease in the 940 cm^{-1} band, and a new α-helical band appears at higher frequencies. In addition, the band near 900 cm^{-1} broadens due to β-sheet formation. According to the spectral profiles in this region, one can observe significant differences between fresh and frozen stored samples in the sense that the fresh ones contained more α-helical structure and fewer β-sheets (Figure 4). However, these structural differences were not apparent between fresh and frozen, nonstored samples.

Other Features. Exposure of buried tryptophan residues in proteins of hake fillets, particularly in hake frozen stored at −10 and −30 °C, is observed by the

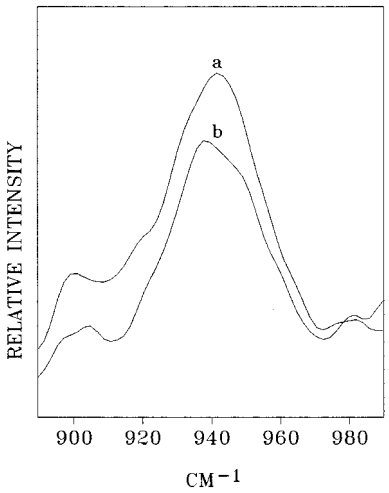

Figure 4. C−C stretching bands of hake muscle: fresh (a) and frozen stored at high temperature (b).

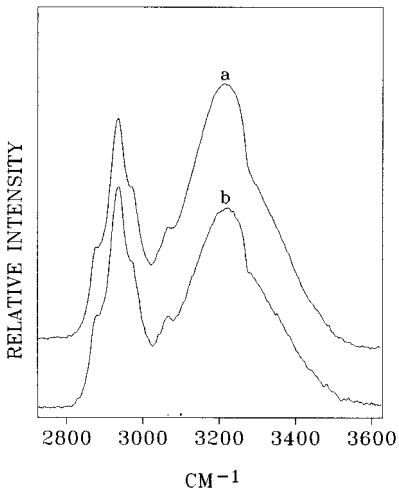

Figure 5. Raman spectra in the C−H and O−H regions of hake muscle: fresh (a) and frozen stored at high temperature (b).

Table 6. Changes in the Normalized Intensities of the 1450 cm^{-1} ($\delta_{as}CH_3$, δCH_2, δCH) Band, the 759 cm^{-1} (Tryptophan) Band, and the Tyrosyl Doublet at 855/828 cm^{-1} (Mean ± SEM)[a]

conditions	$\delta_{as}CH_3$, δCH_2, δCH band	Trp band	Tyr doublet $I_{855\ cm^{-1}}/I_{828cm^{-1}}$
FR	8.3 ± 0.1 (5)	4.5 ± 0.3	0.62 ± 0.05
LA	8.3 (1)	4.8	0.68
BF	7.7 ± 0.1 (3)	3.8 ± 0.6	0.64 ± 0.06
LTS	7.4 ± 0.0 (2)	3.4 ± 0.0	0.72 ± 0.06
HTS	7.9 ± 0.0 (2)	2.9 ± 0.3	0.62 ± 0.01

[a] Abbreviations as in Table 1.

Table 7. One-Way Analysis of Variance of the Normalized Bands from Table 6[a]

conditions	1450 cm^{-1} band	Trp band	Tyr $I_{855\ cm^{-1}}/I_{828cm^{-1}}$
fresh (FR)	a (5)	a	a
frozen (BF + LA)	b (4)	a	a
frozen stored (LTS + HTS)	b (4)	b	a

[a] Different letters in the same column indicate significant differences ($P < 0.05$). Abbreviations as in Table 1.

decrease of peak intensity at 759 cm^{-1} (Tables 6 and 7). The exposed tryptophan may play a role in the protein−protein interactions accompanying the detrimental changes generated upon frozen storage in the above conditions. On the other hand, the ratio of the tyrosyl doublet at 855 and 828 cm^{-1} is known as a good indicator of the hydrogen bonding of the phenolic hydroxyl group. Standard values for normal, strongly hydrogen-bonded, and ionized tyrosins have been proposed in a previous work (Siamwiza et al., 1975). No significant changes in the Tyr doublet ratio in the frozen stored specimens were observed (Tables 6 and 7).

There was a change in the intensity of the 1450 cm^{-1} strong band assigned to the CH_2 and CH_3 bending vibrations (Tables 6 and 7), which may have resulted from hydrophobic interactions of aliphatic residues (Lippert et al., 1976; Arêas et al., 1989). Such decrease has been observed for myosin isolated from cod, particularly after frozen storage in the presence of formaldehyde (Careche and Li-Chan, 1997).

The bands generated by disulfide and SH bonds, falling in the 500−650 cm^{-1} range, are masked by the librational broad band of water and consequently they cannot be observed in the present spectra.

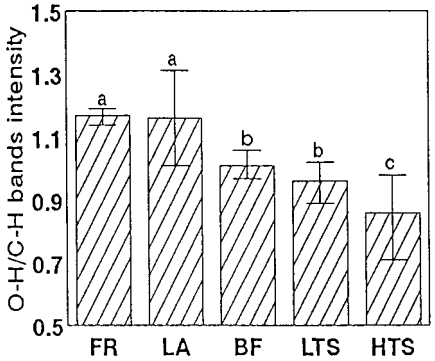

Figure 6. O−H/C−H stretching band ratio of hake muscle: (from left to right) fresh (FR), blast frozen (BF), and liquid air (LA) frozen samples and low- (LTS) and high-temperature (HTS) stored samples. Different letters indicate significant differences ($P < 0.05$). Error bars: standard deviation of the mean. Numbers of samples: FR, 15; BF, 9; LA, 3; LTS, 6; HTS, 6.

Comparison of the normalized spectral profiles in the 2800−3600 cm^{-1} region (Figure 5) showed that there was no change in the intensity of the C−H stretching bands (2800−3100 cm^{-1}) among conditions. It has been suggested that exposure of aliphatic hydrophobic side chains of proteins to the aqueous environment would lead to an increase of the intensity of this band (Verma and Wallach, 1977). The absence of this increase in the present work does not necessarily mean that there is no exposition of those groups, because hake muscle has ∼1% lipid content, the C−H of which will give a strong signal in this region, thus masking the C−H signal from proteins.

There was a decrease of the O−H stretching band of water (3100−3500 cm^{-1}), which is indicative of water loss (Figure 5). When all of the spectra are compared, it could be seen that for fresh samples the intensity of the O−H stretching band was higher than that of the C−H stretching band and that the opposite occurred in the samples stored at −10 °C, the rest of the curves falling between these two. The intensity ratio of O−H/C−H bands is shown in Figure 6. There is a trend of this ratio to decrease the harsher the condition of freezing or storage. These results suggest that this ratio could be used to determine quality loss due to freezing and frozen storage.

DISCUSSION

One of the advantages about Raman spectroscopy is that it gives the possibility of extracting information from complex solid systems. It has been used to study structural changes occurring as a result of the addition of Ca^{2+}, Mg^{2+}, and ATP for internally perfused fibers (Caillé et al., 1983). With regard to possible interferences in the amide I band, release of amino acids and peptides may occur for white fish only upon long ice storage, which is not the case for the samples studied here. Therefore, it is not expected that these substances interfere with protein secondary structure measurements through the amide I band. As to other constituents that could interfere in this sense, for example, amines, it is well-known that the intrinsic intensity of the νNH interfering bands is very weak and their frequencies fall below 1630 cm^{-1}. Therefore, the contribution of amines to the amide I band can be neglected. On the other hand, nucleic acids could also interfere in the amide I band region, but these compounds can also be neglected due to the fact that other typical Raman bands of nucleobases are absolutey absent in these spectra.

Results in this paper show that there are changes in secondary and tertiary structure of hake fillets when muscle is affected by freezing or frozen storage, these being more evident at the highest storage temperature. All of the spectral changes occurring under storage at detrimental conditions point out that protein denaturation takes place to some extent, this process involving a decrease of α-helices and concomitant increases of β-sheets and random coiled polypeptide backbone. In addition, increase of hydrophobic interaction is higher upon freezing. Among all of the parameters, changes in the O−H/C−H stretching band ratio were the most sensitive to show differences between conditions.

Changes related with the O−H stretching band found in this work are consistent with different water losses expected due to the ice formation and crystal growth pattern during freezing and storage in the studied conditions. The results obtained from the O−H/C−H stretching bands ratio look promising, and work is in progress to further explore this ratio for quality purposes. The use of Raman spectroscopy is becoming more feasible because Fourier transform spectrometers are currently compact and computerized, which makes this technique relatively simple and fast.

For both structural and functional measurements, the most prominent changes are between fresh and frozen stored samples and between both storage temperatures. The structural/functional correspondence will have to be further studied with more times of storage to see which of the structural changes are responsible for the functionality loss. In fact, there are some structural changes not necessarily reflected as loss of functionality or textural deterioration: the loss of α-helix and increase of β-sheets that occur in the samples stored at −30 °C have very little effect on apparent viscosity and no effect on Kramer shear resistance values.

It has previously been observed that there is an increase of hydrophobic residues exposed to solvent as evidenced by an increase of surface hydrophobicity of fish actomyosin upon freezing (Niwa et al., 1986) or upon short frozen storage periods (Del Mazo et al., 1999) with subsequent modification at more prolonged storage times (Del Mazo et al., 1999). Raman spectroscopy supported these results because an increase of exposure of hydrophobic groups was evidenced by a decrease of the 759 cm^{-1} band of tryptophan; however, no changes were observed in the Tyr doublet ($I_{855cm^{-1}}/I_{828cm^{-1}}$). With regard to the importance of hydrophobic interactions, which are accepted as playing an essential role in fish protein aggregation, a hypothesis based on a model experiment with cod myosin treated with formaldehyde and frozen stored (Ang and Hultin 1989) has been proposed. It was suggested that by interacting with the side-chain groups of the fish proteins, formaldehyde could increase the rate of protein denaturation during frozen storage, which would be mainly due to increased exposure of hydrophobic groups, and this would lead to subsequent aggregation by hydrophobic forces. These authors postulated that any reaction which modified side chains would cause increased rates of denaturation when the proteins were stressed. Careche and Li-Chan (1997), with Raman spectroscopy of myosin treated with formaldehyde and frozen stored, and results of this paper in whole muscle also show a decrease in the C−H bending band near 1450 cm^{-1}, attributing this decrease to hydrophobic interactions of aliphatic residues.

However, after the denaturing process produced by frozen storage, one can obtain some protein aggregates that are not extractable by detergents which break hydrophobic interactions (Tejada et al., 1996; Careche et al., 1998). This may in part be explained by the formation of interchain β-sheet structures shown in the above spectroscopic results. In fact, protein aggregates from other sources that were found to be water insoluble and insoluble in the presence of SDS showed high proportions of β-sheet structures (Jaenicke, 1987; Carmona et al., 1988). Examination of the estimates of protein secondary structures obtained under different storage conditions revealed that turn percentages are very similar. On the other hand, two mechanisms of β-sheet formation are conceived to occur upon denaturation, that is, by folding of polypeptide chains or by hydrogen bonding of adjacent polypeptide chains. As β-sheet formation was not accompanied here by simultaneous appearance of turns, the only possibility is that β-sheets stemmed from bonding of adjacent polypeptide chains by interchain contacts of C═O and NH groups. Consequently, if these interchain bonds are formed to great extent during protein denaturation, their corresponding aggregates would not be extractable by detergents capable of breaking hydrophobic intermolecular bonds.

A remarkable difference between high- and low-temperature storage is that the formation of random coil structure appears only at −10 °C. The reason for this result needs further investigation to take into account other factors involved in the denaturation and aggregation processes.

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