Characteristics of the Salt-Soluble Fraction of Hake (*Merluccius merluccius*) Fillets Stored at −20 and −30 °C

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Natural actomyosin (NAM) extracted in 0.6 M NaCl from hake fillets stored at −20 and −30 °C for up to 49 weeks was studied. The extracted protein decreased as storage progressed and became poorer in myosin while the proportion of actin remained constant. Two major peaks composed of myosin plus actin and actin plus tropomyosin plus troponins were obtained by size exclusion chromatography. SDS−PAGE analysis of the protein retained in the precolumn filter showed that there was protein aggregated by covalent bonding. Surface hydrophobicity increased while Ca^{2+}-ATPase activity, apparent viscosity, and SH groups decreased as storage progressed. The loss of Ca^{2+}-ATPase activity was due mainly to denaturation of the extracted myosin, whereas the minimum viscosity values occurred earlier and were not directly due to the lower proportion of myosin in the extracts. Thus, the extracted NAM exhibited changes during frozen storage. The temperature-dependent difference was mainly quantitative due to a smaller amount of protein extracted at −20 °C.

Keywords: *Hake; fillets; frozen storage; salt soluble; functionality; actomyosin*

INTRODUCTION

Myofibrillar proteins are very important in fish muscle for technological purposes, but they are the most prone to alteration in frozen storage. The changes these proteins undergo during freezing and frozen storage cause protein−protein bonds to form, making proteins difficult to extract in NaCl solutions. Hydrophobic interactions have been identified as the chief cause of the lower extractability and reduced functionality of myofibrillar proteins (Connell, 1965; Kinsella, 1982). The proportion of protein linked by disulfide and nondisulfide covalent bonds also increases with storage time (Owusu Ansah and Hultin, 1986; Tejada et al., 1996), varying according to species and muscle integrity (Tejada et al., 1995; Careche et al., 1996) and frozen storage temperatures (Careche et al., 1998). In species that form formaldehyde (FA) during frozen storage, these changes speed up as the FA reacts with the myofibrillar proteins and accelerates denaturation and aggregation (Ang and Hultin, 1989; Del Mazo et al., 1994; Tejada et al., 1997). The major myofibrillar proteins have different stabilities during frozen storage (Owusu-Ansah and Hultin, 1992; Huidobro and Tejada, 1995). In previous studies on cod muscle we found that although the greatest changes were apparent in the myosin heavy chain (MHC) molecules, myosin and actin became less extractable in 0.6 M NaCl in frozen storage as different kinds of protein−protein bonds formed, in amounts and percentages that changed with storage time, and produced protein aggregates. These aggregates grew in size, so that as frozen storage progressed they could no longer be extracted in 0.6 M NaCl and passed into the fraction not extractable in salt solutions (Tejada et al., 1996; Careche et al., 1998). This indicates that not only does the fraction extracted from frozen stored fish with saline solutions decrease with time but also the percent protein composition of the extracts differs.

Salt soluble proteins are a major contributor to muscle functionality, with regard to both hydration properties and surface properties. For this reason changes in muscle functionality are used to evaluate alterations of proteins when they undergo denaturation or aggregation during frozen storage. Protein changes in frozen fish muscle are detectable at an early stage through loss of Ca^{2+}-ATPase activity in muscle (Matsumoto, 1980; Huidobro and Tejada, 1994) and isolated protein (Careche and Tejada, 1991, 1994), through apparent viscosity in muscle homogenates (Borderías et al., 1985; Careche and Tejada, 1990a,b) and extracted protein (Jiménez Colmenero et al., 1988; Chen et al., 1989; Careche and Tejada, 1991, 1994; Del Mazo et al., 1994), and through alterations in surface hydrophobicity (Li-Chan et al., 1985; Del Mazo et al., 1994; Cofrades, 1994; Torrejón, 1996).

Frozen storage temperature is considered very important for the preservation of fish, in that lower temperatures prolong its practical storage life. As the extractability of different myofibrillar proteins alters depending on storage time and temperature (Tejada et al., 1995; Careche et al., 1998), the changes observed in frozen muscle during storage at different temperature could be due to a decrease in the amount of protein extracted in salt solutions, to changes in the composition of the extract, and/or to changes in protein stability during frozen storage. It is therefore important to know whether changes in functionality observed in frozen stored muscle at various temperatures are due only to changes in the reaction rate or whether there are also changes in the reaction mechanisms.

The aim of this paper was to determine the effect of storage temperature on extractability of natural actomyosin (NAM) in 0.6 M NaCl during frozen storage of hake fillets and what modifications occur in the functional and structural characteristics of this fraction.

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MATERIALS AND METHODS

Fish Source. Hake (*Merluccius merluccius* L.) fillets were supplied by the Instituto Português de Investigação Marítima (IPIMAR), Lisbon, Portugal. Fillets were prepared from gutted fish in post-rigor condition from fish caught in December 1993, frozen on board at −30 °C in a blast freezer, and vacuum-packed in plastic bags [transmission rates (23 °C and 75% RH) for O_2, CO_2, and $N_2 = 34$, 83, and 12 $cm^3/m^2/24$ h, respectively] in lots of five fillets. The fillets were air-freighted with solid CO_2 to our laboratory, divided into two lots, and stored in chambers at -20 ± 1 and -30 ± 1 °C. Samples were received in the laboratory 15 days after catch. Time 0 has been considered the time of arrival. The average length of the fillets was 22 ± 4.6 cm, and the average weight was 78 ± 16 g. Sixty-five individuals were used in the experiment, and periodic controls were carried out at 0, 5, 8, 14, 22, 36, and 49 weeks.

For each control and storage temperature, fillets from five individuals were taken from the vacuum-packed bags, cut in pieces of similar weight, and chopped until a homogeneous mince was obtained. Separate samples were taken for analyses, which were performed in triplicate unless stated otherwise.

Homogeneous mince obtained from the dorsal muscle of three unfrozen hake individuals was used to get the chromatographic pattern of fresh hake. The fresh fish, in early post-rigor condition, was purchased from a local supplier.

Proximate Analyses and pH. Crude protein content was analyzed according to the Kjeldahl method (AOAC, 1984) using a conversion factor of 6.25 (Lillevik, 1970). Crude fat was measured according to the method of Bligh and Dyer (1959) as modified by Knudsen et al. (1985). Moisture and ash were measured according to AOAC recommended methods (1984). The results were expressed as percentage of the muscle. The pH was determined at room temperature according to the method of Vyncke (1981).

NAM Extraction. NAM was extracted with 0.6 M NaCl from 100 g of the mixture according to the method of Kawashima et al. (1973), as modified by Careche et al. (1998): Thawed homogeneous minced muscle was washed with 5 volumes of phosphate buffer, pH 7.5 (3.38 mM potassium dihydrogen phosphate/15.5 mM disodium hydrogen phosphate, $\mu = 0.05$). The mixture was centrifuged at 5000*g* for 15 min (0−5 °C) (RC 5B refrigerated centrifuge, Sorvall Instruments, DuPont Co., Wilmington, DE), and the precipitate was washed twice following the same process as before. The resulting precipitate was homogenized in an Omnimixer (Omni International, Waterbury, CT) with 3 volumes of 0.8 M NaCl pH 7.5 (3.38 mM potassium dihydrogen phosphate and 15.5 mM disodium hydrogen phosphate) for 3 min at setting 6 in an ice−water bath. The homogenate was transferred to a beaker, and the homogenizer vase was rinsed with 0.8 M NaCl solution and added to the homogenate in the beaker. After standing for 2 h in an ice−water bath, the homogenate was centrifuged for 20 min at 5000*g* (0−5 °C). The supernatant was diluted with 10 volumes of cold water (0−2 °C) and left to stand in ice−water for ∼20 min until the protein precipitated. The top layer was siphoned off, and the rest, containing the protein suspended in water, was centrifuged for 15 min at 5000*g* (0−5 °C); 3 M NaCl (50 mM Tris−maleate, pH 7.0) was added to the precipitate to bring the concentration up to 0.6 M NaCl. The mixture was filtered through nylon gauze to remove any adhering traces of connective tissue and then dialyzed against 0.6 M NaCl (50 mM Tris−maleate, pH 7.0) overnight in a refrigeration chamber. The extracted NAM obtained after dialysis was denominated salt-soluble fraction. Protein concentration was determined according to the Lowry method Lowry (Lowry et al., 1951; Peterson, 1979) and verified with Kjeldahl (AOAC, 1984). Results were expressed as grams of extracted NAM per gram of total protein content in the muscle.

Polyacrylamide Gel Electrophoresis. The salt-soluble fractions were analyzed by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) in a Phastsystem horizontal apparatus (Pharmacia LKB Biotechnology, Uppsala, Sweden), using 12.5% polyacrylamide gels. Samples were treated according to the method of Hames (1985) as described by Tejada et al. (1996). Electrophoresis conditions were 4 mA/gel, 250 V, and 3 W. Aliquots of 1 μL containing 1 mg/mL were applied in the gels. Protein bands were stained with Coomassie Brilliant Blue (Pharmacia LKB Biotechnology) except for the proteins eluted from size exclusion chromatography, for which silver staining was used (*Phast-System User's Manual*, 1990). Myosin heavy chain (MHC) and actin (Ac) bands [measured as integrated optical density (IOD) per microgram of protein extracted] and electrophoretic profiles were analyzed on an Image Analyzer (Bio Image and Visage, Millipore Corp., Ann Arbor, MI) according to the method of Tejada et al. (1996).

Size Exclusion Chromatography (SEC). The extracted fractions were stored at −18 °C in glycerol (1:1) (v/v) until SEC analysis. They were used after overnight dialysis [0.6 M NaCl, pH 7.0 (Tris−maleate), 1 mM phenylmethanesulfonyl fluoride (PMSF)] and analyzed by SEC according to the method of Tejada et al. (1996). The column (Pharmacia LKB Biotechnology) filled with Bio-Gel A-50 m gel (medium) filler (55 cm × 2.5 cm) (Bio-Rad Laboratories, Richmond, CA) was equilibrated with 0.6 M NaCl, pH 7.0 (Tris−maleate). Aliquots were filtered (0.8−8.0 μm, AP 20, Millipore Corp.), and 2 mg of protein was applied to the column. Fractions of 5.5 mL were collected. SEC was performed in a cold room (4 ± 1 °C). The A_{280} detector sensitivity (UV detector, model UV-1, Pharmacia LKB Biotechnology) was adjusted to 0.1, and the flow rate was held constant at 0.5 mL min^{-1}. The molecular masses (MM) of the peaks were estimated by comparing their mobility with that of dextran blue (2000 kDa), thyroglobulin (669 kDa), and aldolase (158 kDa) (Pharmacia LKB Biotechnology). The proteins eluted in the major peaks were separated by electrophoresis (SDS−PAGE), and bands were visualized by silver staining. The amount of protein retained in the filtrate was calculated by the difference in protein as measured by Lowry (Lowry et al., 1951; Peterson, 1979) before and after filtering. Composition was determined by SDS−PAGE using Coomassie Brilliant Blue for staining.

Ca^{2+}-ATPase Activity. This was measured according to the method of Kawashima et al. (1973) at 25 °C with 1 mg mL^{-1} of extracted NAM. Assay conditions were 60 mM KCl, 25 mM Tris−maleate, pH 7.0, 5 mM $CaCl_2$, 1 mM ATP, and 1 mg mL^{-1} protein. Organic phosphorus (P_i) release was measured by using the method of Fiske and SubbaRow (1925). Results were expressed as micromoles of P_i released per minute per milligram of protein.

Surface Hydrophobicity. Aliphatic hydrophobicity was measured in the extracted NAM with *cis*-parinaric acid (CPA) according to the method of Kato and Nakai (1980). Aromatic hydrophobicity was measured with 8-anilino-1-naphthalenesulfonic acid (ANS) by using the method of Hayakawa and Nakai (1985). Results were expressed in arbitrary units (S_0) as the initial slope of fluorescence intensity versus protein concentration in milligrams per milliliter.

Apparent Viscosity. This was determined in the extracted NAM (10 mg mL^{-1}) (0.6 M NaCl, pH 7.0) (w/v) at 1−3 °C according to the method of Careche and Tejada (1991). Measurements were made with a rotary viscometer Brookfield model LVTD (Brookfield Engineering Labs, Inc., Stoughton, MA), at 12 rpm, using a small sample adapter (8 mL) with circulating water. Results were expressed in centipoise (cP).

Sulfhydryl (SH) Groups. Total SH groups were determined in the extracts according to the method of Beveridge et al. (1974). Results were expressed as micromoles of SH per gram of NAM.

Statistical Analysis. To determine the effect of temperature, one-way analysis of variance was applied, taking the storage time as cofactor. Given the existence of temperature/time interactions, one-way analysis of variance was likewise applied for each storage temperature. The level of significance was set at $P < 0.05$. These analyses were performed using the programs BMDP7D and BMDP2V (BMDP Statistical Software, Inc., Los Angeles, CA).

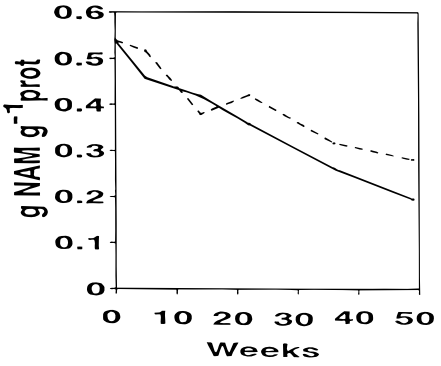

Figure 1. Extractability in 0.6 M NaCl of proteins from hake fillets stored at −20 °C (—) and −30 °C (- - -) (g of NAM g^{-1} of protein).

Table 1. IOD of MHC and Ac from NAM Extracted in 0.6 M NaCl from Frozen Hake Fillets (−20 and −30 °C) at Different Times

storage temp (°C)	weeks	IOD MHC	IOD Ac
−30	0	0.56	0.28
	49	0.29	0.28
−20	0	0.53	0.26
	22	0.34	0.27
	49	0.27	0.28

RESULTS AND DISCUSSION

Proximate analyses of the fillets (180.6 ± 1.4 g kg^{-1} of crude protein; 13.3 ± 2.0 g kg^{-1} of crude fat; 796.9 ± 2.6 g kg^{-1} of moisture; 13.9 ± 0.2 g kg^{-1} of ash) and **pH** (6.65 ± 0.01) were within the expected value ranges for hake (Careche and Tejada, 1990a; González, 1991).

At both temperatures **extractability** of NAM in 0.6 M NaCl decreased with time (Figure 1). The decrease was more pronounced in fillets stored at −20 °C. Similar results have been found in cod fillets (Careche et al., 1998).

The proportion of **MHC** in the extracts decreased with frozen storage time, down to values in the region of 50% after 49 weeks, while the proportion of **Ac** remained constant. There were no temperature-dependent differences in the proportions of MHC or Ac in the extracts (Table 1). In cod fillets stored at −20 and −30 °C likewise, no temperature-dependent differences were found, although the relative decrease of MHC in the extract after 49 weeks was greater than in the hake fillets in the present study (Careche et al., 1998). However, in minced hake stored at −20 °C, a decrease in the proportion of both MHC and Ac with storage time was found (Torrejón et al., 1999). Nevertheless, the total amount of MHC and Ac extracted from hake fillets in saline solutions decreased at both temperatures as storage time progressed because the amount of NAM extracted decreased, mainly at −20 °C (Figure 1). Thus, the temperature-dependent differences found were only quantitative, given that there was practically no difference in composition of the extracts obtained in each control.

The **chromatographic pattern** of unfrozen hake (Figure 2) showed three peaks: **peak 1**, which eluted at an apparent MM >2000 kDa (MM of the dextran blue used to determine the volume of exclusion); **peak 2** with an MM maximum around 1500 kDa; and **peak 3** with an MM maximum between 355 and 100 kDa. SDS−PAGE electrophoresis showed that peak 1 was composed mainly of myosin and actin, peak 2 of myosin, actin, and tropomyosin, and peak 3 of actin, tropomyosin, and troponins. No myosin was detected in peak 3. Peak 1 is considered to be actomyosin (Umemoto et al., 1971). Wakameda and Arai (1986) found peak 2 largely composed of myosin, probably due to the different extraction conditions that favor separation of myosin from the actomyosin complex.

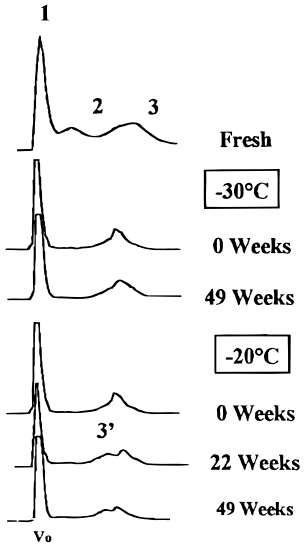

Figure 2. SEC chromatograms of NAM extracted in 0.6 M NaCl from fresh and frozen hake fillets stored at −20 and −30 °C at different extraction times: peak 1, MM ≥ 2000 kDa; peak 2, MM maximum around 1500 kDa; peak 3, MM maximum between 355 and 100 kDa.

Table 2. Percentage of the Peaks Eluted by SEC Corresponding to Figure 2

storage temp	weeks	peak 1 (%)	peak 2 (%)	peak 3′ (%)	peak 3 (%)
fresh		47	19	−[a]	34
−30	0	66	−	−	34
	49	60	−	−	40
−20	0	66	−	−	34
	22	57	−	22	21
	49	56	−	20	24

[a] No peak observed.

In NAM extracted from frozen fillets, peak 2 was not detected and the percentage of peak 1 with respect to the total area increased proportionally (Figure 2; Table 2). This suggests that part of the major proteins in peak 2 were modified at an early stage by freezing, becoming part of the soluble aggregates that eluted at higher MM values. So in peak 1 not only did NAM elute, as suggested by Umemoto et al. (1971), but also soluble aggregates coeluted with NAM.

At both temperatures, the percentage of peak 1 in the extract decreased as storage time progressed. This suggests that the molecular species increased in size and shifted to the fraction not extracted in salt (Figure 1), the extracts becoming poorer in myosin as frozen storage progressed (Table 1), and/or that despite being extracted in salt solutions they were retained in the precolumn filter as observed in minced cod (Tejada et al., 1996). SDS−PAGE of the protein retained in the precolumn filter at 22 weeks of storage at −20 °C showed a major protein peak retained in the stacking gel (Figure 3, peak

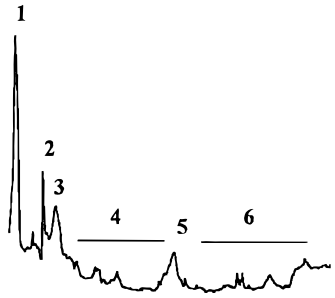

Figure 3. SDS−PAGE (12.5%) of the protein retained in the precolumn filter in the preparation of extracted NAM (−20 °C, 22 weeks) for SEC: peak 1, application zone; peak 2, peak in the stacking/resolving interface; peak 3, MHC; peak 4, proteins of MM between 200 and 45 kDa; peak 5, actin; peak 6, tropomyosin, troponins, and myosin light chains.

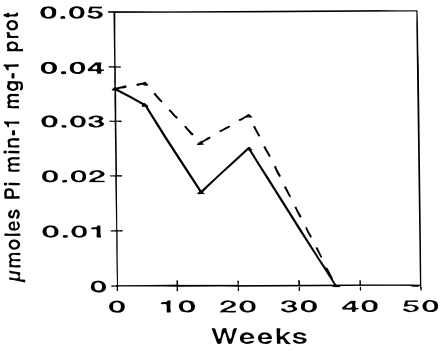

Figure 4. Ca^{2+}-ATPase activity of NAM extracted in 0.6 M NaCl from hake fillets stored at −20 °C (—) and −30 °C (- - -) (μmol of P_i min^{-1} mg^{-1} of protein in the extract).

Table 3. Analyses of Variance of the Variables Listed[a]

	temp	weeks							
	(°C)	0	5	14	22	36	49	*t*	*T*
Ca-ATPase	−20	a/x	a/x	b/x	ab/x	c/x	c/x	***	NS
	−30	a/x	a/x	a/x	a/x	b/x	b/x	***	
So CPA	−20	a/x	bc/x	b/x	bc/x	c/x	a/x	**	NS
	−30	a/x	bc/x	c/x	b/x	ab/x	ad/x	**	
So ANS	−20	a/x	bc/x	d/x	be/x	de/x	c/x	**	NS
	−30	a/x	b/x	b/x	b/x	b/x	c/y	**	
viscosity	−20	a/x	b/x	c/x	d/x	d/x	d/x	***	NS
	−30	a/x	b/y	c/x	de/x	b/x	e/x	***	
SH	−20	a/x	b/x	a/x	b/x	ab/x	c/x	***	NS
	−30	a/x	bc/x	a/y	b/x	c/x	d/x	***	

[a] For each variable different letters (a, b, ...) in the same row indicate significant differences in time (*t*); different letters (x, y) in each column indicate significant differences by storage temperature (*T*). ***, $P < 0.001$; **, $P < 0.01$; NS, not significant.

1). This confirms that in frozen hake fillets high-MM salt-soluble aggregates formed in which the proteins were partly linked by non-disulfide covalent bonds, but it does not exclude the possible involvement of other kinds of bonding. In all controls the retentate was greater at −20 °C than at −30 °C.

After 22 weeks, a peak 3′ (apparent MM maximum between 600 and 700 kDa and composed by actin, tropomyosin, and troponin), observed as a shoulder of peak 3, was detected at −20 °C but not at −30 °C (Figure 2).

Ca^{2+}-ATPase activity of the extracted NAM (Figure 4; Table 3) decreased significantly during frozen storage, with no activity detected at either temperature at 36 and 49 weeks despite extraction of MHC (Table 1). Although the initial values are lower than in other species, they lie within the range previously observed for hake caught in December (Careche and Tejada, 1991). Loss of Ca^{2+}-ATPase activity has been associated with denaturation of myosin (Sano, 1988). This suggests that from 22 weeks in frozen storage, the extracted myosin was denatured.

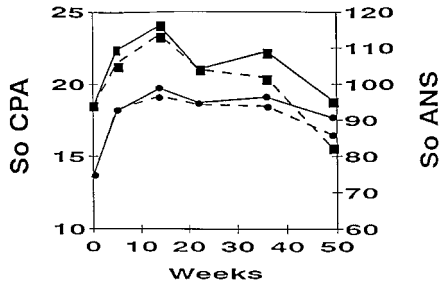

Figure 5. Surface hydrophobicity of NAM extracted in 0.6 M NaCl from hake fillets stored at −20 °C (—) and −30 °C (- - -): aromatic hydrophobicity (So ANS) (●); aliphatic hydrophobicity (So CPA) (■); S_0, fluorescence intensity vs protein concentration (mg/mL) (arbitrary units).

Aliphatic and aromatic surface hydrophobicity of the NAM extracted in each control, measured with CPA and ANS, respectively, also revealed significant differences with respect to time, but no differences were found in the extracts with respect to temperature of frozen storage (Figure 5; Table 3). Both aliphatic hydrophobicity (So CPA) and aromatic hydrophobicity (So ANS) increased in extracts from the first weeks of frozen storage. Aromatic hydrophobicity then remained practically stable until the end of storage. However, there was a slight decrease in aliphatic hydrophobicity, which returned to its initial levels.

It has been suggested that the initial increase of surface hydrophobicity of frozen-stored isolated actomyosin is due to a greater exposition of hydrophobic groups when actomyosin is denatured by freezing (Niwa et al., 1986). Del Mazo et al. (1994) and Cofrades (1994) have also reported an initial increase in surface hydrophobicity during frozen storage of isolated hake NAM. Subsequent evolution will depend on the balance between denaturation and aggregation of the proteins in the extract, because the protein−protein bonds that form may be broken or not during sample preparation for measurement of hydrophobicity depending on their type, as proposed by Careche and Li-Chan (1997).

In NAM extracted from hake fillets, at both temperatures aggregates were detected in which the proteins were linked by covalent bonds from the first weeks of storage. Changes in extracts composition also had to be taken into account because at both temperatures myosin was the major protein in the initial extracts but decreased over time to values similar to those for actin (Table 1). All of these factors could modify the exposed protein surface and hence surface hydrophobicity of the extracts decreases or remains constant.

The **apparent viscosity** of saline extracts (10 mg mL^{-1}) from hake fillets stored at −20 and −30 °C peaked at 5 weeks and then declined with only minor variations until the end of storage (Figure 6a; Table 3). An initial increase of viscosity has been reported in hake NAM stored at −18 °C (Careche and Tejada 1991) and in homogenates from hake fillets stored at −18 °C (Jiménez-Colmenero et al., 1988). This increase is thought to be due to initial protein aggregation, producing particles that are larger but still retain high water

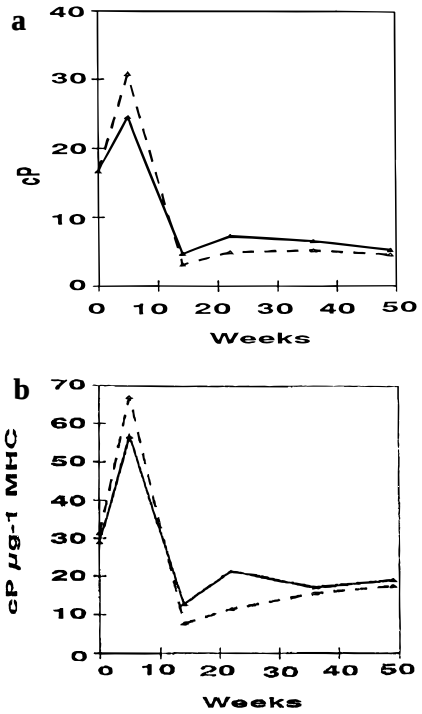

Figure 6. Apparent viscosity of NAM (10 mg mL^{-1}) extracted in 0.6 M NaCl from hake fillets stored at −20 °C (—) and −30 °C (- - -): (a) cP; (b) cP μg^{-1} of MHC.

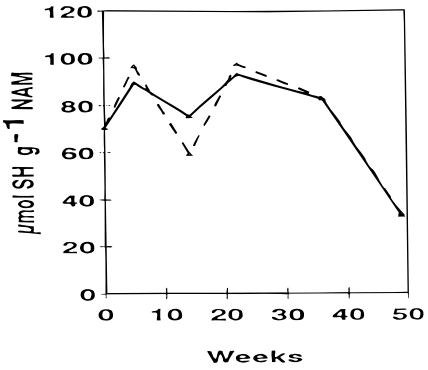

Figure 7. SH groups of NAM extracted in 0.6 M NaCl from hake fillets stored at −20 °C (—) and −30 °C (- - -) (μmol of SH g^{-1} of NAM).

binding capacity. Having taken into account that MHC decreased in the extracts as frozen storage progressed (Figure 6b), the changes in viscosity of the extracts were not directly related to the proportion of MHC at different storage times but to other parameters that affected the viscous behavior of protein solutions or dispersions, such as changes in the conformation and/or formation of intermolecular bonds with formation of aggregates, which altered the number and size of the particles, their axial relationship, and their water binding capacity.

At both temperatures the **SH groups** (Figure 7; Table 3) remained within a given range until the end of storage, when there was a sharp decrease. There were no significant differences in extracts with respect to storage temperature. These results suggest greater involvement of S−S-bonded proteins in the aggregates extracted at the end of storage, although differences due to changes in composition of the extracts as storage progressed are not dismissed.

Conclusions. NAM periodically extracted in 0.6 M NaCl from frozen hake fillets exhibited changes as storage progressed. These changes were reflected by a decreasing amount of NAM extracted and changes in the composition of the extracts. The composition changes consisted of a lower percentage of MHC, increased size of soluble aggregates with some proteins linked even by covalent bonds, loss of Ca^{2+}-ATPase activity, and changes in apparent viscosity values that were not directly proportional to the myosin in the extracts. The effect of storage temperature on the characteristics of the salt-soluble fraction from hake fillets was mainly quantitative, given that less NAM was extracted at the higher storage temperature. However, there were scarcely any qualitative differences in the extracts obtained at both temperatures.

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