Importance of Frozen Storage Temperature in the Type of Aggregation of Myofibrillar Proteins in Cod (*Gadus morhua*) Fillets

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Cod fillets were examined to determine the effect of two frozen storage temperatures (−20 and −30 °C) on the formation of aggregates and if this relates to changes in texture and functionality. The evolution of apparent viscosity during frozen storage was similar at either storage temperature. Loss of extractability in 0.6 M NaCl was slightly greater at −20 °C. The aggregate which formed at −30 °C was extractable in solutions which cleave secondary interactions [2% sodium dodecyl sulfate (SDS)] whereas at −20 °C the role of non-disulfide covalent bonds was more important, so that this aggregate was not totally extracted in 2% SDS plus 5% β-mercaptoethanol. The aggregates consisted largely of myosin and actin, but myosin was the most important constituent, contributing more to the formation of covalent bonds, particularly at the higher temperature. This difference in aggregation was accompanied with a difference in the evolution over time of shear resistance, which was higher at −20 °C.

Keywords: *Cod fillets; frozen storage; aggregation; actomyosin; temperature*

INTRODUCTION

Cod is a commercially valuable species whose annual total worldwide catch amounts to 1 239 522 metric tons (FAO, 1994), a large part of which is consumed in the form of frozen fillets (∼20%; FAO, 1992). Although frozen storage is an excellent means of preservation, fish can undergo a number of alterations, largely affecting texture in lean species such as cod. This means that by the end of frozen storage the product is hard and fibrous and has lost juiciness, so that it is liable to be rejected by the consumer. These changes, which are attributed largely to alterations in the myofibrillar proteins, depend not only on species but also on technological factors such as processing prior to freezing, or storage conditions (for reviews, see Haard, 1990; Mackie, 1993; Sikorski and Kolakowska, 1994). For this reason, a lot of attention has been paid in the literature to the effect of technological factors on loss of functionality and texture and how this relates to the appearance of compounds such as formaldehyde which are thought to accelerate the deterioration of certain species and are closely linked to storage temperature (Haard, 1990; Hultin, 1992). Work has also been done on the types of interaction responsible for aggregation of myofibrillar proteins, and attention has been drawn to the importance of secondary interactions and disulfide bridges although the authors differ as to the importance of the part played by each bond type and protein involved, probably owing to differences in species, storage conditions, and methodology (Connell, 1975; Dingle et al., 1977; Gill et al., 1979; Matthews et al., 1980; Laird et al., 1980; Lim and Haard, 1984; Rehbein and Karl, 1985; Owusu-Ansah and Hultin, 1986; Tejada et al., 1996). We have found at our laboratory that in minced cod, the aggregates forming during frozen storage are mostly composed of myosin and actin linked by secondary interactions and disulfide bridges (Tejada et al., 1996). These aggregates tended to grow in number and size as storage progressed, becoming insoluble in salt solutions but still extractable in sodium dodecyl sulfate (SDS) or SDS plus β-mercaptoethanol (ME). Evidence has also been found for the formation of non-disulfide covalent bonds, some extracted as aggregates in salt, SDS, or SDS + ME solutions, others appearing in the insoluble residue remaining after all three treatments. This unextracted residue has a structure resembling the sarcomere, which tends to become more pronounced the longer the storage time.

However, most research on the nature of aggregates has concerned the conditions which accelerate deterioration, such as mincing of fish muscle or storage at relatively high temperatures (Connell, 1965, 1975; Lim and Haard, 1984; Matthews et al., 1980; Laird et al., 1980; Tejada et al., 1996). On the other hand, it is believed that the changes detected in the texture or functionality of fish muscle are smaller the lower the temperature. We therefore think it is important to study the formation of aggregates in technological storage conditions which are as close as possible to the conditions prevailing in habitually consumed products.

The aim of this work was to examine the effect of two storage temperatures (−20 and −30 °C) on the formation of aggregates in cod fillets and if this relates to changes in texture and functionality.

MATERIALS AND METHODS

Fish Source. Cod (*Gadus morhua*) fillets were supplied by Torry Research Station, (TRS) Aberdeen, U.K. Fillets were prepared from fish in *post rigor* condition, caught in October 1992 and transported in ice in expanded polystyrene boxes to TRS. The fillets were blast-frozen at −40 °C for 4 h and the two fillets from each individual identified and individually packed in laminated vacuum pouches, 30-μm nylon + 120-μm polyethylene. Frozen fillets thus prepared were air-freighted with solid CO_2 in insulated containers to our laboratory, where

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the fillets from each individual were vacuum-packed in Cryovac BB-1 bags (80 torr of pressure), and stored at −20 and −30 °C for up to 62 weeks. Samples were received in the laboratory 7 days after catch. Time 0 has been considered the time of arrival in the laboratory. The average length of the fillets was 28 ± 3.7 cm, and the average weight was 209 ± 68 g.

For each control and storage temperature, fillets from five individuals (paired fillets at −20 and −30 °C) were taken out from the vacuum-packed bags and cut in five pieces of similar weight. A piece of each fillet was mixed with a piece of a different part of the other four fillets so that five pools were obtained. For each of the techniques, one pool was taken out, thawed (only if the technique required it), and chopped until a homogeneous mince was obtained, and then separated samples were taken for performing the analyses in triplicate, unless stated otherwise.

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

Apparent Viscosity. This was determined in a homogenate of muscle in 5% NaCl, pH 7 (1:4) (w/v), according to Borderías et al. (1985). Measurements were made with a Rotary Viscometer Brookfield Mod LVTD (Brookfield Engineering Labs. Inc., Stoughton, MA). Results were expressed in centipoises (cP).

Shear Resistance. The samples were thawed for 15 h at 4 °C, chopped, and then heated at 100 °C for 5 min in a water bath in cylindrical stainless-steel containers (height 30 mm × diameter 30 mm). These were hermetically sealed with screw-fitting tops and bottoms and each contained approximately 25 g of muscle. The samples in the containers were cooled for 30 min in ice/water and then kept refrigerated until the measurements were made. Each determination was performed on two 15-mm-thick slices, and six determinations were performed per lot. Determinations were performed on an Instron Universal Testing Machine, Model 4501 (Instron Engineering Corp., Canton, MA), fitted with a shear cell developed by Kramer et al. (1951). The assays were performed with a head which exerted a maximum force of 5 kN at a speed of 100 mm/min. The results were analyzed using the Instron Series IX program. Maximum load was measured and expressed as N g^{-1}.

Dimethylamine. The amine extracts were prepared from 20 g of muscle extracted with 60 mL of 6% trichloroacetic acid (TCA), homogenized for 1 min in an Omnimixer (Omni International, Waterbury, CT) at setting 4. One milliliter of benzene and 1 mL of KOH 65% (w/v) were added to 1 mL of TCA extract. The tubes were heated at 60 °C for 10 min and shaken for 2 min. Dimethylamine (DMA) was quantified using a modification of the gas chromatographic methods of Lundstrom and Racicot (1983) and Pérez Martín et al. (1987). The gas chromatograph (Perkin-Elmer 8500, Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England) was equipped with a glass column (1.75 m × 2 mm i.d.) and a flame ionization detector (FID). The column was packed with 25 cm of untreated 80−100 mesh Chromosorb 103 and 150 cm of 4% Carbowax 20 M + 0.8% KOH on Carbopack B. After conditioning (10 mL of N_2 min^{-1}, 210 °C), deionized water (10 mL) was injected several times, as recommended by Supelco Inc. (1971). The operating conditions were the following: initial temperature 115 °C; final temperature 200 °C; rate of temperature rise 30 °C min^{-1}; initial hold 5 min; time at final temperature 12 min; injection port and detector temperature 250 °C; N_2 flow rate 25 mL min^{-1}; FID sensitivity 10^{-10}; sample size 2 μL. Peaks were identified on the basis of sample coincidence with the relative retention times of standards (DMA). Peak areas were corrected by calculating the response factors of the standards in relation to the internal standard (*N*-propylamine). Results were expressed as μmol of DMA g^{-1}.

Free Formaldehyde. This was determined by the method described by Castell and Smith (1973). The colorimetric reaction was measured by the method of Nash (1953). Results were expressed as μmol of formaldehyde (FA) g^{-1}.

Natural Actomyosin Extraction. Samples were thawed, and natural actomyosin (NAM) was extracted with 0.6 M NaCl from 100 g of the mixture by the method of Kawashima et al. (1973) as follows: The mixed and homogenized muscle was washed with 5 volumes of phosphate buffer, pH 7.5 (3.38 mM potassium dihydrogen phosphate−15.5 mM disodium hydrogen phosphate). The mixture was centrifuged at 5000*g*, 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−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 2 volumes of the above 0.8 M NaCl solution and added to the previous protein extract. After allowing to stand for 2 h in an ice−water bath, the protein extract was centrifuged for 20 min at 5000*g* (0−5 °C). The supernatant and the precipitate were separated. The precipitate was denominated P1 and kept for further extractions in SDS and ME as described in the next section. The supernatant was diluted with 10 volumes of cold water (0−2 °C) and left to stand in ice−water for about 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. This dialyzed fraction was denominated S1. One single extraction was performed per lot. Protein concentration in the supernatants was determined by Lowry (Lowry et al., 1951; Peterson, 1979) and verified with Kjeldahl (AOAC, 1984). Results were expressed as g of extracted actomyosin g^{-1} total protein content in the muscle.

Extractability of Aggregates. The aggregates insoluble in 0.6 M NaCl (P1) which formed during frozen storage were treated with 4 volumes of 2.5% SDS (Merck, Darmstadt, Germany) and stirred in a magnetic stirrer for 10 min at room temperature. After centrifuging for 15 min at 5000*g*, a supernatant was collected, and the pellet was washed again with 1 volume of 2% SDS and centrifuged in the above conditions. Both supernatants were mixed, and, thus, a fraction extracted in 2% SDS (fraction S2) was obtained. Any remaining precipitate (P2) was treated with 2% SDS plus 5% ME in the same conditions as before, to obtain an extracted fraction (S3) and in some cases an insoluble precipitate (P3). The purpose of these two steps was to break down noncovalent bonds and disulfide bonds, respectively. The amount of soluble protein in fractions S2 and S3 and in P3 was determined by Kjeldahl (AOAC, 1984). Results were expressed as percentage of P1.

Polyacrylamide Gel Electrophoresis. All fractions extracted (S1, S2, and S3) 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 Hames (1985) (2% SDS, 5% ME, and 0.002% bromophenol blue), heated for 5 min in a boiling water bath, and centrifuged (Sorvall Microspin 24S, Sorvall Instruments, DuPont Co., Wilmington, DE) at 10000*g* for 1 min. One-microliter aliquots containing 1 mg/mL were applied in the gels. Electrophoresis conditions were 4 mA/gel, 250 V, and 3 W. Protein bands were stained with Coomassie Brilliant Blue (Pharmacia LKB Biotechnology) (*Phast-System users manual,* 1990). Myosin heavy chain (MHC) and actin (Ac) bands [measured as integrated optical density (IOD)] and electrophoretic profiles were analyzed on an Image Analyzer (Bio

Table 1. Proximate Analyses and pH (Mean ± Standard Deviation, $n = 3$)

% crude protein	% crude fat	% moisture	% ash	pH
19.00 ± 0.58	1.16 ± 0.16	80.22 ± 0.07	1.45 ± 0.12	6.82 ± 0.02

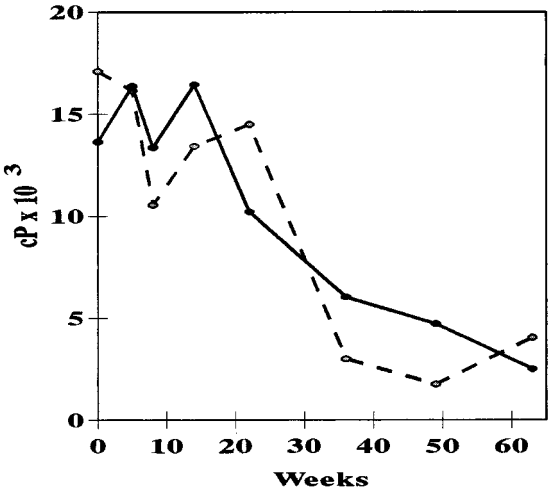

Figure 1. Apparent viscosity [centipoise (cP)] of a homogenate of cod fillets stored at −20 °C (—) and −30 °C (- - -).

Image and Visage, Millipore Corp., Ann Arbor, MI). The molecular masses (MW) of the main component proteins in the samples were estimated by comparing their mobility with that of a standard high molecular mass protein mix (ferritin, 220 kDa subunit; albumin, 67 kDa; catalase, 60 kDa subunit; lactate dehydrogenase, 36 kDa subunit; and ferritin, 18.5 kDa subunit) (Pharmacia LKB Biotechnology). For the quantitative measurement of myosin heavy chain and actin bands, IOD was previously checked for linearity. The composition of the various protein fractions extracted (S1, S2, and S3) in the course of storage of fillets at the two temperatures was measured on the basis of the changes occurring in the proportion of the majority proteins (MHC and Ac), by means of the IOD μg^{-1} protein in each fraction. The MHC/Ac ratio was also calculated for each fraction. The qualitative changes occurring in the rest of the extracted proteins were reflected in the electrophoretic profiles.

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).

RESULTS

The proximate analyses and pH (Table 1) of the cod fillets were found to be within the expected value ranges for lean fish species (Suzuki, 1987).

The initial values of apparent viscosity were similar to those of fresh cod muscle (Chalmers et al., 1992). There was a significant and pronounced time-dependent decline (Figure 1; Table 2) as from week 22 at −30 °C and week 14 at −20 °C, which continued until the end of storage. There was no clear tendency reflecting differences between the two storage temperatures.

In fillets stored at −30 °C, shear resistance displayed no clear tendency to increase over storage. In fillets stored at −20 °C, on the other hand, an increase was detected from week 8 up to week 36, declining thereafter until the end of storage (Figure 2; Table 2). This decrease was attributed to loss of cohesiveness, so that the sample passed more easily through the slits in the Kramer shear cell.

Table 2. One-Way Analyses of Variance of the Variables Listed Below as a Function of Time (a, b, c, ...) and Temperature (x, y)[a]

	temp (°C)	weeks 0	5	8	14	22	36	49	62
apparent viscosity	−20	a/x	b/x	a/x	b/x	c/x	d/x	e/x	f/x
	−30	a/y	a/x	b/y	c/y	c/y	d/y	e/y	f/y
shear resistance	−20	ab/x	b/x	b/x	a/x	c/x	c/x	a/x	a/x
	−30	abc/x	ab/x	ab/x	c/x	ad/y	ad/y	cd/x	ad/x
dimethylamine	−20	a/x	b/x	bc/x	b/x	c/x	d/x	−	−
	−30	ab/x	bc/x	ab/y	a/y	ab/y	c/y	−	−
formaldehyde	−20	a/x	b/x	c/x	c/x	d/x	d/x	c/x	c/x
	−30	a/x	bc/y	a/y	a/y	a/y	d/y	b/x	cd/x

[a] Analyses of variance as a function of time: For each of the variables (apparent viscosity, shear resistance, etc.) and storage temperature (−20 °C, −30 °C), different letters a, b, ..., f in the same row indicate significant differences ($P < 0.05$). Analyses of variance as a function of temperature: For each of the variables (apparent viscosity, shear resistance, etc.) and storage times (0−62 weeks), different letters in the same column (x, y) indicate significant differences ($P < 0.05$).

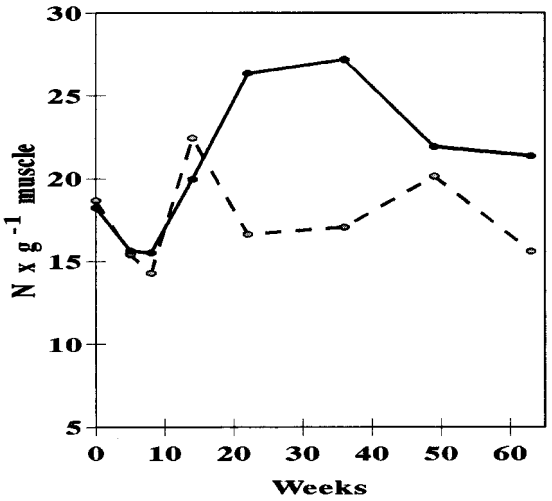

Figure 2. Shear resistance (N g^{-1} muscle) of cod fillets stored at −20 °C (—) and −30 °C (- - -).

DMA levels rose with storage time at either temperature and were significantly higher at the higher storage temperature (Figure 3; Table 2). Increased DMA formation at higher frozen storage temperatures has been reported in cod fillets (LeBlanc et al., 1988).

Free FA levels also differed significantly depending on storage time and temperature (Figure 3; Table 2). The fillets stored at −30 °C generally exhibited lower levels of free FA than did those stored at −20 °C. The decline observed in free FA levels by the end of storage could have been due to increased reactivity of this compound. In both lots, the amount of bound FA, measured as μmol of DMA minus μmol of free FA, increased with storage time, attaining higher values in fillets stored at −20 °C (0.64 and 0.96 μmol of bound FA at −30 and −20 °C, respectively, at 36 weeks). Studies of minced cod muscle stored at −8 °C (Rehbein, 1988) and −20 °C (Torrejón, 1996) have reported larger amounts of bound FA than were found in the present case, indicating more extensive reaction of FA to proteins when the storage temperature is higher or the muscle is minced.

Extractability in 0.6 M NaCl, 2% SDS, and 2% SDS plus 5% ME. Figure 4a shows slight differences

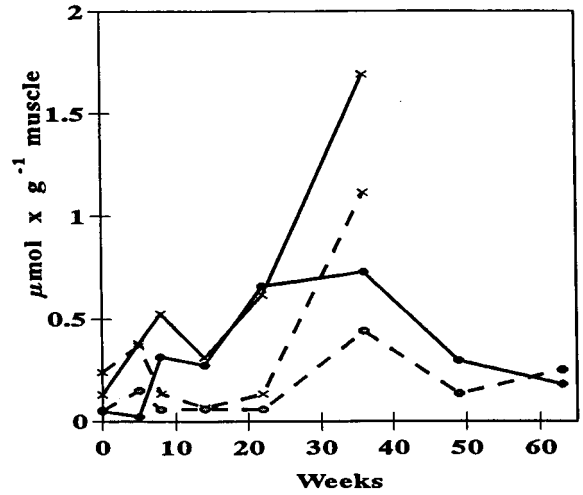

Figure 3. Dimethylamine (×) and free formaldehyde (●) (μmol g $^{-1}$ muscle) of cod fillets stored at −20 °C (—) and −30 °C (- - -).

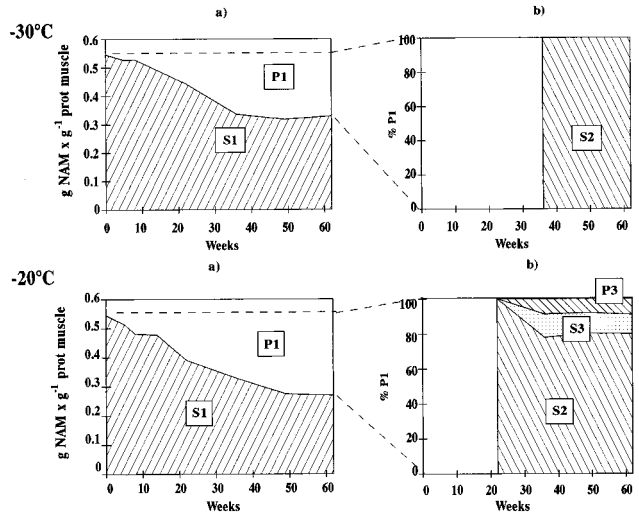

Figure 4. Extractability of cod fillets proteins stored at −30 °C and −20 °C: (a) grams of natural actomyosin (NAM) extracted in 0.6 M NaCl (g g^{-1} protein muscle) (S1); and (b) protein extracted in 2% SDS (S2) and 2% SDS plus 5% β-mercaptoethanol (S3) from the aggregate nonextracted in 0.6 M NaCl of cod fillets stored at −30 °C and −20 °C, and an insoluble fraction [Percentage of aggregate insoluble in NaCl (%)].

in extractability in 0.6 M NaCl (fraction S1) between the two experimental temperatures. Loss of extractability became apparent earlier and was more pronounced in the fillets stored at −20 °C.

Analyses of protein unextractable in 0.6 M NaCl (P1) were carried out from week 36 and week 22, respectively, in fillets stored at −30 and −20 °C (Figure 4b). At −30 °C, all of the aggregate not extracted with 0.6 M NaCl was extracted in all controls when treated with SDS (fraction S2). At −20 °C, on the other hand, although the amount of protein extracted in fraction S2 increased with storage time, it constituted a decreasing proportion of aggregate P1 as the amount of protein linked by covalent bonds increased. At −30 °C, there was no fraction S3, since all the protein was extracted when fraction P1 was treated with 2% SDS. In the fillets stored at −20 °C, fraction S3 was extracted as from week 36 and remained a constant percentage of P1 throughout storage. This suggests that in this lot the incidence of protein−protein disulfide bonds increased with time, although by a smaller amount and in smaller proportion than in minced cod muscle stored at −20 °C (Tejada et al., 1996). Again in the fillets stored at −20 °C, as from week 36 a precipitate P3, which was not extractable in the given conditions, was obtained. The amount of P3 increased, but the proportion remained constant until the end of storage. The percentage of unextractable protein with respect to aggregate not extractable in saline solutions (P1) was similar to that found in minced cod muscle in our laboratory (Tejada et al., 1996).

Protein Composition of Fractions S1, S2, and S3. (A) Fraction S1. The IOD of the MHC decreased significantly as storage progressed in the fractions extracted with 0.6 M NaCl, with no clear trend with respect to storage temperature (Figure 5, Table 3). At both temperatures, values fell below the initial levels as from week 14. This decline in the proportion of MHC extracted in saline solution has been reported by other authors for the same species (Matthews et al., 1980; Laird and Mackie, 1981; Ragnarsson and Regenstein, 1989; LeBlanc and LeBlanc, 1989; Tejada et al., 1996). There was no clear trend in the proportion of Ac in the saline extract (Figure 5; Table 3) over the storage period. However, the total amount decreased over time at both temperatures as extractability of this fraction declined (Figure 4). Greater stability of actin than MHC during frozen storage had previously been reported for cod (LeBlanc and LeBlanc, 1989; Tejada et al., 1996). The electrophoretic profiles of the NAM extracted in 0.6 M NaCl (Figure 6) show that during storage, there were no temperature-dependent differences in the proteins of lower molecular weight than actin extracted in fraction S1. Peaks 1 and 2 correspond to protein aggregates which did not penetrate the gel. These aggregates are believed to be linked by non-disulfide covalent bonds and have been reported in this species (Matthews et al., 1980; Tejada et al., 1996).

(B) Fraction S2. In the fraction extracted with 2% SDS, the proportion of MHC at −30 °C (Figure 5; Table 3) remained virtually unchanged over time. Values were for both temperatures higher than in fraction S1 (0.6 M NaCl) for the same storage time but lower than initially. The proportion of Ac (Figure 5, Table 3) remained unchanged at −30 °C whereas it increased slightly at −20 °C over time. The electrophoretic profile for week 49 (Figure 7) shows that the proportions of peaks 1 and 2 were high, indicative of the formation of protein aggregates with non-disulfide covalent bonding in the fractions extracted with SDS in frozen fillets, as had previously been reported by Matthews et al. (1980) and Tejada et al. (1996) in cod minces stored at −7 and −20 °C, respectively.

(C) Fraction S3. In the fillets stored at −20 °C, the proportion of MHC did not differ significantly as a function of time (Figure 5; Table 3) and was slightly lower than in fraction S2. No data are available for fillets stored at −30 °C, as all the protein was extracted when aggregate P1 was treated with 2% SDS. There were no changes in the proportion of Ac (Figure 5) or in the electrophoretic profile as storage progressed. Scarcely any bands other than MHC and Ac were apparent in the resolving gel at 49 weeks (Figure 7).

MHC/Ac Ratio. The MHC/Ac ratio in fraction S1 decreased considerably with storage time (Figure 8; Table 3). In fraction S2 (Figure 8; Table 3), on the other hand, this ratio was dependent on storage temperature.

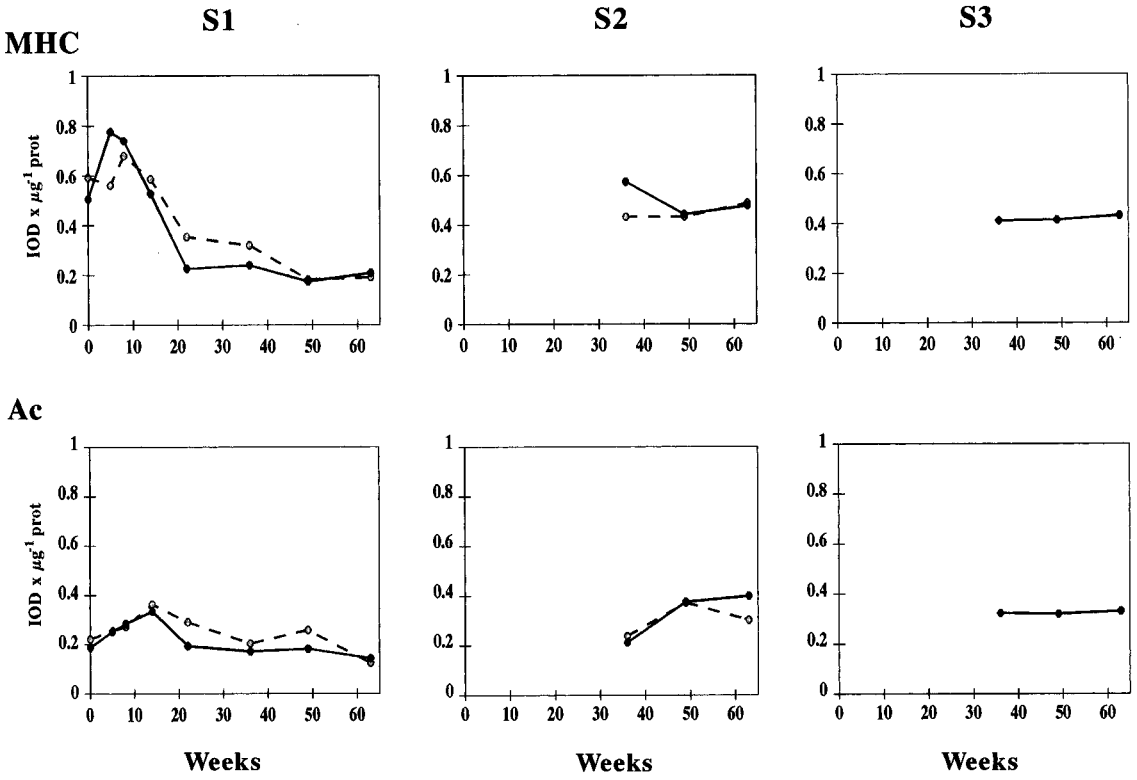

Figure 5. Evolution of myosin heavy chain (MHC) and actin (Ac) obtained from SDS−PAGE (12.5%) in fractions S1, S2, and S3 of cod fillets stored at −20 °C (—) and −30 °C (- - -) [integrated optical density (IOD) μg^{-1} protein extracted].

Table 3. One-Way Analyses of Variance of the Proportion of Myosin Heavy Chain (MHC), Actin (Ac), and MHC/Ac Ratio Obtained from SDS−PAGE (12.5%) of Fractions S1, S2, and S3 in Cod Fillets Stored at −20 and −30 °C[a]

	temp	weeks							
	(°C)	0	5	8	14	22	36	49	62
MHCS1	−20	a/x	b/x	b/x	a/x	c/x	c/x	c/x	c/x
	−30	a/x	a/y	b/x	a/x	c/y	c/y	d/x	d/x
MHCS2	−20	−	−	−	−	−	a/x	b/x	b/x
	−30	−	−	−	−	−	a/y	a/x	a/x
MHCS3	−20	−	−	−	−	−	a	a	a
AcS1	−20	ad/x	ab/x	bc/x	c/x	ad/x	d/x	d/x	d/x
	−30	ab/x	acd/x	ac/x	e/x	c/y	bd/x	acd/y	acd/x
AcS2	−20	−	−	−	−	−	a/x	b/x	b/x
	−30	−	−	−	−	−	a/x	b/x	ab/y
AcS3	−20	−	−	−	−	−	a	a	b
MHC/Ac S1	−20	a/x	a/x	a/x	b/x	bc/x	bc/x	c/x	b/x
	−30	a/x	a/y	a/x	b/x	bc/x	b/x	c/x	cd/y
MHC/Ac S2	−20	−	−	−	−	−	a/x	b/x	b/x
	−30	−	−	−	−	−	a/y	b/x	a/y
MHC/Ac S3	−20	−	−	−	−	−	a	a	a

[a] Analyses of variance as a function of time: For each of the variables (MHCS1, MHCS2, etc.) and storage temperature (−20 °C, −30 °C), different letters a, b, c, d in the same row indicate significant differences ($P < 0.05$). Analyses of variance as a function of temperature: For each of the variables (MHCS1, MHCS2, etc.) and storage times (0−62 weeks), different letters in the same column (x, y) indicate significant differences ($P < 0.05$).

The ratio was constant throughout storage in fillets stored at −30 °C. In the fillets stored at −20 °C, the MHC/Ac ratio declined in both fraction S1 and fraction S2 and remained constant in fraction S3.

DISCUSSION

The results of extractability in NaCl, SDS, and SDS plus ME show the influence of storage temperature on the formation of aggregates for this species. Since

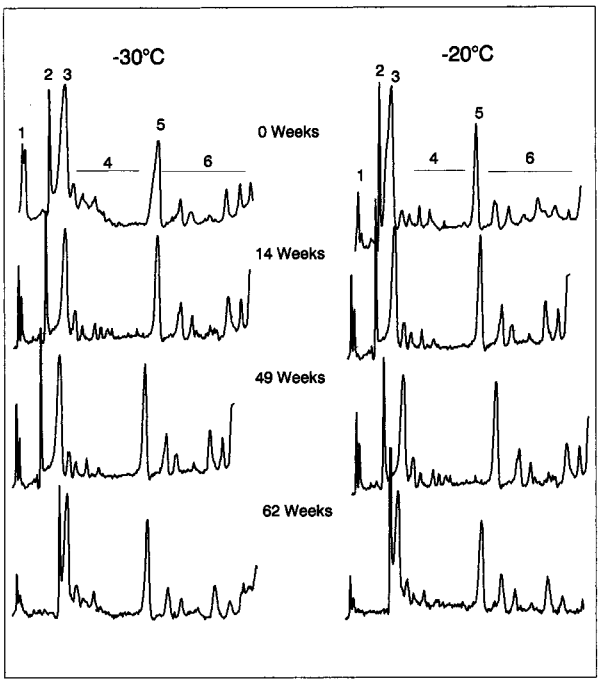

Figure 6. SDS−PAGE (12.5%) of fractions S1 from cod fillets extracted at 0, 14, 49, and 62 weeks' frozen storage at −20 and −30 °C: 1, application zone; 2, peak in the stacking/resolving interphase; 3, myosin heavy chain; 4, proteins of molecular mass between 200 and 45 kDa; 5, actin; 6, tropomyosin, troponins, and myosin light chains.

extractability in 0.6 M NaCl did not differ greatly from one temperature to the other, the main difference was the nature of the aggregate formed over time as determined by extractability in a series of agents which cleave secondary interactions and disulfide covalent bonds. At −30 °C (Figure 4), once the aggregate had

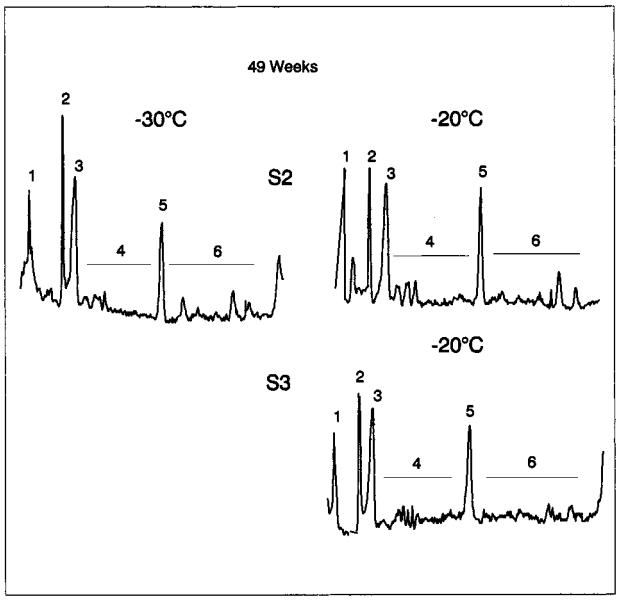

Figure 7. SDS−PAGE (12.5%) of protein extracted in 2% SDS (S2) from cod fillets extracted at 49 weeks' frozen storage at −20 and −30 °C and in 2% SDS plus 5% β-mercaptoethanol (S3) from cod fillets extracted at 49 weeks' frozen storage at −20 °C. Peaks as in Figure 6.

formed, it was totally extracted with 2% SDS. At −20 °C, the involvement of disulfide and non-disulfide covalent bonds increased to the extent that up to 20% of the aggregate unextractable in 0.6 M NaCl was found in fractions S3 or P3. Nevertheless, this amount was much smaller than had been found in minced muscle, where at the same storage temperature the unextractable portion accounted for up to 50% of the total aggregate (Tejada et al., 1996). This means that in cod, the nature of the aggregate changes considerably according to the storage temperature, time, and the intactness of the muscle.

The lack of great differences in extractability in salt as a function of temperature (Figure 4) corresponds with similar protein changes in composition of the majority of proteins, as can be seen in the MHC/Ac ratio for this fraction (Figure 8). The protein most involved in the formation of aggregates is myosin measured as loss of the MHC band, and the evolution in time is similar for both temperatures (Figure 5). Similarly to the results in extractability, the main differences in the relative proportion of proteins as a function of temperature were found when the aggregate P1 was extracted with SDS and ME. The results show that at both temperatures myosin is more involved than actin in the aggregate, although the relative contribution of this myosin to the covalent aggregates differs as a function of temperature. At −30 °C, the evidence for covalent bonding of myosin comes from the lack of recovery of this protein in fractions S1 and S2 during the storage. When there is a loss in the proportion of a certain protein band in fraction S1, for the total recovery of this protein in fraction S2 it would be needed to obtain a higher proportion of this particular protein than initially and a certain given amount of protein extracted in each of the fractions. If the proportion of protein recovered in fraction S2 is lower or equal to the initial one, then there is no total recovery of this protein band, which is the case of MHC. Results of Figure 5 show that the decline of the MHC band in the 0.6 M NaCl soluble fraction does not correspond with a higher proportion of this

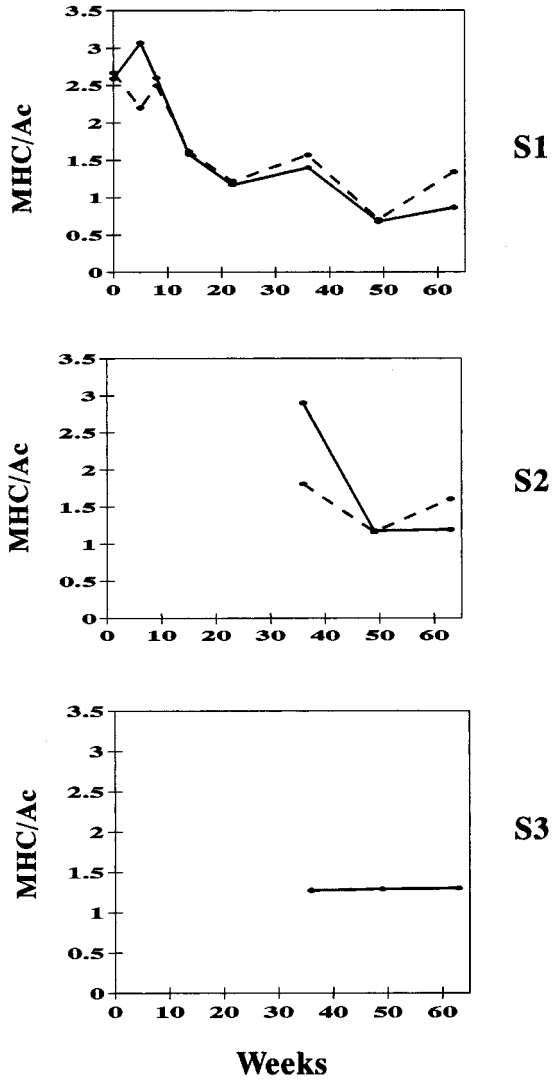

Figure 8. MHC/Ac ratio of fractions S1, S2, and S3 from cod fillets stored at −20 °C (—) and −30 °C (- - -).

band than initially in fraction S2. Since there is no fraction S3 or P3 for this temperature, this would mean that this protein should be in fractions S1 or S2 as a form of other protein band(s). Since no increment of molecular weight bands lower than the MHC band have been found which would make suspect the presence of proteolysis, the "lost" myosin could be forming extractable aggregates in peak 1 or 2 as was previously reported at our laboratory in experiments on minced cod muscle (Tejada et al., 1996). The protein present in these peaks would be linked by non-disulfide covalent bonds in fractions S1 and S2. However, this experiment does not rule out the contribution of disulfide covalent bonds to the cross-linking of these proteins, since they are broken down in the preparation of the sample for electrophoresis. With actin, on the other hand, although the trend of the fraction extracted in 0.6 M NaCl is not as clear as with myosin, at the end of storage, the proportion of this protein band is slightly higher in fraction S2 than initially in fraction S1. By multiplying the relative proportions of this protein with the amount extracted in each fraction at the beginning and the end (data from Figures 5 and 4, respectively), it can be seen that all the actin unextracted in fraction S1 is recovered in fraction S2 [0.2 × 0.55 = 0.11 total IOD at the beginning in S1 fraction, 0.12 × 0.33(S1) + 0.3 ×

0.22(S2) = 0.11 total IOD at the end in fractions S1+S2] and therefore this protein does not seem to be involved in the formation of non-disulfide covalent bonds.

At −20 °C, the contribution of myosin and actin to the formation of aggregates through disulfide covalent bonds is shown in the fact that both proteins are extracted in fraction S3. Also there is evidence of proteins bound through non-disulfide covalent bonds since there is an inextractable fraction P3. Similarly to the reasoning at −30 °C, for MHC there is no more protein concentration in fractions S2 and S3 than in fraction S1 initially, and therefore there must be protein involved in non-disulfide covalent bonds, either in fraction P3 or in the extracted fractions S1, S2, and S3. Also, myosin is more involved than actin in the formation of covalent bonds since the MHC/Ac ratio decreases in fraction S2 without a recovery in fraction S3 (Figure 8).

The consequences of this different aggregation pattern as a function of temperature in functionality and texture are not clear yet. Loss of both extractability and the MHC/Ac ratio in 0.6 M NaCl with time of storage could account for the changes observed in the apparent viscosity. It is known that the apparent viscosity can change over storage as the protein's conformation or composition in the homogenate alters or the amount of protein decreases. The decline in apparent viscosity can be explained at least partly by the decrease in extractability and the MHC/Ac ratio in fraction S1. These results would account for the lack of any clear differences in the apparent viscosity between the two experimental temperatures, since there was no change in the relative protein composition. In fact, there was a correlation between the apparent viscosity and the MHC/Ac ratio (0.7253 at −30 °C and 0.6103 at −20 °C, $P < 0.05$).

Regarding changes in shear resistance, it is tempting to infer that the texture of cod fillets stored at either temperature was related to the type of salt-insoluble aggregate formed, shear resistance being significantly higher where these aggregates contained a larger amount of myosin linked by covalent bonds. Experiments on the effect of temperature on other species such as hake (manuscript in preparation) do not rule out this link; however, they do not confirm a clear cause−effect relationship between both events. Our results show even when disrupting the structure of the fillet in order to make samples of homogeneous size and shape, that this method can show differences between both temperatures in cod, until a moment when the loss of cohesiveness of the sample makes the data difficult to interpret.

It is known that FA in model systems of myosin or actomyosin can act as an aggregating agent (Ang and Hultin, 1989; Del Mazo et al., 1994; Careche and Li-Chan, 1997), and it has been highly correlated to decreases in functional properties and loss of textural attributes in fish muscle (Gill et al., 1979; Tokunaga, 1980; Careche and Tejada, 1990, 1991). In this paper, we have found differences in the formation of DMA or FA and even in bound FA as a function of temperature during frozen storage, corroborating the findings of other authors. However, more experiments will be needed to show a relationship between the formation of aggregates and the amount of FA formed in a real system such as fish muscle.

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