Protein Extracts and Aggregates Forming in Minced Cod (*Gadus morhua*) during Frozen Storage

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Natural actomyosin was extracted from frozen minced cod muscle stored for up to 62 weeks at −20 °C with 0.6 M NaCl, and the insoluble aggregates, when formed, were solubilized successively with 2% sodium dodecyl sulfate (SDS) and 2% SDS + 5% β-mercaptoethanol (ME) solutions, giving extracted fractions S1 (NaCl), S2 (SDS), and S3 (ME + SDS), precipitates insoluble in 0.6 M NaCl (P1) and 2% SDS (P2), and a precipitate not soluble in any of the agents used (P3). SDS polyacrylamide gel electrophoresis (SDS-PAGE) of fraction S1 showed that the proportion of the major proteins changed during frozen storage. Size exclusion chromatography showed a decrease in the peak containing myosin heavy chain (MHC) and actin (Ac). Transmission electron microscopy (TEM) of S1 showed at the outset a filamentous morphology associated with globules interconnected crosswise. As storage progressed, the number and size of aggregates increased. In fractions S2 and S3, the major proteins detected by SDS-PAGE were MHC and Ac. TEM showed a greater abundance of ring-shaped structures than in S1. TEM of the insoluble fractions showed a sarcomere-like structure, more pronounced the milder the solubilizing treatment and the longer the storage time.

Keywords: *Aggregation; frozen storage; actomyosin; cod; mince; Gadus morhua; microstructure*

INTRODUCTION

The myofibrillar proteins of many fish species aggregate during frozen storage (Matsumoto and Noguchi, 1992), with considerable alteration to some functional properties and the texture of the muscle. When this occurs in many species of high commercial value, it spells the end of practical storage life.

In view of the economic concern, research on frozen stored fish muscle aggregation has been extensive. Most of this work has focused on changes in extractability and solubility of myofibrillar proteins in salt and different agents which rupture secondary interactions and disulfide bridges. These studies are supplemented by electrophoretic profiles of proteins soluble or dispersed in these conditions (for reviews, see Haard (1992), Hultin (1992), Mackie (1993), and Sikorski and Kołakowska (1994)). These studies emphasize the importance of secondary interactions and disulfide bridges, which are considered the chief cause of myofibrillar protein aggregation, without ruling out the participation of non-disulfide covalent bonds. There are divergent data as to 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). A number of studies have been carried out using electron microscopy (Jarenbäck and Liljemark, 1975) and gel filtration chromatography (Umemoto et al., 1971; Seki and Arai, 1974; Ohnishi and Rodger, 1980), which enabled observation of the morphological changes in aggregates and proteins present in solution.

The purpose of the present study was to identify the relationships between changes occurring in extractability, composition, and morphology of myofibrillar proteins in minced cod during frozen storage. For this purpose, the changes occurring in natural actomysin (NAM) extractability with different agents capable of selectively breaking different protein bonds were examined periodically during 62 weeks of frozen storage of minced cod muscle. It was hoped to ascertain which bonds are responsible for the formation of aggregates and which are the major proteins extracted with each of the agents and to determine the structures that are formed by extracted myofibrillar proteins and the insoluble aggregates.

MATERIALS AND METHODS

Fish Source. Minced cod (*Gadus morhua*) was supplied by Torry Research Station, Aberdeen, U.K. The mince was made from fillets taken from 20 individuals kept in ice for 3 days, using an Omega TE22 mincer with 5-mm hole size. The blocks of mince were blast frozen at −40 °C for 4 h. They were then packed in laminated vacuum pouches, 30-μm nylon + 120-μm polyethylene and air-freighted with solid CO_2 in insulated containers. Once in the laboratory, the blocks were cut up, vacuum-packed in Cryovac BB-1 bags (80 Torr of pressure), and stored at −20 °C (±1 °C) for up to 62 weeks, during which time periodic analyses were performed initially and at 5, 8, 14, 22, 36, 49, and 62 weeks.

NAM Extraction. NAM was extracted with 0.6 M NaCl from 100 g of thawed mince by the method of Kawashima et al. (1973). The centrifuging force applied was 5000*g* (Sorvall RT6000B; DuPont Co. Wilmington, DE). This was fraction S1. Protein concentration in the supernatants was determined by the Lowry method (Lowry et al., 1951; Peterson, 1979) and Kjeldahl (AOAC, 1984).

Extractability of Aggregates. The insoluble materials from the 0.6 M NaCl (P1) extractions were treated with four

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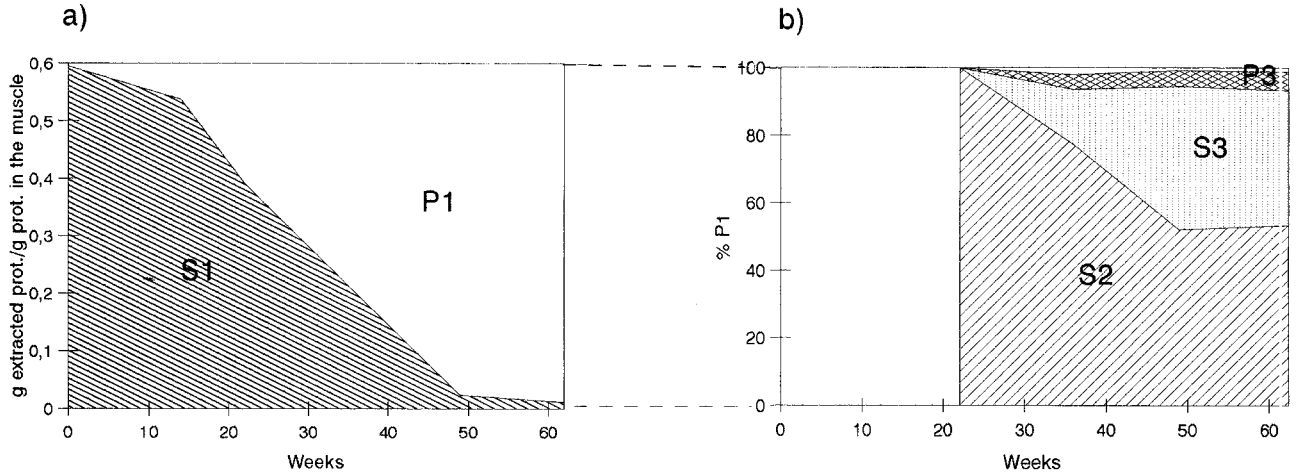

Figure 1. (a) Grams of protein extracted with 0.6 M NaCl (S1) per gram of protein in the muscle; (b) protein extracted in 2% SDS (S2), 2% SDS + 5% β-mercaptoethanol (S3), and an insoluble fraction (P3) as percentages of total aggregate insoluble in 0.6 M NaCl (P1), from minced cod stored at −20 °C. Crude protein in the mince: 17.8%. Protein nitrogen (N × 6.25) in the mince: 14.4%.

volumes of 2.5% sodium dodecyl sulfate (SDS) (Merck, Darmstadt, Germany) stirred with a magnetic stirrer for 10 min at room temperature. After centrifugation (Sorvall RT6000B, DuPont) for 15 min at 5000*g*, it was washed again with one volume of 2% SDS and recentrifuged. This was fraction 2. Any remaining aggregate (P2) was treated with 2% SDS plus 5% β-mercaptoethanol (ME) (Merck) as before, to obtain fraction S3 and in some cases an insoluble precipitate (P3). The purpose of these two extractions was to break down non-covalent bonds and the disulfide bond, respectively. The amount of soluble protein in fractions S2, S3, and P3 was determined by Kjeldahl (AOAC, 1984).

Polyacrylamide Gel Electrophoresis. All extracted fractions were analyzed by SDS-PAGE in a Phast-system 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 (Merck)) and then heated for 5 min in a boiling water bath. Samples were then centrifuged (Sorvall Microspin 24S, DuPont) at 10,000*g* for 1 min. Aliquots of 1 μL each, 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); Phast-system users manual, 1990). The protein gels were scanned on a 3CX image analyzer (Bio Image and Visage, Millipore Corp., Ann Arbor, MI). Electrophoretic profiles and the integrated optical density (IOD) of the Myosin heavy chain (MHC) and actin (Ac) bands were obtained. The molecular weights (MWs) of the main proteins in the samples were estimated by comparing their mobility with that of a standard high-MW protein mix (ferritin, 220K subunit; albumin, 67K; catalase, 60K subunit; lactate dehydrogenase, 36K subunit and ferritin, 18.5K subunit; Pharmacia). For the quantitative measurement of the MHC and Ac bands, the integrated optical density was previously checked for linearity.

Size Exclusion Chromatography. The 0.6 M NaCl soluble fractions (S1) were analyzed by size exclusion chromatography (SEC) using a Pharmacia column (length, 55 cm; diameter, 2.5 cm) filled with Bio-Gel A-50 m gel (medium) filler and an UV detector (Model UV-1, Pharmacia). The column was equilibrated with 0.6 M NaCl, pH 7.0 (trismaleate). Pending analysis, S1 fractions were stored at −18 °C in 50% glycerol (v/v) and used after overnight dialysis [0.6 M NaCl, pH 7.0 (trismaleate), 1 mM phenylmethanesulfonyl fluoride]. A 2-mg sample of dialyzed protein (2−3 mL) was filtered (0.8−8 μm) and applied onto the column, collecting fractions of 5.5 mL. The A_{280} detector's sensitivity was adjusted to 0.1, and the flow rate was held constant at 0.5 mL/min. The molecular weights of the peaks was estimated by comparing their mobility with that of dextran blue (2000K), aldolase (158K), and tiroglobuline (669K) (Pharmacia).

Transmission Electron Microscopy. Precipitates P1, P2, and P3 and extracted fractions S1, S2, and S3 were examined by transmission electron microscopy (TEM). The precipitates were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, postfixed in 1% osmium tetroxide, washed, dehydrated on acetone, and finally embedded in Epon 812 (Electron Microscopy Sciences, Washington). Thin sections were stained with 2% uranyl acetate followed by lead citrate. Electron micrographs were obtained with a Zeiss Model 902 electron microscope (Carl Zeiss, Oberkochen, Germany). Extracted fractions S were diluted with 0.05 M tris buffer, pH 7.2, at 0.15 mg/mL protein concentration. Staining with 1% uranyl acetate was performed using the method described by Jarenbäck and Liljemark (1975).

Statistical Analysis. A one-way analysis of variance was performed using an *F* test. The differences of means between pairs were resolved by a LSD test to obtain the confidence intervals. The level of significance was set for $P < 0.05$.

RESULTS AND DISCUSSION

Extractability. The initial amount of NAM extracted from the frozen cod mince with 0.6 M NaCl (S1) was 85 mg/g of mince, and this changed very little until week 14 (Figure 1). Thereafter, extractability decreased rapidly, and by the end of the storage period there was virtually no solubilization with 0.6 M NaCl. As storage progressed there was an increase in the amount of protein extracted upon addition of 2% SDS, but a decrease in the proportion as a percentage of aggregate P1 (Figure 1b). The SDS insoluble residues (P2) were partially extracted with 2% SDS + 5% ME (fraction S3). A residue (P3) was left, which increased with storage time, although it remained relatively stable as a percentage of the aggregate insoluble in 0.6 M NaCl (Figure 1b).

The protein extracted with non-covalent disrupting agents (fractions S1 + S2) fell about midway between the values reported by Laird et al. (1980) and Connell (1975) for cod frame mince and whole cod, respectively. Treatment with a combination of a disulfide bridge-reducing agent and SDS produced a substantial increase in the amount of protein extracted during the later stages of frozen storage, thus highlighting the importance of disulfide covalent bonds in aggregation of minced cod, as previously pointed out by Lim and Haard (1984) in minced Greenland halibut and Owusu-Ansah and Hultin (1986) in red hake fillets.

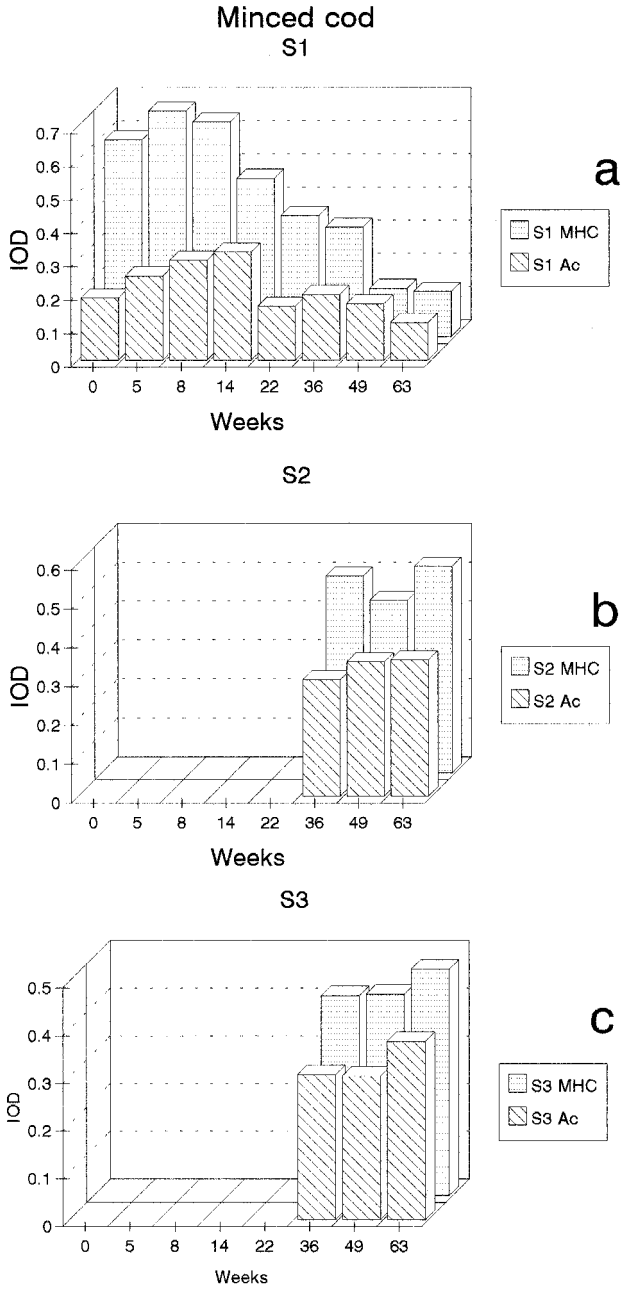

Figure 2. Integrated optical density (IOD) per microgram of protein extracted of myosin heavy chain (MHC) and actin (Ac) obtained from SDS-PAGE (12.5%) of fractions S1 (a); S2 (b), and S3 (c). S fractions obtained as in Figure 1.

SDS-PAGE. When the fractions extracted in the various agents were analyzed by SDS-PAGE, it was found that the IOD of MHC decreased significantly per microgram of protein in extract S1 (Figure 2a; Table 1) while the proportion of Ac proved more stable, although it peaked around week 14. As frozen storage advanced, the salt-extracted protein became enriched with proteins of lower molecular weight than actin [tentatively troponins (TNs) and tropomyosins (TM)] (Figure 3) and part of the protein extracted with 0.6 M NaCl did not enter the resolving gel. Loss of salt-extractability was accompanied by a gradual change in the protein composition of the supernatant, which became increasingly pronounced with storage. In fractions S2 and S3, MHC and Ac were found in larger proportion than in S1 and this proportion remained more stable over storage (Figure 2b,c; Figure 4a,b).

Table 1. Analysis of Variance of the IOD of MHC and Ac Obtained from the Electrophoresis of the Protein Solubilized in the Fractions S1, S2, and S3[a]

		week							
		0	5	8	14	22	36	49	62
MHC	S1	a	b	a, b	c	d	d	e	e
	S2						a, b	a	b
	S3						a	a	b
Ac	S1	a, b	b, c	c, d	d	a, e	a, b	a, e	e
	S2						a	a	a
	S3						a	a	b

[a] Different letters in the same row indicate significant differences in time ($P < 0.05$).

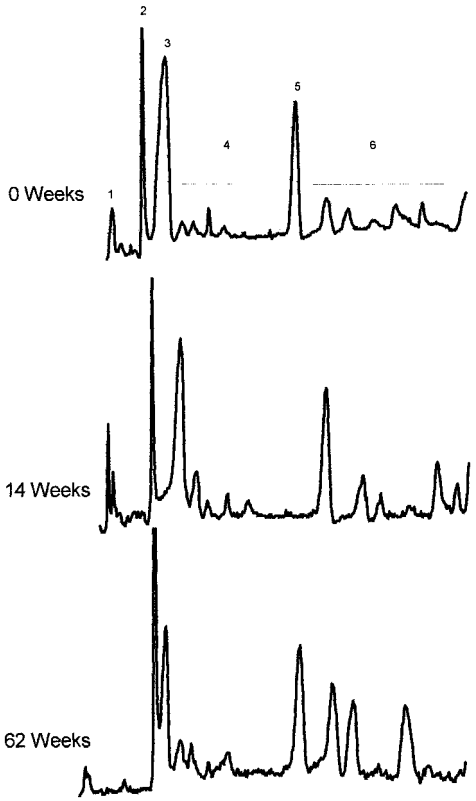

Figure 3. SDS-PAGE of fraction S1 from minced cod extracted at 0, 14, and 62 weeks of frozen storage: (1) application zone; (2) peak in the stacking/resolving interphase; (3) myosin heavy chain; (4) proteins of MW between 200- and 45K; (5) actin; (6) tropomyosin, troponins, and myosin light chains. S1 fraction obtained as in Figure 1.

A decrease in the proportion of MHC in the salt-extracted fraction during frozen storage has been detected by other authors (Dingle and Hines, 1975; Laird and Mackie, 1981; Matthews et al., 1980; Lim and Haard, 1984; Owusu-Ansah and Hultin, 1986; Ragnarsson and Regenstein, 1989; Leblanc and Leblanc, 1989; among others). Some authors have also found that during frozen storage there was a concomitant increase of high-MW bands which did not enter the gel (Matthews et al., 1980; Lim and Haard, 1984; Owusu-Ansah and Hultin, 1986). Laird and Mackie (1981) and Leblanc and Leblanc (1989) reported an increase in low-MW bands in cod at the end of storage, coinciding with our findings. The present results showed that although the proportion of actin in S1 remained relatively unchanged over the experimental period, the total amount of this protein tended to decrease, and more was found in the S2 and S3 fractions. This confirms that actin also becomes insolubilized during frozen storage.

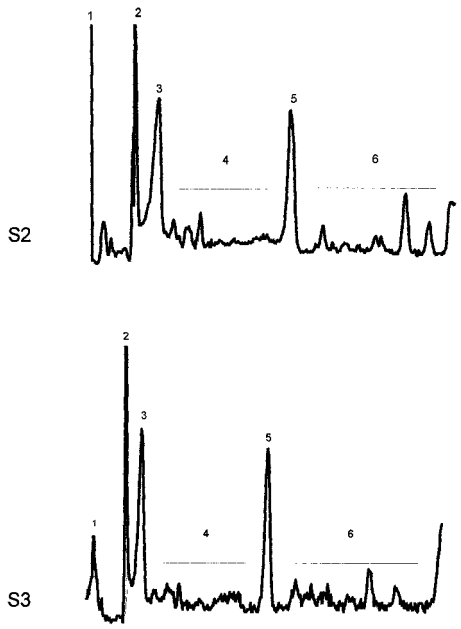

Figure 4. SDS-PAGE of fractions S2 and S3 from minced cod extracted at 49 weeks of frozen storage. Fractions were obtained as in Figure 1. Peaks are as in Figure 3.

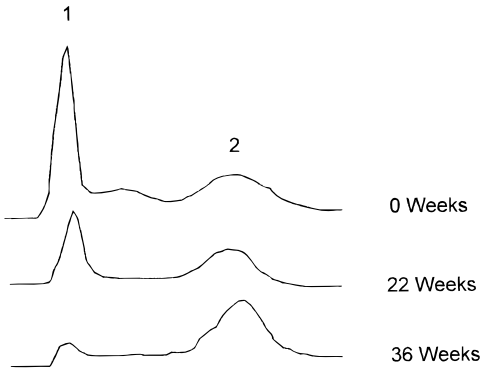

Figure 5. Size exclusion chromatograms of fraction S1 from minced cod extracted at 0, 22, and 36 weeks of frozen storage. Peak 1: MW ≥ 2000K. Peak 2: maximum MW 355K. S1 fraction obtained as in Figure 1.

SEC. Figure 5 shows the S1 fractions examined by SEC up to 36 weeks of frozen storage. At the outset, two major peaks were observed: peak 1, MW > 2000K; and peak 2, a MW maximum between 100 and 355K. With SDS-PAGE, peak 1 was initially predominantly MHC and Ac and peak 2 Ac and lower MW bands (results not shown). Peak 1 declined as frozen storage progressed and by week 36 was considerably lower than peak 2. Electrophoretic analysis of extract S1 retained in the filter at week 22 revealed bands of MHC, Ac, proteins of MW lower than actin [tentatively TM, TNs, myosin light chains (MLCs)], and an aggregate not incorporated in the gel. Under TEM, the proteins found in the material retained in the filter were aggregated (results not shown). Using SEC, Umemoto et al. (1971) also found that actomyosin (corresponding to peak 1 in our results) in frozen Alaska pollack surimi declined as storage progressed, coinciding with the present work.

TEM. *Aggregate Morphology.* The TEM micrographs of the insoluble aggregates (P1) obtained at week 22 of frozen storage at −20 °C (Figure 6) show visible muscle fiber residues, exhibiting sarcomere-like structures. There was a visible A band, but thick myosin filaments were not visible at the H line, and there was no sign of a Z line. Residues from the sarcoplasmic reticulum was also apparent, as previously found by Jarenbäck and Liljemark (1975).

Figure 6. Precipitate insoluble in 0.6 M NaCl from muscle stored for 22 weeks showing myofibril residues with remains of A band (−A−). Sarcoplasmic reticulum remains (*) are indicated. Bar = 0.6 μm.

Parts a−c of Figure 7 show the precipitates after extraction with 0.6 M NaCl (P1), 2% SDS (P2), and 2% SDS + 5% ME (P3), respectively, at week 36. The insoluble fraction (P1) (Figure 7a) contained muscle fiber residue displaying sarcomere-like structures; the A band appeared more intact than in the sample stored for 22 week (Figure 6). Typical individual thick filaments were not apparent. Material in the H zones was scarce. Moreover, broken thin filaments appeared anchored to a disorganized Z line. In fraction P2 (Figure 7b) part of the protein, mainly from the A band, had been extracted, and the Z line had also disappeared. However, from the material remaining in the A band after SDS treatment, it would seem that the number of bonds remaining intact after 2% SDS treatment was sufficient to retain a structure reminiscent of the original sarcomere. In insoluble fraction P3 (Figure 7c) no organized structure was observed. This suggests that in minced cod muscle stored for 8 months, disulfide bridge formation played a part in the loss of muscle extractability.

Fractions P1, P2, and P3 from samples stored for 62 weeks (Figure 8a−c, respectively) were comparable to those obtained after 36 weeks of frozen storage. The main difference was that the sarcomere structures were more marked. The residual myofibrils remaining after actomyosin extraction with 0.6 M NaCl (P1) (Figure 8a) exhibited a highly organized A band in which individual thick filaments were apparent. These filaments were detected through and beyond the H zone. Intact actin filaments anchored to a broken Z line were also more abundant at 62 weeks. After treatment of P1 with SDS and SDS + ME, the ultrastructure is shown in Figure 8b,c, respectively. Protein extraction from the sarcomere was less effective than in muscle stored for 36 weeks, so the sarcomere structure was retained even after treatment with 2% SDS + 5% ME (P3). Ag-

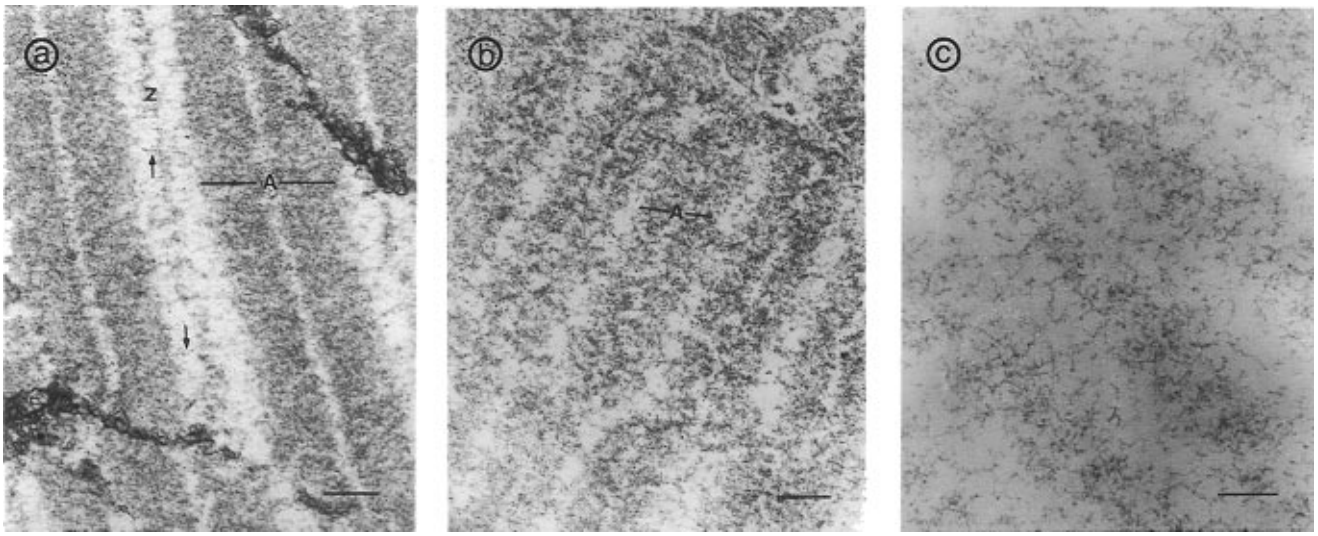

Figure 7. Precipitates from muscle stored for 36 weeks. (a) Precipitate insoluble in 0.6 M NaCl (P1). The A band (−A−) shows a better definition than in Figure 6. Broken thin filaments (→) appear anchored to a broken Z line (Z). Bar = 0.4 μm. (b) Precipitate insoluble in 2% SDS (P2) showing remains of A band (−A−). Note that the sarcomere structure is not so clear as in Figure 7a. Bar = 0.4 μm. (c) Precipitate insoluble in 2% SDS + 5% ME (P3) showing no sarcomere residue. Note the scarce material retained in the A band. Bar = 0.25 μm.

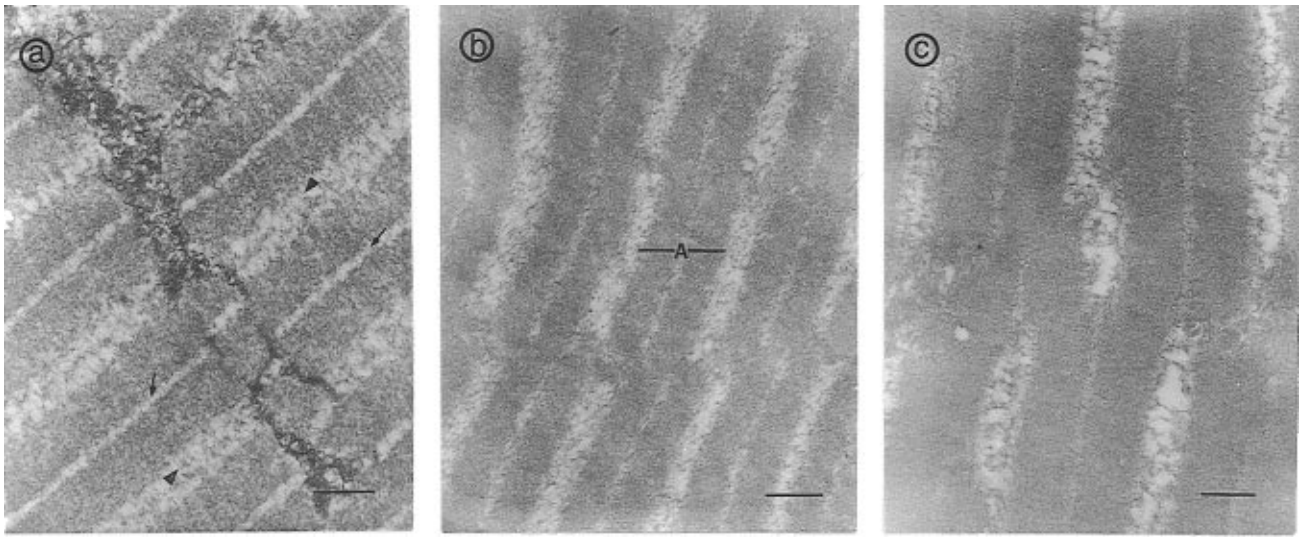

Figure 8. Precipitates from muscle stored for 62 weeks obtained as in Figure 7. (a) Precipitate P1 shows sarcomere structure more marked than in Figure 7a. Individual thick filaments (→). Thin filaments (▼) are more abundant than in samples stored for 36 weeks. Bar = 0.4 μm. (b) Precipitate P2. The amount of material retained in the A band is greater than in Figure 7b. Bar = 0.4 μm. (c) Precipitate P3. Sarcomere structure is similar to that in b, although the A band is more disorganized. Note the greater amount of proteins in the A band compared with Figure 7c. Bar = 0.4 μm.

gregates from muscle stored for 49 weeks exhibited intermediate states between that of muscle stored for 36 and 62 weeks (figures not shown).

These results suggest that as storage progressed, disulfide bridges and other covalent bonds were formed, thus giving rise to precipitates of varying composition. At the outset it was shown that much of the myosin and actin in the precipitates had been extracted by the different agents with loss of sarcomere-like structure, the more so the harsher the treatment, and by week 36 there was scarcely any appreciable structure in the aggregate treated with 2% SDS + 5% ME. However, at 62 weeks of frozen storage the sarcomere structure was retained in part even after treatment with 2% SDS + 5% ME.

Supernatant Morphology. Figure 9 shows the protein extracted with a solution of 0.6 M NaCl (S1) at 0 time. In this supernatant numerous actin filaments, decorated with other proteins, were found having an average length of 5 μm; these were grouped in sheaves of two or three parallel filaments, alternating with other, randomly associated and generally shorter filaments. In some cases filaments were found crossed at angles ranging from 94° to 108°, which suggests the presence of covalent bonds. These filaments coexisted alongside protein microaggregates, with which they formed associations.

Fraction S1, extracted with 0.6 M NaCl solution from minced cod muscle after 36 weeks of frozen storage (Figure 10a), exhibited shorter filaments in association with globules mutually interconnected. Also visible were numerous microaggregates. This ultrastructure has been previously observed by Jarenbäck and Liljermark (1975). Filaments cross-linking at angles ranging from 94° to 108° were also detected more often than in the initial supernatant and could provide the basis for the ring-shaped structures found in incipient form in fraction S1 at 0 time. In fraction S2 (Figure 10b) the ring-shaped structures were more clearly appreciable than in S1. The S3 fractions (Figure 10c) displayed

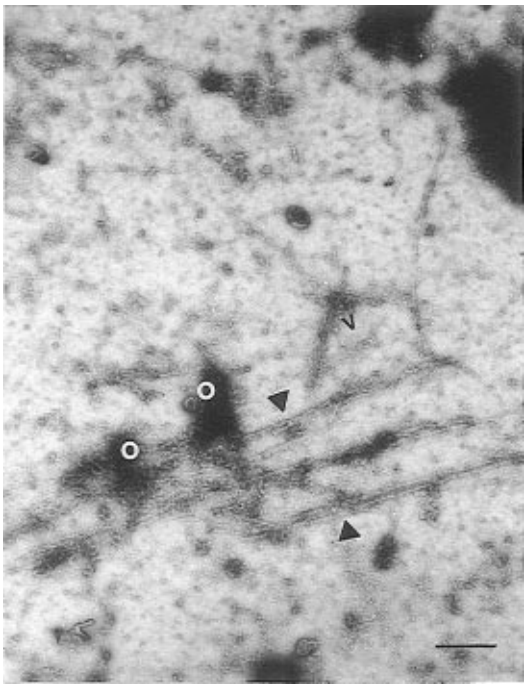

Figure 9. Fraction S1 at zero time. Actin filaments (▼), angles ranging from 94° to 108° (>), and microaggregates (○) are indicated. Bar = 0.6 μm. Fraction obtained as in Figure 1.

ring-shaped structures similar to the S2 fractions but not as well-defined, perhaps due to the fact that the P2 aggregates from which they were extracted were less highly organized.

S1 fractions from minced cod muscle extracted with 0.6 M NaCl solution after 49 weeks of storage are shown in Figure 11. In this extract loose filaments having an average length of 1 μm were detected (Figure 11a), along with many more ring-shaped structures and microaggregates (Figure 11b) larger than those shown in Figure 10a. Fractions S2 and S3 displayed the ring-shaped structures and hardly differed at all from extracts taken at 36 weeks of storage.

Therefore, TEM showed that from the onset of storage, the morphology of the proteins in solution generally tended to alter, with formation over time of more and larger aggregates and increasingly shorter filaments.

Taken together, the results show that the natural actomyosin fraction of minced cod stored at −20 °C altered over time in terms of the amount, proportion, and morphology of proteins extractable in 5% NaCl, 2% SDS, and 2% SDS + 5% ME.

In the weeks of maximum extractability in 0.6 M NaCl (i.e., up to 14 weeks; Figure 1), there was no significant variation in the proportion of extracted proteins as determined by SDS-PAGE (Figure 2, Table 1). As extractability in salt declined, the morphology of the proteins in solution altered. This was directly observed with TEM (increased aggregate size: Figures 9, 10a, and 11b) and indirectly in SEC (Figure 5) as a decrease in the actomyosin peak. There was a corresponding increase in protein retained in the filter. The proportion of proteins in solution also altered, although more gradually, so that by the end of storage there was a smaller percentage of myosin and a larger percentage TM and TNs (Figure 3).

The change in the proportion of proteins in 0.6 M NaCl can be attributed to two main causes: On the one hand, part of the protein becomes insolubilized (P1) and passes into the fractions extracted with SDS (fraction S2) and SDS + ME (fraction S3) or forms an aggregate that is insoluble (P3); and on the other hand, a percentage of the protein that is extracted in fraction S1 forms covalent bonds and hence peaks of high molecular weight which do not enter the electrophoretic resolving gel.

In the fractions extraced in SDS and SDS + ME, chiefly myosin and actin were detected (Figure 4), which also tended to form aggregates visible under TEM (Figure 10).

Although greater in number and size throughout the storage period, most of the aggregates observed in all solutions under TEM break down under electrophoresis conditions.

CONCLUSIONS

The aggregates formed during frozen storage of minced cod are mostly linked by secondary interactions and disulfide bridges. Myosin and actin are the proteins mainly involved. These aggregates tend to grow in number and size, becoming insoluble in salt but still extracted in SDS and SDS + ME. There is also evidence

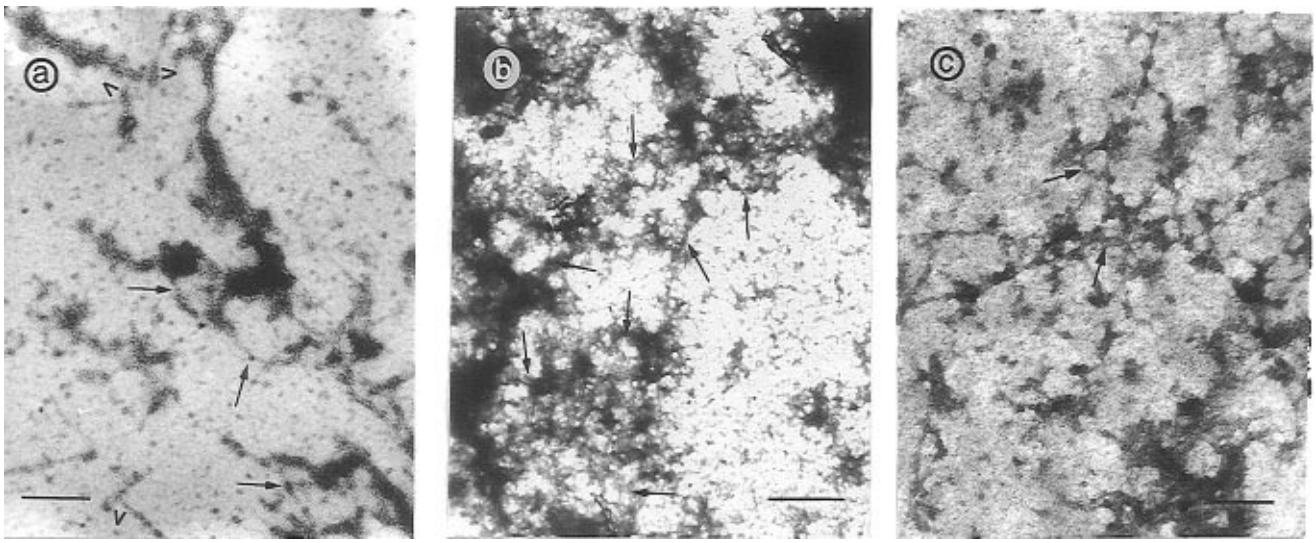

Figure 10. S fractions from muscle stored for 36 weeks obtained as in Figure 1. Ring shaped structures (→). (a) Fraction S1 showing angles ranging from 94° to 108° (>). Note the incipient ring shaped structures. Bar = 0.6 μm. (b) Fraction S2. Bar = 2.5 μm. (c) Fraction S3. Bar = 0.15 μm.

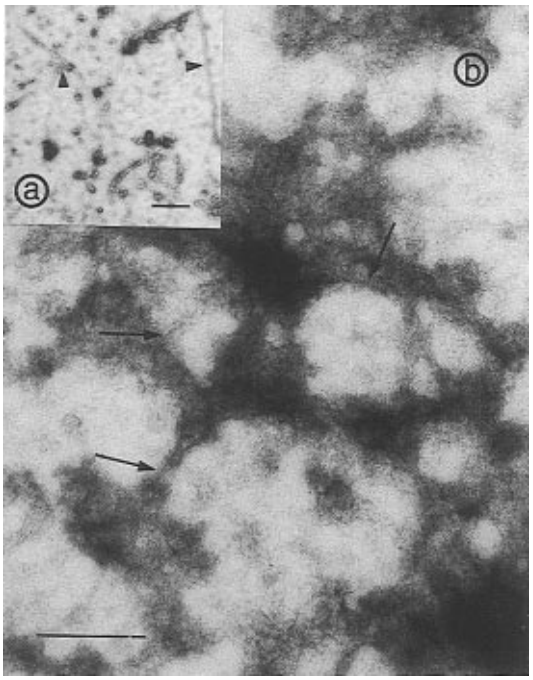

Figure 11. Fraction S1 extracted with 0.6 M NaCl from muscle stored for 49 weeks. (a) Actin filaments (▼) are shorter than at 0 time (Figure 9). Bar = 0.25 μm. (b) Note that the ring shaped structures (→) are more abundant compared with Figure 10a. Bar = 0.15 μm.

of the formation of non-disulfide covalent bonds, some occurring in aggregates extracted in salt, SDS, and SDS + ME, others appearing in the insoluble residue remaining after all three treatments.

The insoluble fractions showed a resemblance to sarcomere structure, which tended to become more pronounced the milder the solubilizing treatment and the longer the storage time. At the end of the storage period, an insoluble aggregate remained in which the sarcomere structure was partially preserved.

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