Aggregation of Myofibrillar Proteins in Hake, Sardine, and Mixed Minces during Frozen Storage

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The effect of frozen storage on the loss of extractability of natural actomyosin (NAM) with 0.6 M NaCl (P1) was studied in hake (*Merluccius merluccius*), sardine (*Sardina pilchardus*), and mixed minces in varying proportions stored for up to 1 year at −20 °C. The objective was to ascertain the types of bonds established among the proteins. The unextracted NAM was treated with different extracting agents to cleave secondary bonds (2% SDS, 2% SDS + 8 M urea) and secondary and disulfide bonds [2% SDS + 5% β-mercaptoethanol (ME)]. There was more NAM extracted from the mixes with higher percentages of sardine, although this effect was weaker than might have been expected from the percentages of sardine in the mixes. In hake, for up to 8 months of storage, the bulk of the proteins not extracted in 0.6 M NaCl was extracted with SDS + urea or SDS + ME, with extraction declining as storage progressed. This suggests that the amount of protein linked by covalent bonds increased with time. In sardine and mixed lots, a high percentage of proteins linked by covalent bonds was detected earlier, although no clear pattern emerged in terms of hake/sardine ratios. SDS−PAGE showed that treatment with SDS + urea and SDS + ME extracted protein in the form of small aggregates that were retained in the stacking gel. These aggregates were also found in the SDS extracts from the sardine and mixed minces.

Keywords: *Hake; sardine; frozen storage; aggregates; solubilization*

INTRODUCTION

Fish species differ in their susceptibility to protein denaturation and aggregation during frozen storage. There have been numerous reports of a relationship in muscle of lean species of the gadiform order between changes in muscle texture and myofibrillar protein extractability and the formation of formaldehyde (FA) and dimethylamine (DMA) from trimethylamine oxide (TMAO) [see review by Haard (1990); Careche and Reece, 1992; Hultin, 1992; Sotelo et al., 1995]. In recent studies we have observed changes in the absolute and relative amounts of different bonds formed by proteins in lean species during frozen storage, leading over time to an increase in covalent bonds. Depending on species and the time and temperature of storage, the proteins linked by covalent bonds form aggregates that remain in solution or suspension or are unextractable in 0.6 M NaCl (Tejada et al., 1996; Del Mazo et al., 1997).

In model systems made with isolated natural actomyosin (NAM), addition of FA reduced the solubility of NAM in saline solutions and Ca^{2+}-ATPase activity. Both decreased more when the system was frozen (Del Mazo et al., 1994). Loss of NAM solubility and differences in the percent of covalent bonds involved in protein aggregation induced by added FA have been previously reported (Ang and Hultin, 1989; Cofrades, 1994), and they have been found to vary according to species (Tejada et al., 1997).

Changes in muscle proteins have also been associated with lipid oxidation occurring during frozen storage (Takama, 1974a,b; Jarenback and Liljemark, 1975; Sikorski, 1977; Shenouda, 1980; Lilliard, 1987; Jahncke et al., 1992). It has been suggested that free radicals can react with protein side chains (Srinivasan and Hultin, 1995) and that the carbonyl groups of the oxidized lipids may participate in covalent bonding to form stable protein−lipid aggregates (Sikorski and Kolakovska, 1994). However, changes in texture during frozen storage are less apparent in fatty species (Careche and Tejada, 1991a).

When free fatty acids are added to isolated NAM, which is then stored frozen, the lipids interact with both the proteins soluble in 0.6 M KCl and those that become insoluble during storage; this has a detrimental effect on protein functionality and Ca^{2+}-ATPase activity (Careche and Tejada, 1994). However, when triolein was added, no detrimental effect was found, although there were lipid−protein interactions (Careche and Tejada, 1991b). When neutral and oxidized lipids are added to hake muscle, there is less DMA and FA formation and less loss of functionality during frozen storage (Careche and Tejada, 1990a). In model systems, we found that DMA formation differed according to lipid oxidation and that the ability of trimethylamine *N*-oxide demethylase (TMAO-ase) to catalyze the reaction is influenced by the redox potential (Joly et al., 1997).

The objective of this study was to determine what types of aggregates form during frozen storage of minced muscle of hake, sardine, and a mixture of the two, on the assumption that in mixed minces the formation of FA and DMA from TMAO and the generation of lipid oxidation products would occur simultaneously during frozen storage. The ultimate aim of this work is to find a natural system in which it is possible to interfere with

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Table 1. Proximate Analysis and pH (Mean ± Standard Deviation, $n = 5$)

lot	% crude protein	% crude fat	% moisture	% ash	pH
A	16.13 ± 0.04	0.77 ± 0.13	82.67 ± 0.15	1.04 ± 0.05	6.91 ± 0.03
B	16.85 ± 0.22	2.89 ± 0.22	79.10 ± 0.13	1.11 ± 0.07	6.75 ± 0.10
C	17.63 ± 0.32	5.58 ± 0.21	75.82 ± 0.26	1.12 ± 0.02	6.47 ± 0.07
D	18.18 ± 0.52	7.51 ± 0.31	72.31 ± 0.18	1.16 ± 0.04	6.34 ± 0.12
E	18.53 ± 0.35	11.33 ± 0.59	69.03 ± 0.30	1.24 ± 0.08	6.15 ± 0.15

the reactions that cause protein aggregation during frozen storage.

MATERIALS AND METHODS

Species Used. Hake (*Merluccius merluccius*) and sardine (*Sardine pilchardus*) were caught in June 1994 off the northwest coast of Spain and brought to the laboratory by refrigerated transport, packed in ice in expanded polystyrene boxes. Maximum time before processing at the laboratory was 48 h. Average fish weights were 1160 and 56.2 g for hake and sardine, respectively, and average sizes were 50.8 (±7.2) cm and 15.4 (±1.81) cm. The fish were headed, gutted, and washed with iced water to remove blood, etc. The flesh was minced in a Baader model 694 deboner (Baader, Lübeck, Germany) using a drum with 3 mm diameter holes to remove skin and bones. The mince was kept below 7 °C during subsequent handling. For each species, 36 kg of fish was used to prepare the minces.

Preparation of Lots. Five lots were prepared with varying proportions of minced hake and sardine muscle: lot A (100% hake); lot B (75% hake + 25% sardine); lot C (50% hake + 50% sardine); lot D (25% hake + 75% sardine); and lot E (100% sardine). Samples were homogenized in a Stephan Universal model UM5 homogenizer (Stephan u Söhne GmbH & Co., Hameln, Germany), coolant temperature −2 °C, for 30 s at setting I. All lots were placed in trays 5 cm deep and immediately frozen at −30 °C in a Sabroe Benjamin (SMC 4-65) model horizontal plate freezer (Sabroe, Aahrus, Denmark) for 5 h. Each lot weighed 6.4 kg. The frozen minces were cut in portions, vacuum-sealed (10.66×10^3 Pa pressure) individually in plastic bags (Cryovac BB-1, Duncan, SC; oxygen permeability at 23 °C = 60 $cm^3/24\ h/m^2/atm$) and kept for up to 1 year at −20 °C.

Proximate Analyses and pH. Crude protein content was analyzed according to the method of Kjeldahl (AOAC, 1995), 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 (1984) recommended methods. The results were expressed as percentage of the mince. The pH was determined according to the procedures of Vyncke (1981) at room temperature. All determinations were performed in quintuplicate.

Extraction of Protein. NAM was extracted with 0.6 M NaCl from 100 g of the minces according to the method of Kawashima et al. (1973) as follows: The mince 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* for 15 min (0−5 °C) (RC5B 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, which was added to the previous protein extract. After standing 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, urea, and β-mercaptoethanol (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 an ice−water bath 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). NaCl (3 M; 50 mM tris maleic, pH 7.0) was added to the precipitate to bring the concentration up to 0.6 M NaCl. The mixture was filtered through three layers of cheesecloth to remove any adhering traces of connective tissue and then dialyzed against 0.6 M NaCl (50 mM tris maleic, pH 7.0) overnight in a refrigeration chamber. This dialyzed fraction was denominated extracted NAM in 0.6 M NaCl.

For each lot, the protein remaining after the NAM was isolated (fractions P1, formed during frozen storage) was isolated was divided into aliquots and treated with different extracting agents [1:4 (w/v) P1/extracting agent]. To cleave secondary interactions, aliquots from P1 fractions were subjected to different treatments: 2% SDS (Merck, Darmstadt, Germany) with magnetic stirring for 10 min; 10% SDS with 20 h of magnetic stirring; and 2% SDS + 8 M urea (Panreac, Barcelona, Spain) with 20 h of magnetic stirring. Secondary interactions and disulfide bonds were cleaved with 2% SDS + 5% ME (Merck) with 20 h of magnetic stirring. Samples were then centrifuged (18000*g*, 10 min) to separate the proteins extracted with each treatment (fractions S: S_{SDS}, $S_{SDS+urea}$, and S_{SDS+ME}) and the unextracted fractions (precipitates: P2). For rapid measurement of protein concentration in fractions S, protein was determined according to the Lowry method (Lowry et al., 1951); for samples treated with ME, the Bradford (1976) method was used. As the amount of protein measured by these two methods may vary when proteins aggregate in frozen storage, preventing the reagents from reaching some locations in the protein, the final amount of protein was contrasted in SDS and SDS + ME lots by Kjeldahl (AOAC, 1984). Protein in the P1 precipitates was measured by Kjeldahl (AOAC, 1984).

Polyacrylamide Gel Electrophoresis (PAGE). All S fractions were analyzed by SDS−PAGE in a Phast system horizontal apparatus (Pharmacia-Biotechnology, Uppsala, Sweeden), using 12.5% polyacrylamide gels. Samples were treated according to the method of 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 Co) at 10000*g* for 1 min. One microliter aliquots containing 1 mg/mL of protein were applied to the gels. Electrophoresis conditions were 4 mA/gel, 250 V, and 3 W. Electrophoretic profiles were analyzed on a 3CX image analyzer (Bio Image and Visage, Millipore Corp, Ann Arbor, MI). The molecular weight (MW) of the main proteins in the samples was estimated by comparing their mobility with a standard high MW protein mix (ferritin, 220 000 subunit; albumin, 67 000; catalase, 60 000 subunit; lactate dehydrogenase, 36 000 subunit; and ferritin, 18 500 subunit; Pharmacia).

RESULTS AND DISCUSSION

Proximate Analyses and pH. The proximate analyses and pH of the hake, sardine, and mixed lots are shown in Table 1. The values for hake and sardine were found to be within the expected values for these species and the season.

NAM Unextracted in 0.6 M NaCl. The percentage of NAM not extracted in 0.6 M NaCl (P1) during frozen storage of the minces is shown in Figure 1. In hake, practically none of the NAM was extracted by the fifth

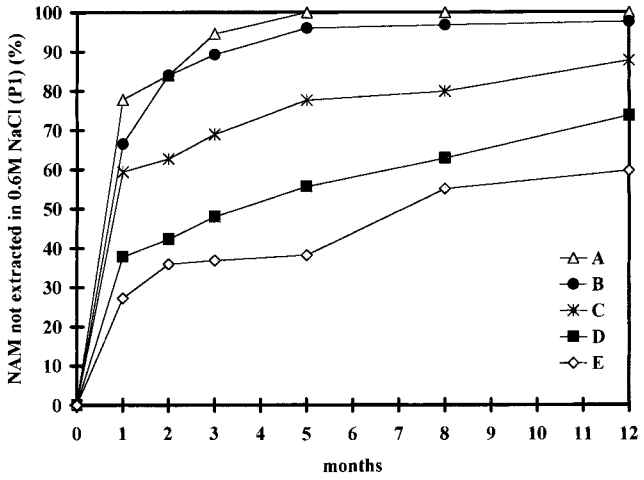

Figure 1. Percentage of NAM not extracted in 0.6 M NaCl (P1) from minces stored at −20 °C for 12 months: (A) 100% hake; (B) 75% hake + 25% sardine; (C) 50% hake + 50% sardine; (D) 25% hake + 75% sardine; (E) 100% sardine.

month of storage, whereas 40% of the NAM in sardine was still extractable at the end of a year. Such species-specific differences in extractability have been reported in previous studies of protein solubility in 5% NaCl (pH 7) during frozen storage (Careche and Tejada, 1990a,b, 1991a; Huidobro and Tejada, 1992). The losses of NAM extractability in lean species were similar to those found in hake, bearing in mind that myofibrillar protein in muscle is estimated to be 66−77% of the total proteins in skeletal muscle (Suzuki, 1981). The fact that there is more insoluble protein in hake than in sardine muscle may be attributed to the reaction of FA in lean species during frozen storage (Owusu-Ansah and Hultin, 1987; Tejada and Careche, 1988; Huidobro and Tejada, 1992). In hake/sardine minces, the percentage of NAM not extracted in 0.6 M NaCl was greater the higher the proportion of hake in the mix (B > C > D). The aggregated protein in lots B and C was higher than might have been expected from the percentage of hake in the mixed minces.

Extraction of Proteins from P1 with Extracting Agents. The amount of protein extracted from fractions P1 with 10% SDS for 20 h (results not shown) was not significantly different from that achieved with 2% SDS and 10 min of stirring. Therefore, the latter was considered adequate for cleavage of secondary protein−protein bonds.

The percentage of protein extracted from fraction P1 with 2% SDS (S_{SDS}) after 5 months of storage was 26.7% in hake (lot A) (Figure 2), as compared to 44.4% in sardine (lot E). Both percentages were much lower than for minced cod stored at the same temperature, for which SDS extracted all of the protein up to 22 weeks of storage (Tejada et al., 1996). In the mixed lots the percentages of NAM extracted with SDS from P1 were 40.2, 36.7, and 44.4% for lots B, C, and D, respectively. The P1 fractions therefore behaved more like sardine. In all lots the percentage of protein extracted from P1 decreased with storage time; practically no protein was extracted by SDS after 12 months.

When P1 fractions were treated with 2% SDS + 8 M urea ($S_{SDS+urea}$), after 5 months of storage nearly all of the protein was extracted from lots A and C (95.6 and 95.2%, respectively)—far more than with 2% SDS. The percentage of protein extracted was comparable to extraction with 2% SDS in lot B (37.0%) and lower in lots D and E (22.5 and 28.8%, respectively). The amount of protein extracted with 2% SDS + 8 M urea also decreased in all lots as storage progressed. The highest percentage of protein extracted at 1 year was found in lot E (11.5%) and the lowest in lot A (6.8%). Intermediate values were found for lots containing both types of muscle (7.7% in lot B, 7.5% in lot C, and 7.3% in lot D). The percentage of protein extracted from the aggregates with 2% SDS + 8 M urea was similar in lots A and C, whereas in lots B and D the pattern was closer to that of lot E.

Both 2% SDS (Tejada et al., 1996) and 8 M urea (Tsuchiya et al., 1979) are thought to cleave secondary interactions. Urea is considered a denaturant and a solubilizing agent and is widely used for solubilization and unfolding of proteins by destabilizing the hydrogen bonding and hydrophobic interactions. In β-lactoglobulin and bovine serum albumin, Xiong and Kinsella (1990) reported a decrease in SH content when urea was present, suggesting that the disappearance of SH, presumably via oxidation, was induced or facilitated by urea. This interchange of SH−SS reactions induced by urea is accompanied by polymerization of the proteins, which may result in the formation of artifacts during extraction. This could account for the fact that less protein was extracted with SDS + urea than with SDS alone from minces with high percentages of sardine (lots D and E). The differences in protein extraction in SDS and SDS + urea among lots suggest that the type of P1 fraction was initially different in lots A and C from that in lots B, D, and E.

When the P1 fractions were treated with 2% SDS + 5% β-ME (S_{SDS+ME}), it was found with lot A that, although all of the NAM was not extractable in 0.6 M NaCl from 5 months onward (Figure 1), P1 fractions changed during storage, so that after 8 months, 98% of the protein was extracted by cleavage of secondary and disulfide bonds, whereas after 12 months this was the lot from which the least percentage of protein was extracted (20%). This points to the initial importance of disulfide bonds in the loss of salt extractability during frozen storage of hake minces and to the further formation of non-disulfide covalent bonds, which prevent the extraction of protein. Similar results have been reported in cod by Tejada et al. (1996) and Careche et al. (1998) and in hake by Torrejón (1996) and Del Mazo (1997). In sardine the amount of P1 formed was smaller than in lot A (Figure 1); however, only 33.1% of the protein was extracted from P1 with this treatment after 8 months. In the mixed lots there was very little change with time in the percentage of protein extracted from P1, which remained higher in lots C and D than in lot B. In all mixed lots the cleavage of secondary and disulfide bonds with this treatment increased the amount of protein extracted from P1 compared to the other treatments after both 8 and 12 months of storage. The increase was most evident in lot D, followed by lots C and B. For lot B (75% hake + 25% sardine), although the NAM extracted by 0.6 M NaCl was close to 0 as in lot A (100% hake) (Figure 1), 2% SDS + 5% β-ME extracted much less protein from P1 and was closest to lot E.

SDS−PAGE. The electrophoretic profiles of the proteins extracted from P1 by 2% SDS (fractions S_{SDS}) are shown in Figure 3. In the hake (lot A), after 5 months of storage, S_{SDS} was largely myosin heavy chain (MHC) (peak 3) and actin (Ac) (peak 5). As storage progressed, MHC decreased in the extract. In the sardine (lot E),

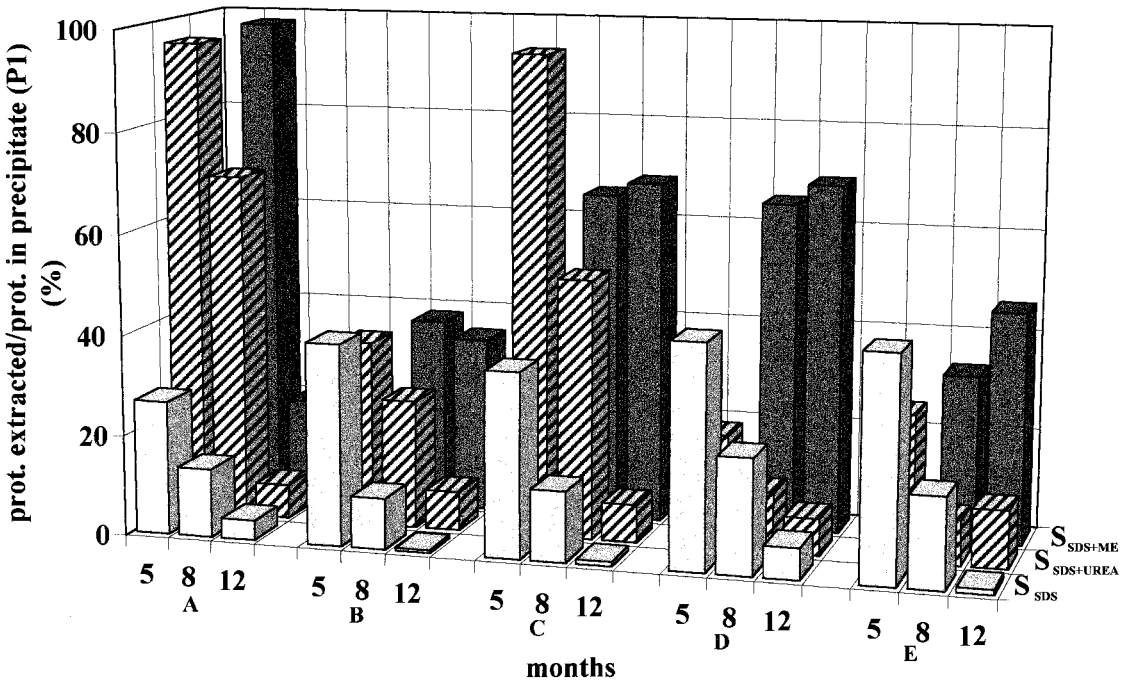

Figure 2. Percentage of NAM extracted from P1 in 2% SDS (S_{SDS}), 2% SDS + 8 M urea ($S_{SDS+urea}$) at 5, 8, and 12 months, and 2% SDS + 5% β-mercaptoethanol (S_{SDS+ME}) at 8 and 12 months. Lots are as in Figure 1.

after 5 months of storage the protein extracted with 2% SDS from P1 was composed largely of high MW bands, which did not enter the stacking gel (peak 1) or the resolving gel (peak 2). This indicates that the extracted protein formed microaggregates composed of proteins presumably linked by non-disulfide covalent bonds given that they remained after the sample was treated with ME for electrophoresis. After 12 months of storage, the major band for all samples was composed of aggregates that did not enter the resolving gel (peak 2). Such an increase in the intensity of the high MW protein bands not entering the gel during frozen storage has been reported previously (Matthews et al., 1980; Lim and Haard, 1984; Owusu-Ansah and Hultin, 1986; Tejada et al., 1996). Values in the mixed lots (B−D) were intermediate between those of lots A and E. At the outset there were protein aggregates that did not enter the gel (peaks 1 and 2) and there were MHC and Ac bands in the resolving gel. By the end of storage the major band was Ac, although MHC and peak 2 were visible in all lots.

Figure 4 shows the electrophoretic profiles of the fractions extracted from P1 by treatment with 2% SDS + 8 M urea (fractions $S_{SDS+urea}$). In lots A and C the profiles at 5 months of storage showed differences from the profiles for the S_{SDS} extracts (Figure 3). In these two lots the largest peak was the high MW band (peak 1) that did not enter the gel, whereas in lots B, D, and E the profile was similar to the fraction extracted with SDS alone. The MHC and Ac peaks were visible in lots B and D. The protein extracted with SDS + urea formed high MW microaggregates composed of proteins linked by covalent bonds; these microaggregates were presumably held together by secondary bonds. Extraction of these microaggregates would explain why more protein was extracted with SDS + urea than with SDS alone from P1 at 5 and 8 months of storage in lots A and C (Figure 2). As storage progressed, peak 1 diminished in all lots, which suggests that another type of protein−protein bond had formed, producing higher MW aggregates which were not extracted. Because of this, after 12 months, the electrophoretic profiles of the extracts obtained with 2% SDS + 8 M urea were more like those obtained by extraction with 2% SDS alone.

Figure 5 shows the electrophoretic profiles at 8 and 12 months of fractions S_{SDS+ME}. In lot A, although the amount of protein extracted decreased sharply between months 8 and 12 (Figure 2), the electrophoretic profile of the extract scarcely altered, with peak 1 the major peak. In both profiles the MHC peak appeared joined to peak 2, whereas the Ac peak was visible at both 8 and 12 months of storage. This electrophoretic pattern closely resembled those for 2% SDS + 8 M urea (Figure 4), despite the fact that less protein was extracted with the latter (Figure 2). This suggests that in minced hake, at up to 8 months of frozen storage, part of the protein not extracted in 0.6 M NaCl (P1) formed microaggregates held together by secondary bonds (extracted by SDS + urea) or by disulfide bonds (extracted by SDS + ME). At 12 months, covalent bonds were formed preventing extraction. In sardine, although the percentage of peak 1 was higher at 8 months than at 12 months, the major peaks in both were 1 and 2; the Ac peak was evident in both profiles, whereas the MHC peak was not obvious (Figure 5). In the mixed lots likewise there were no major differences in the peaks at 8 and 12 months. Although the percentages of protein extracted from P1 were similar in lots C and D (Figure 2), the types of aggregate formed were different, given that at the end of storage the MHC and Ac peaks were barely visible in lot C but were clearly observed in lot D.

It was deduced from the results that the type of protein aggregation occurring during frozen storage differed from one lot to another in terms of both percentage of protein unextracted in 0.6 M NaCl (Figure 1) and type of aggregates (Figures 2−5). In hake (lot A) most of the remaining mince was extracted with 2% SDS + 8 M urea for up to 5 months of storage and with 2% SDS + 5% β-ME for up to 8 months. This is indicative of the importance of secondary and disulfide

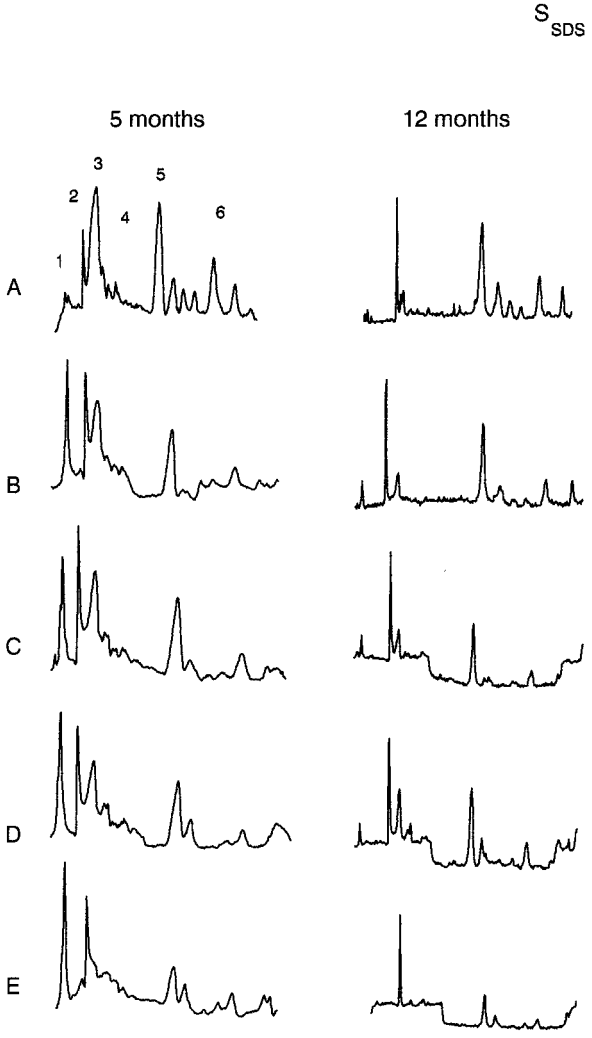

Figure 3. SDS−PAGE (12.5%) of proteins extracted in 2% SDS (S_{SDS}) from P1 at 5 and 12 months: (1) application zone; (2) peak in the stacking/resolving interphase; (3) myosin heavy chain; (4) proteins of molecular weight between 200 000 and 45 000; (5) actin; (6) tropomyosin, troponins, and myosin light chains. Lots are as in Figure 1.

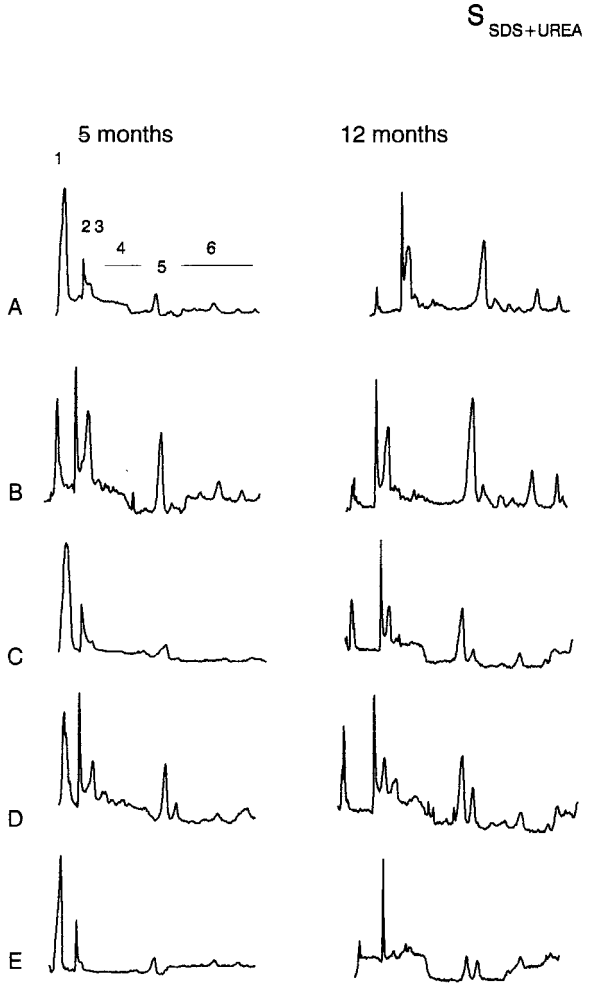

Figure 4. SDS−PAGE (12.5%) of proteins extracted in 2% SDS + 8 M urea ($S_{SDS+urea}$) from P1 at 5 and 12 months. Peaks are as in Figure 3. Lots are as in Figure 1.

bonds in the loss of extractability in 0.6 M NaCl. By the end of 12 months, however, extraction had fallen drastically, even in treatments with ME. This suggests that the protein−protein interactions responsible for nonextractability in 0.6 M NaCl changed during storage; thus, initially most of the proteins were extracted by cleavage of secondary and disulfide bonds. Subsequently, covalent bonds were formed, which prevented extraction of 80% of P1. The electrophoretic profiles of the extracts obtained by treating P1 with SDS + urea or SDS + ME showed that at 5 and 8 months, respectively, there were microaggregates composed of proteins linked by non-disulfide covalent bonds (given that they were not broken during electrophoresis sample preparation). These appeared as high MW bands retained in the stacking gel. The presence of these microaggregates in hake muscle has been confirmed by Del Mazo et al. (1997). When these microaggregates link together, they may produce aggregates of higher molecular weight, which would not be extractable under the experimental conditions.

In sardine muscle (lot E), although the amount of protein not extracted in 0.6 M NaCl was much smaller than in lot A (Figure 1), protein aggregates involving non-disulfide covalent bonding appeared earlier. This was deduced from the fact that although a higher percentage of protein was extracted from P1 by cleavage of secondary and disulfide bonds than by treatments that cleave only secondary bonds (SDS or SDS + urea), a high percentage was not extracted. The maximum percentage of protein extracted from P1 at 8 months was far lower than for hake (Figure 2), which suggests that the formation of non-disulfide covalent bonds, producing higher MW aggregates which cannot be extracted, occurs earlier in sardine. The electrophoretic profile of the extracts at 5 months (S_{SDS} and $S_{SDS+urea}$) and 8 months (S_{SDS+ME}) showed the major peaks were retained in the stacking gel (Figures 3−5), indicating that a percentage of the extracted proteins consisted of protein microaggregates composed of proteins linked by non-disulfide covalent bonding.

The difference in extractability of the aggregates in these species may be indicative of the different types of aggregation occurring in FA-forming species versus species with higher lipid content. In hake, the formation of FA in the muscle is associated with early formation of aggregates that are not extractable in 0.6 M NaCl, in which the proteins initially form protein−protein secondary interactions and disulfide bonds, leading over time to an increase in covalent bonds as previously observed in hake and other lean species (Torrejón, 1996; Tejada et al., 1997). In sardine the presence of lipids seems to induce earlier formation of

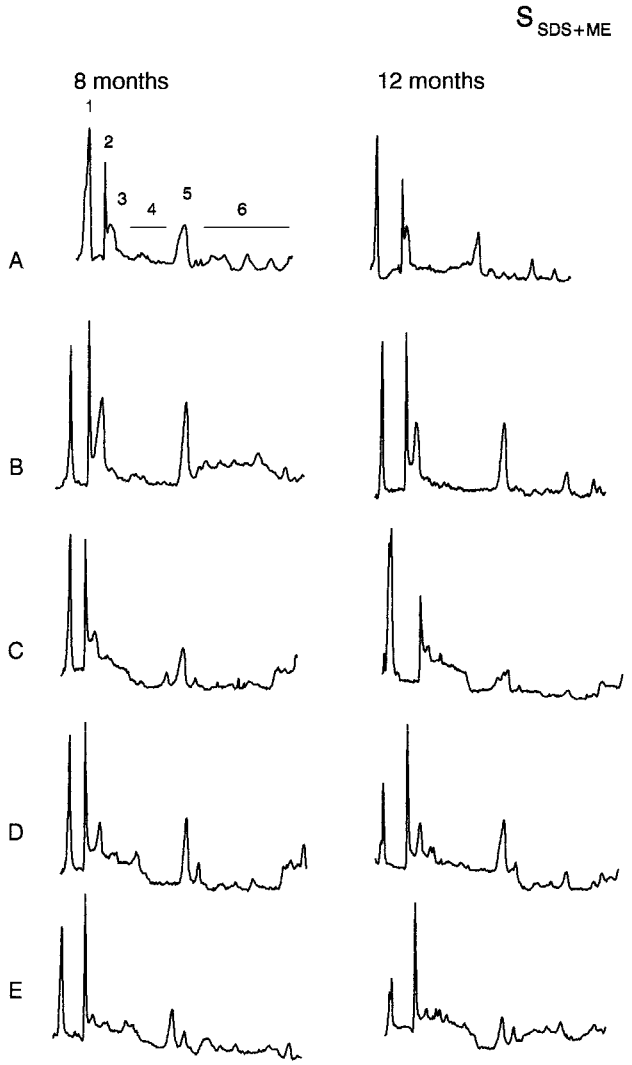

Figure 5. SDS−PAGE (12.5%) of proteins extracted in 2% SDS + 5% β-mercaptoethanol (S_{SDS+ME}) from P1 at 8 and 12 months. Peaks are as in Figure 3. Lots as in Figure 1.

aggregates in which a high percentage of the protein is linked by non-disulfide covalent bonds.

Contrary to expectations, addition of sardine to hake muscle did not slow protein insolubilization or aggregation in the mixed-species lots. In these lots the amount of protein extracted in 0.6 M NaCl was greater the higher the percentage of sardine in the mixes, although the effect was equal to (lot D) or less than (lots B and C) what might have been expected from the hake/sardine ratio. With regard to the type of bonding in the mixed lots, there was practically no variation in the percentage of aggregated NAM linked by secondary and disulfide bonds with storage time, although in lot B this was much lower that in lots C and D and similar to the percentage in sardine (lot E).

Addition of 25% sardine to hake muscle proved the most detrimental; the percentage of protein unextracted in 0.6 M NaCl increased and values were comparable to those found in the hake lot. Moreover, protein linked by non-disulfide covalent bonds was detected earlier and in larger amounts, similar to what was observed in sardine. The highest FA formation (measured as DMA) and FA reaction (measured as the difference between FA formed and free FA) of all lots were detected in lot B and were higher even than had been found in hake (Mohamed, 1997). In a model system made with hake kidney it has been found that depending on the amount and the oxidation conditions, lipids and other compounds can enhance or inhibit the capacity of TMAOase to catalyze DMA and FA formation during frozen storage (Joly et al., 1997). This suggests that conditions in lot B were such that the lipids, or other muscle components in the sardine muscle, accelerated or enhanced FA formation in the mix; this in turn would accelerate the initial conformational changes in the protein and subsequent formation of covalent bonds as observed by Del Mazo et al. (1994) and Tejada et al. (1997). At the same time, the presence of lipids in the medium would favor the formation of covalent bonds to build up stable protein−lipid aggregates, as stated by Sikorsky and Kolakovska (1994).

CONCLUSIONS

The amount of protein not extractable in 0.6 M NaCl during frozen storage differs in hake and sardine muscle. As sardine is more stable, the amount of protein extracted was greater the higher the percentage of sardine in the minces, although the effect is proportional or less than expected given the percentage of sardine in the mixes. The type of protein−protein bonds formed depends on the species, not on the amount of protein not extracted by 0.6 M NaCl. In hake, for up to 8 months of storage there was considerable formation of secondary and S−S protein−protein bonds, producing high MW aggregates that were not extractable in 0.6 M NaCl; thereafter, extraction was prevented by the formation of non-disulfide covalent protein−protein bonds. In sardine or mixed minces, the percentage of protein extracted from the 0.6 M insoluble mince by cleavage of secondary and disulfide bonds hardly varied with storage time, although the percentage of protein extracted was unrelated to the relative proportions of hake and sardine in the mix. In all lots protein microaggregates formed of proteins linked by covalent bonds were extracted.

Although in all cases the cleavage of S−S bonds allowed more protein to be extracted from the 0.6 M NaCl-insoluble mince, higher MW aggregates were formed by covalent bonding in sardine muscle (lot E) and in lot B (75% hake + 25% sardine), preventing extraction.

In all cases the addition of varying proportions of sardine muscle to hake muscle altered the nature of the bonds involved in protein aggregation, and non-disulfide covalent bonds were detected earlier in the process.

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