Structural and Functional Changes in Myofibrillar Proteins of Sea Salmon *(Pseudopercis semifasciata)* by Interaction with Malonaldehyde (RI)

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ABSTRACT: Changes on the myofibrillar proteins of Sea Salmon *(Pseudopercis semifasciata)* induced by malonaldehyde were investigated. Electrophoretic patterns, solubility, and differential scanning calorimetric studies were performed after incubation of proteins with malonaldehyde (MDA) at 27 °C. Results obtained showed a different thermal behavior, evidencing a decrease in thermal stability, changes in denaturation enthalpies values (ΔH_{total} and ΔH_{myosin}), and the appearance of new molecular species with a loss in the cooperativity of the myosin denaturation. A decrease in solubility and SDS-PAGE profiles revealed the participation of myosin and other proteins in the formation of aggregates involving nondisulfide covalent linkages for myosin heavy chain (MHC) and disulfide bridges in some other proteins.

Lipid oxidation, malonaldehyde, myofibrillar proteins, sea salmon, protein functionality

Introduction

LIPID OXIDATION IS ONE OF THE MAIN PROCESSES RESPONSIble for the loss of food quality since it affects different components such as lipids, proteins, vitamins, and other compounds. This oxidative deterioration is related to negative changes in flavor, texture, appearance, nutritional value, and protein functionality (Erickson 1997). Protein reactions with oxidized lipids can occur by 2 different mechanisms: 1) reactions with free radicals produced by cleavage of hydroperoxides (primary oxidation products), forming protein free radicals and subsequent formation of polymers and protein scission, and 2) reactions of secondary products from lipid oxidation (aldehydes, ketones, epoxydes, carbonyl compounds) with ε-amino groups causing cross-linking (Frankel 1998). A major secondary product from lipid oxidation is malonaldehyde (MDA). This substance can react with proteins, phospholipids, and nucleic acids, producing covalent links and cross-linking of large molecules (Aubourg 1993). Enzymes containing sulfhydryl groups are inactivate, whereas others are denatured (Funes and Karel 1981; Nakhost and Karel 1983).

Myofibrillar proteins are relevant muscle components susceptible to these oxidative reactions. Functional (water holding capacity, gelation, and emulsifying properties) and textural characteristics of meat products depend on these proteins; this fact is more important in fish muscle than in mammalian muscle, because of fish low collagen content (Hultin 1985). Interactions of proteins from different animal species with free radicals have been studied using free-radical-generating-systems, exhibiting chemical, physical, functional, and structural changes. It was possible to detect protein polymerization and polypeptide chain scission by SDS-PAGE, resulting in a decrease in solubility, gel strength, and amino acid destruction (Decker and others 1993; Srinivasan and Hultin 1995, 1997). There was also observed a decrease in Ca^{+2}-ATPase activity, conformational changes measured by sulfhydryl content, tryptophan fluorescence and 1-anilino-8-naphtalenesulfonate (ANS) fluorescence (Li and King 1996) and changes in thermal denaturation (Liu and Xiong 2000).

The reaction between myosine and MDA was studied by Buttkus (1967). The results obtained indicated that MDA reacted preferentially with basic amino acids, and the reaction rate was greater at room and frozen temperature than at 0 °C. Li and King (1999) showed that MDA causes cross-linking and modifications in the native structure of rabbit myosin subfragment 1.

The aim of this work was to study the influence of malonaldehyde on myofibrillar proteins of sea salmon (*Pseudopercis semifasciata*) by examining electrophoretic patterns, differential scanning calorimetry profiles and solubility in order to evaluate possible deleterious effects.

Materials and Methods

Materials

Sea salmon (*Pseudopercis semifasciata*) was obtained at a local fish market. 1,1,3,3-tetraethoxipropane (TEP) was purchased from Fluka AG (Paris), albumin and electrophoretic grade chemicals from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Protein standards (Prosieve Protein Markers) were obtained from Biowhittaker Molecular Application–FMC Division (Rockland, Me., U.S.A.). All other chemicals were of analytical grade.

Preparation of myofibrillar proteins

Myofibrillar proteins were prepared according to the procedure of Wagner and Añón (1985). Purified proteins were suspended in 0.6 M KCl, 0.03 M Tris, pH = 7.0. Protein concentration was determined by a modified Biuret method (Robson and others 1968).

Preparation of malonaldehyde

Malonaldehyde (MDA) solution was prepared by acid hydrolysis of 1,1,3,3-tetraetoxipropane (TEP) according to Kakuda and others (1981). The stock solution (100 mM) was adjusted to pH 7.0. Dilutions were done with buffer 0.6 M KCl, 0.03 M Tris, pH = 7.0.

Treatment of myofibrillar proteins with MDA

Myofibrillar proteins (5 mg/mL) were incubated at 2 different concentrations of MDA: 20 and 30 mM (protein: MDA ratio 3.5 : 1 and 2.3 : 1, respectively) at 27 ± 1 °C from t = 0 to 8 hours, (under moderate agitation). After incubation, samples were kept in an ice bath until further analysis (DSC and electrophoresis). For the solubility assays, the myofibrillar protein and MDA concentrations used were 10 mg/mL and 40 and 60 mM, respectively; the corresponding protein: MDA ratio was kept constant.

Protein solubility

Determinations were carried out by measuring the protein concentration using the Biuret method in supernatant obtained by centrifugation at 5000 g at 4 °C for 15 min. Protein solubility was calculated using the formula:

$$\% \text{ solubility} = \frac{\text{protein concn. in supernatant}}{\text{original protein concn}} \times 100$$

Solubility in respect to solubility control was calculated according to:

$$\% \ S/So = \frac{\text{sample solubility}}{\text{control solubility at t = 0}} \times 100$$

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to monitor changes in the myofibrillar proteins incubated with MDA, and to analyse samples of soluble and insoluble fractions obtained after centrifugation at 5000 g at 4°C for 15 min. Soluble fraction: an aliquot of supernatant was mixed with buffer 8 M urea, 0.3 % SDS, pH = 9.0 (with or without β - mercaptoethanol ME). Insoluble fraction: Proteins were redissolved in 0.6 M KCl, 0.03 M Tris, pH = 7.0 and buffer 8 M urea, 0.3 % SDS, pH = 9.0 (with or without β - ME) was added. Slab SDS-PAGE was carried out using the Laemmli discontinous buffer system (Laemmli 1970) in a Mini Protean II Dual Slab Cell (BIO-RAD). A stacking gel of 3 % acrylamide and a gradient resolving gel with 3% to 15 % were used. Gels were stained with Coomasie Brilliant Blue R-250 (0.2 % p/v).

Additional information about the insoluble fractions was obtained by 2-dimensional SDS-PAGE. Three to fifteen percent gradient resolving gels were used. First dimension was run in the absence of ME, and the second in the presence of ME. First dimension lanes were cut off, treated according to Petruccelli and Añón (1995) and placed on top of a second gel. Silver dye was used to second dimension gels.

Gels were analyzed using a Molecular Analyst Software (Bio-Rad, Calif., U.S.A.). Then the corresponding densitometric patterns and molecular weights were estimated.

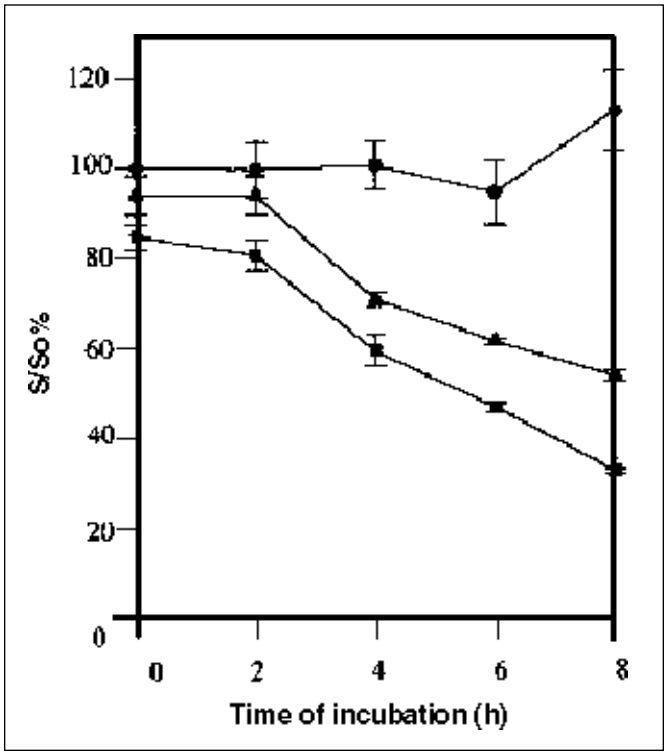


Figure 1—Solubility of Sea Salmon myofibrillar proteins (MP) as a function of time of incubation at 27 ± 1 °C: (h) MP controls, (t) (MP + MDA) (3.5:1) and (g) (MP + MDA) (2.3:1). S: sample solubility, So: solubility of control system at t = 0. Plotted values are the mean ± standard deviation of two determinations.

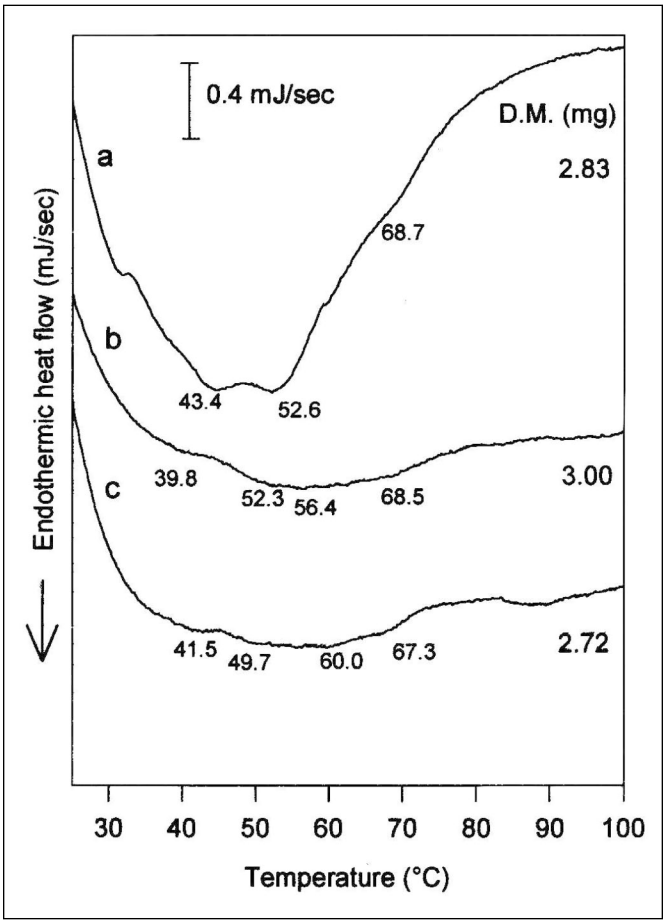


Figure 2—DSC thermograms of Sea Salmon myofibrillar proteins (MP) incubated at 27 ± 1 °C with MAL (MP + MDA) (2.3:1) for different periods of time: (a) control systems, (b) (MP + MDA), t = 4 h and (c) (MP + MDA), t = 8h. Heating rate β: 10 8C/min. D.M.: dry matter (mg).

Differential Scanning Calorimetry (DSC)

Differential scanning calorimetric studies were performed in a Polymer Laboratories calorimeter (Rheometric Scientific Ltd. (Surrey, U.K.) driven with a Plus V 5.41 software. The equipment was calibrated at a heating rate of 10 °C/min using indium as standard. After incubation, samples were centrifuged at 5000 g for 10 min at 4 °C and the supernatant was discarded. Samples (20 to 22 mg wet weight) were placed in DSC hermetic pans, ensuring good contact between the sample and the capsule bottom. Triplicate samples were analyzed. All the samples were scanned over the range of 15 to 100 °C. As reference, a capsule with distilled water was used. Dry matter weight was obtained. Denaturation enthalpies (ΔH_{total} and ΔH_{myosin}) were estimated by measuring the area under the DSC transition curve (a baseline was constructed as a straight line from 33 to 80 °C). Specific area (partial area/total mg of dry sample) for each peak was calculated.

Statistical Analysis

Solubility and denaturation enthalpies (ΔH_{total} and ΔH_{myosin}) data were analyzed using analysis of variance (ANOVA) according to the General Linear Model Procedure. When differences were significant ($P < 0.05$) mean values were evaluated by Least Significant Differences (LSD) using a SYSTAT statistical package (Wilkinson 1990).

Results and Discussion

Protein solubility

Myofibrillar proteins incubated at 27 °C for 8 h showed significant effect on solubility in the absence of MDA. When these proteins were incubated at the same temperature in the presence of MDA there was a significant decrease in solubility. Figure 1 shows the evolution of solubility as a function of incubation time. A sharp decrease in this functional property was observed between 2 and 4 h of incubation, then the decrease slowed down. At each time, there were recorded significant differences ($P < 0.05$) between the solubility measurements of the systems at different concentrations of MDA. This change in solubility could be explained by the formation of insoluble aggregates by MDA reaction with myofibrillar proteins. At t = 0, it was possible to see a decrease in solubility with respect to myofibrillar protein controls, indicating that it does not necessarily take too long to start the reaction of myofibrillar proteins with MDA.

Differential Scanning Calorimetry (DSC)

Calorimetric studies were carried out on the pellets obtained by centrifugation. DSC thermograms of myofibrillar proteins control showed 3 endothermic transition peaks with maximum temperatures ($T_{máx}$) at 43.4 °C, 52.6 °C and 68.7 °C (Figure 2a). First and second peaks were correlated with myosin denaturation and the last peak to actin denaturation (Beas and others 1990; Paredi and others 1998). In the case of systems constituted by myofibrillar proteins incubated with MDA, changes in the transition peaks can be observed, being more evident with increasing MDA concentration and time of incubation. Thermograms became wider and flatter, exhibiting a displacement to lower temperatures of the first transition (Figure 2). These changes evidenced a decrease in thermal stability, associated primarily with myosin. Also, some small new peaks appeared with a variety of denaturation temperatures; this fact could be explained by the formation of new protein species due to the reaction between MDA and myosin, with different thermal stabilities. In addition, the changes in shape of the myosin peaks suggest a loss of cooperativity in the thermal denaturation of this protein. Significant modifications ($P < 0.05$) in the total and myosin

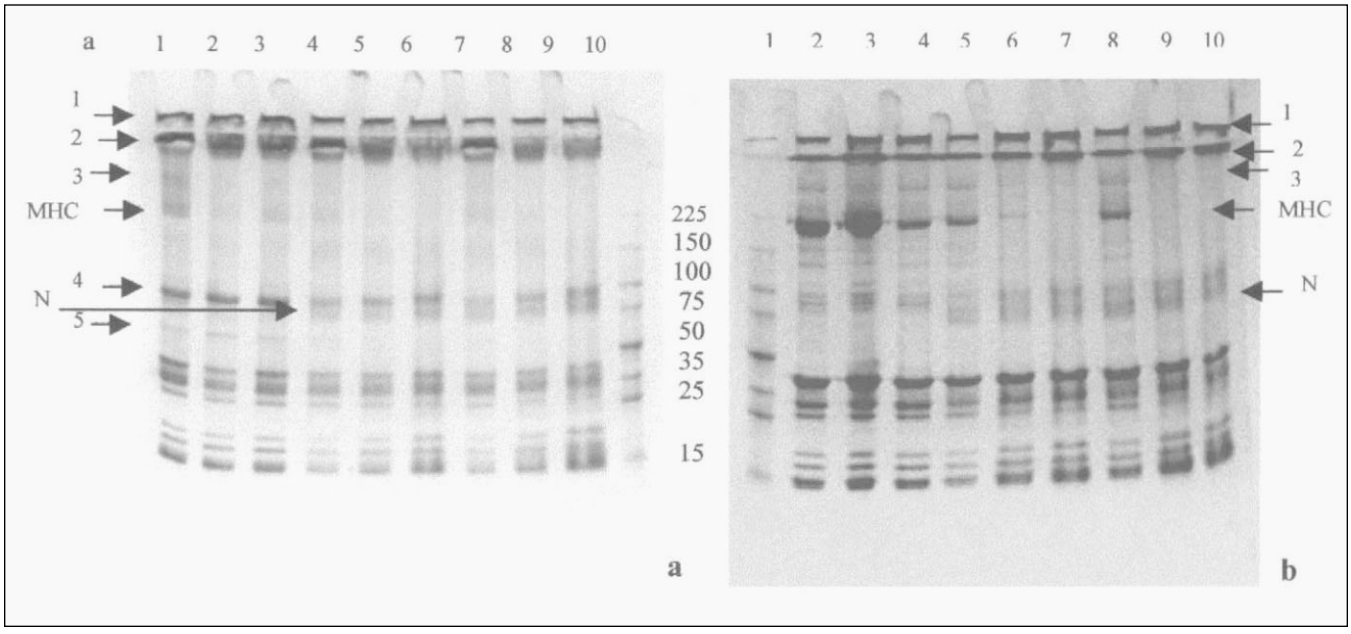


Figure 3—SDS-PAGE patterns of the soluble fraction of myofibrillar proteins (MP) incubated with MDA at 27 ± 1 °C. Electrophoresis was performed on a 3 to 15% gradient gel in the absence (a) and in the presence (b) of ME. (a) Lane 10: molecular mass standard; lane 1 to 3: control systems, t = 0, 4, and 8 h respectively; lane 4 to 6: (MP + MDA) (3.5:1), t = 0, 4 and 8 h, respectively; lane 7 to 9: (M + MDA) (2.3:1), t = 0, 4, and 8 h, respectively. (b) Lane 1: molecular mass standard; lane 2 to 4: control systems, t = 0, 4, and 8 h respectively; lane 5 to 7: (MP + MDA) (3.5:1), t = 0, 4 and 8 h, respectively; lane 8 to 10: (M + MDA) (2.3:1), t = 0, 4, and 8 h, respectively. Each lane contained 30 μg of protein.

Table 1—Denaturation enthalpies (ΔH_{total} and ΔH_{myosin}) of Sea Salmon myofibrillar proteins incubated with MDA (MP + MDA) (2.3:1)

Time of incubation (h)	ΔH_{total} (J/g)	ΔH_{myosin} (J/g)
0	40.3 ± 1.6[a]	33.3 ± 1.1[a]
4	20.5 ± 1.9[b]	15.4 ± 1.3[b]
8	16.4 ± 3.0[b]	12.5 ± 2.9[b]

Each value is represented as mean ± SD (n + 3). Different letters (a, b) in the same column indicate significant differences among times of incubation ($P < 0.05$).

denaturation enthalpies (ΔH_{total} and ΔH_{myosin}) were recorded (Table 1). The values corresponding to the actin denaturation enthalpy were constant; for this reason the decrease in ΔH_{total} could be related to the reduction in ΔH_{myosin} . The results obtained by DSC showed an important effect of MDA on the thermal behavior of the myofibrillar proteins; they became more sensitive to thermal treatment. On the other hand, this information showed the structural changes of the myofibrillar proteins –mainly myosin- incubated with MDA, with no modifications of the system (M + MDA) at t = 0.

Electrophoresis

Electrophoretic studies were performed in order to analyze the structural changes occurring in myofibrillar proteins due to the interaction with MDA.

Soluble fractions. Figure 3a (lane 1) and Figure 3b (lane 2) show SDS-PAGE patterns of myofibrillar proteins of Sea Salmon (without and with β – ME, respectively). In those samples without ME, the band corresponding to myosin heavy chain (MHC) was slightly intense; the intensity of this band (210 ± 6 kDa) showed a very important increase when the samples were treated with ME. This fact suggests that MHC extracted with 0.6 M KCl would be associated to disulfide bridges. Myofibrillar proteins exhibited some changes such as a small decrease in the intensity of MHC band (with and without ME) when they were incubated at 27 °C (Figure 3a – lanes 1 to 3, Figure 3b – lanes 2 to 4). Regarding myofibrillar proteins incubated at different concentrations of MDA, additional effects in the electrophoretic patterns of soluble fraction could be observed. Samples without ME (Figure 3a, lanes 4 to 9) showed a decrease in the intensity of MHC at t = 0 with respect to control, being more important as a function of incubation time. Also, there was a decrease in other bands such as band 3 (292 ± 6 kDa), band 4 (81 ± 3 kDa), band 5 (53 ± 1 kDa), and in band 2, indicating that the constituent proteins of this band became insoluble. On the other hand, a new band (band N, 71 ± 1 kDa) appeared in samples treated with MDA, whose intensity increased with the incubation time. Samples with ME (Figure 3b, lanes 5 to 10) showed also a strong decrease in MHC, and band 3 (292 ± 6 kDa) diminished slightly with the increasing interaction time. In addition, a little increment in band 2 and in those on top of stacking gel (band 1) was shown. Also, the new band of 71 kDa (band N) could be observed. In all cases, the effects were more evident at the increasing MDA concentration. The results previously mentioned would indicate that the interaction with MDA produces the aggregation of MHC and other proteins (3, 4, and 5) and the appearance of a new protein species (band N). The decrease in MHC at t = 0 is in agreement with the reduction of solubility.

Insoluble fractions. In the case of samples obtained by redissolving the pellets after centrifugation, the same volume of sample was loaded in each lane. Total protein mass in each lane decreased as a function of incubation time with MDA due to a lesser redissolution of the myofibrillar proteins. This fact was in agreement with the increase observed in the consistence of the pellets after centrifugation. Electrophoretic patterns of these samples without ME (Figure 4a, lane 6 to 10) showed a decrease in the intensity of those species of high molecular weight (bands 1 and 2) as a function of the interaction time, indicating that these proteins form aggregates that can not be redissolved. Protein 5 was not de-

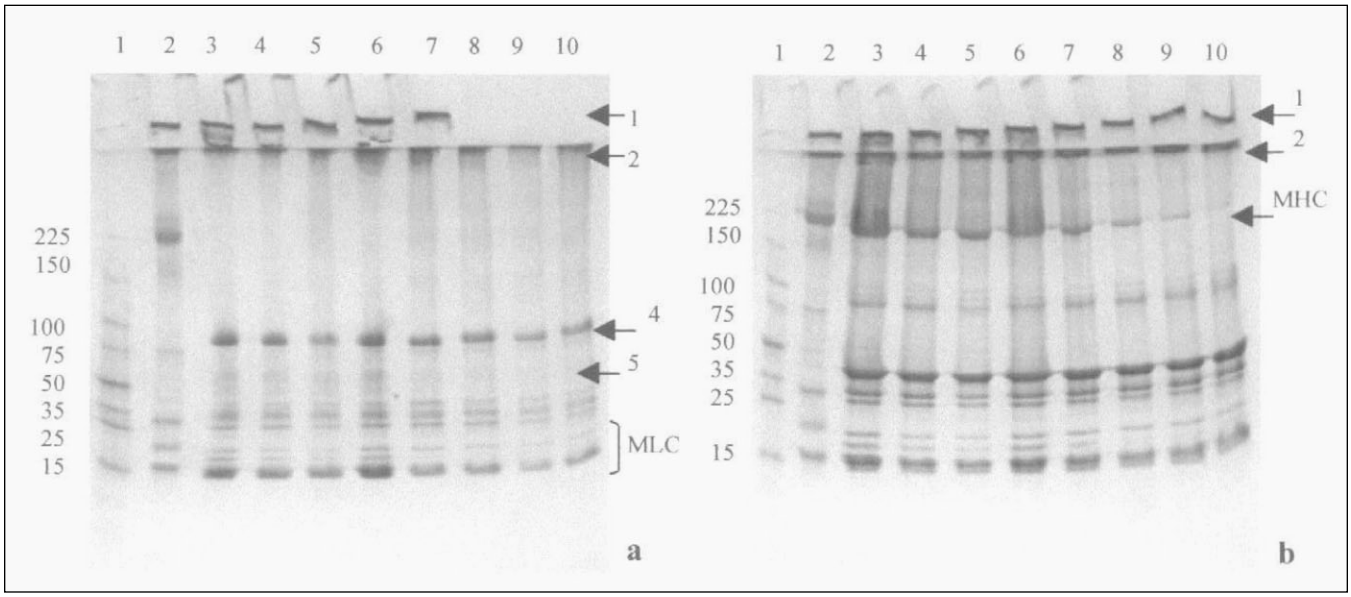
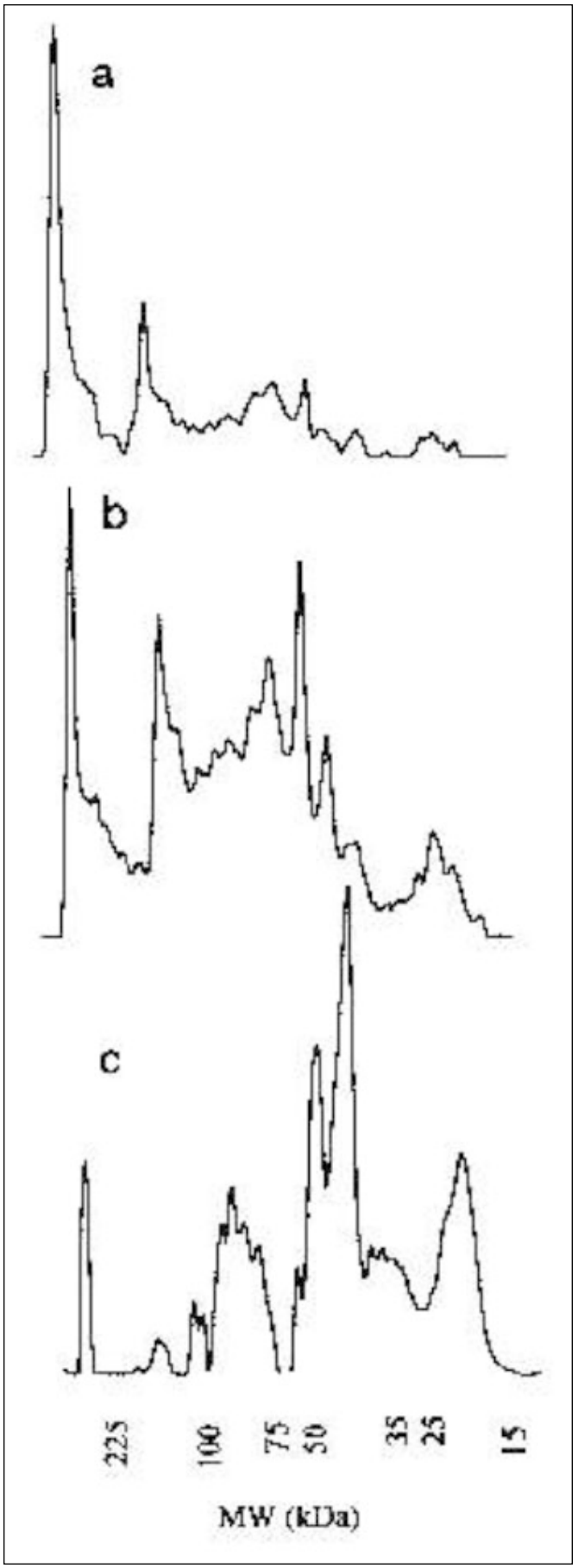


Figure 4— SDS-PAGE patterns of the insoluble fraction of myofibrillar proteins (MP) incubated with MDA at 27 ± 1 °C. Electrophoresis was performed on a 3 to 15 % gradient gel in the absence (a) and in the presence (b) of ME. Lane 1: molecular mass standard; lane 2: rabbit myosin standard; lanes 3 to 5: control system, t = 0, 4, and 8 h, respectively; lanes 6 to 10: (M + MDA) (2.3:1), t = 0, 3, 4, 5, and 8 h, respectively.



tected after 3 h of incubation, while protein 4 and myosin light chains (MLC) (13 – 20 kDa) decreased. Similarly, in samples with ME (Figure 4b, lanes 6 to 10) band 1 decreased slightly, evidencing that the corresponding proteins could not be thoroughly redissolved; band 2 increased, and MHC presented a sharp decline. On the other hand, control systems (Figure 3a and 3b – lanes 1 to 4) did not show important modifications in the profiles as a function of the incubation time.

Further information about the nature of bonds involved in protein species was obtained by 2-dimensional electrophoresis. Densitograms of the second dimension corresponding to the protein species involved in bands 1 and 2 of insoluble fractions can be observed in Figure 5. Profile corresponding to the control sample (Figure 5a) showed that these bands were constituted by polypeptides of 35 to 100 kDa and MHC. In the case of samples incubated with MDA (Figure 5 b and c), it was possible to detect an increment of species from 35 to 100 kDa and 15-20 kDa (MLC) and a decrease of MHC.

Results obtained by electrophoresis suggest that the interaction of myofibrillar proteins with MDA produces the formation of aggregates by cross-linking via covalent linkage different of disulfide bridges. This conclusion correlate with the difficulty to redissolve them after the treatment with chaotropics agents (SDS and urea) and ME. These aggregates involved mainly MHC, whose intensity decrease even after the treatments previously mentioned. This information is in agreement with the results obtained by Li and King (1999), who reported the cross-linking of rabbit myosin subfragment 1 incubated with MDA at 37 °C. On the other hand, the increase in band 2 in samples with ME could be attributed to the formation of aggregates by disulfide bridges, which might be constituted by protein species such as bands 4 (81 kDa) and 5 (53 kDa) and MLC (13 to 20 kDa).

Conclusions

AN APPROACH OF A REAL SYSTEM WITH LESS COMPLEXITY, constituted by fish myofibrillar proteins and the major byproduct generated from lipid oxidation (MDA), was used to simulate their interaction. Experimental results would indicate that, under these conditions, the main proteins suffered denaturation, functional, and structural changes. The most important effect observed was the insolubilization of the myosin heavy chain due to the formation of aggregates of high molecular weight via nondisulfide covalent linkages. Aggregation involved denaturation of this protein as suggested by the decrease in its thermal denaturation entalphy. New protein species—products of the interaction with MDA—appeared, changing the thermal stability of myosin. Besides, other myofibrillar proteins could be involved in the polymerization by disulfide bridges.

So far, it should be taken into account that Sea Salmon (*Pseudopercis semifasciata*) contains a significant amount of polyunsaturated fatty acids (PUFAs), which favor the occurrence of a similar deterioration in the whole muscle

Figure 5—Two-dimensional electrophoresis of the insoluble fraction of myofibrillar proteins (MP) incubated with MDA at 27 ± 1 °C, (MP + MDA) (2.3 : 1). First (without ME) and second (with ME) dimension electrophoresis were performed on 3-15 % gradient gels. Densitometric patterns corresponding to protein species involved in band 1 and 2 at different times of incubation: (a) t = 0, (b) t = 4 h and (c) t = 8 h.

during the storage time under inappropriate conditions. In conclusion, MDA is a promoting agent which action can play an important role in nutritional and technological aspects of this food.

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