Effect of Malonaldehyde on the Gelation Properties of Myofibrillar Proteins of Sea Salmon

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ABSTRACT: Myofibrillar proteins incubated with malonaldehyde (27 °C, t = 0 to 8 h) were used to prepare gels by thermal treatment (80 °C, 45 min). Malonaldehyde did not produce significant modifications (P > 0.05) in the storage modulus (G') and complex viscosity (η*) except for t = 0. Texture analysis showed a significant increase (P < 0.05) in elasticity and cohesiveness with hardness approximately constant. Relaxation test presented a marked increase in the elasticity index . A significant decrease (P < 0.05) in water holding capacity (WHC) was recorded. Ultrastructure showed a different network with the appearance of big filaments. Electrophoretic patterns of protein dispersions remainder after gel formation showed a decrease of 81, 53, 25 to 50, and 20 kDa polypeptides.

Keywords: malonaldehyde, myofibrillar proteins; sea salmon, *Pseudopercis semifasciata*, thermal gelation

Introduction

HEAT-INDUCED GELATION OF MYOFIBRILLAR PROTEINS IS A VERY important functional property, particularly in fish, playing a fundamental role in the preparation of a large variety of seafoods made from surimi. The formation of a protein network in the gel contributes to textural characteristics and to other functional properties of the product, such as water and fat retention (Sharp and Offer 1992). Three steps are required for the thermal gelation process: (1) dissociation of the myofibril structures by treatment with high concentrations of salts; (2) unfolding by thermal denaturation with the exposure of functional groups; and (3) aggregation to form a 3-dimensional network (Roussel and Cheftel 1990).

Malonaldehyde (MDA) is one of the major secondary products of lipid oxidation, which can interact with proteins. Kwon and others (1965) have demonstrated a nucleophilic reaction of MDA with food proteins at pH 6.5 to 7.1. Buttkus (1967) studied the interaction of trout myosin with MDA, observing that this compound reacted preferentially with basic amino acids; its reaction rate was found to be much faster at room and freezing temperatures rather 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.

In a previous work, a model system constitued by myofibrillar proteins of sea salmon and MDA was used to study their interaction (Tironi and others 2001). Those results demonstrated the formation of aggregates via nondisulfide covalent linkages involving the myosin heavy chain (MHC) and via disulfide bridges in some other proteins, with the consecutive solubility decrease. Thermal behavior of myosin was altered, leading to a decrease in its stability.

Taking into account these facts, the effect of MDA on the structure of the myofibrillar proteins, mainly myosin, would affect the gel matrix formation as well as their characteristics such as rheological and texture parameters, water holding capacity, and microstructure.

Our objective was to study the thermal gelation properties of myofibrillar proteins of sea salmon treated with MDA in order to elucidate possible alterations in the whole muscle due to lipid oxidation.

Material and Methods

Materials

Sea salmon *(Pseudopercis semifasciata)* was caught by commercial vessels in the Southwest Atlantic Ocean during the whole year. Fish were kept in ice before and after being filleted. 1,1,3,3–Tetraethoxypropane (TEP) was purchased from Fluka AG (Paris, France), 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, Maine, U.S.A.). All other chemicals were of analytical grade.

Preparation of myofibrillar proteins

Myofibrillar proteins (MP) were prepared according to Wagner and Añón (1985). 8 samples of around 4 g of mince muscle were used in each experience. They were homogenized with 0.25 M sucrose, 1 mM EDTA, 0.05 M Tris–HCl, pH 7.0 buffer and kept on ice for 30 min with agitation. Homogenates were centrifuged (2500 × *g*, 10 min, 4 °C) and the pellet was resuspended in the same buffer. After a new centrifugation, pellet was resuspended in 1 mM EDTA, 0.05 M Tris–HCl, pH 7.0 buffer; the suspension was filtered to remove collagen and later centrifuged. Myofibrils were resuspended in 0.15 M KCl, 0.03 M Tris–HCl, pH 7.0 buffer and then purified by 2 successive steps of resuspension and centrifugation with 1 mM EDTA, pH 7.0 solution and distilled twice water, respectively. Purified proteins were suspended in 0.6 M KCl, 0.03 M Tris, pH = 7.0 and their concentrations were determined by a modified biuret method (Robson and others 1968). Isolation yield was around 7 to 8 % (p/p). Protein solution was diluted to 25 mg / mL.

Preparation of malonaldehyde

Malonaldehyde (MDA) solution was prepared by acid hydrolysis of 1,1,3,3–tetraethoxypropane (TEP) according to Kakuda and others (1981). The solution was adjusted to pH 7.0 and 300 mM with buffer 0.6 M KCl, 0.03 M Tris, pH 7.0.

Treatment of myofibrillar proteins

MP were placed in glass tubes (2.2 cm i.d. × 6 cm height) and incubated with and without MDA (MP: MDA ratio 5 : 1, final protein concentration approximately 20 mg/mL) at 27 ± 1 °C, under moderate agitation with an orbit Environ shaker (Lab-line) (Li and King 1999; Tironi and others 2001). Samples corresponding to t = 0, 4, and 8 h of incubation were used for further gelation process.

Thermal gelation

Gels were prepared by thermal treatment at 80 °C for 45 min. Then, gels were quickly cooled in a water bath at room temperature and stored at 4 °C for 24 h (Puppo and Añón 1998; Lupano 2000).

Rheological measurements

Viscoelastic analysis of the gels was performed in a Haake CV20 rheometer (Gebrueder Haake GmbH, Karlsruhe, Germany) using a 1-mm-gap parallel plate sensor. Gel sections (1 mm height) were placed in the lower plate, which was kept at 20 °C. The rheometer was controlled through the Haake software osc. 2.0. Linear viscoelasticity range was determined measuring the complex modulus (G*) as a function of deformation (f = 1 Hz). From these results, 5% deformation was chosen for the frequency (f) scans, recording the development of the storage modulus (G′) and complex viscosity (η*) as functions of oscillation frequency (Puppo and Añón 1998).

Texture analysis

Determinations were performed on gel sections (1 cm height) using a TA-TX2i Texture Analyzer (Stable Micro Systems, Godalming, U.K.), with the data analysis software package Texture Expert for Windows, version 1.2. Compression was exerted by a cylindrical probe with a flat contact surface (7.5 cm dia). Texture profile analysis (TPA) was performed applying 20% (2 mm) compression, a compression rate of 1 mm/s, and a 5-s interval between 2 compression cycles. Values for hardness (H), instantaneous recoverable springiness (S_{ins}), retarded recoverable springiness (S_{ret}) and cohesiveness (C) were obtained (Fiszman and others 1998).

Relaxation tests were carried out under the same conditions; but were mantained at this compression for 15 min. Relaxation curves were normalized using the equation:

$$Y(t) = \frac{F_o - F(t)}{F_o} \times 100$$

where Fo and F(t) are the force recorded at t = 0 and at t = t min, respectively (Peleg 1979).

Water holding capacity (WHC)

Gel samples (0.6 to 1.4 g), equilibrated at room temperature, were placed on a nylon plain membrane (5.0 μm pores, Micronsep) and centrifuged at 120 g for 10 min. Water loss was obtained by weighing gels before and after centrifugation (Lupano 2000). WHC was calculated as follows:

$$WHC = \frac{\text{water remaining in gel after centrifugation}}{\text{initial water content}} \times 100$$

Initial water content was obtained by heating gels at 110 °C during 24 h to constant weight.

Scanning electron microscopy (SEM)

Gel samples were fixed in phosphate buffer, pH 7.2, with 2.5% glutaraldehyde, gradually dehydrated in acetone, and finally dried using carbon dioxide-critical point drying. Samples were coated with a gold layer in a sputter coater Pelco 91000 and examined with a Jeol 35 CF scanning electron microscope at 5-6 kV (Lozano and Morales 1983).

Electrophoresis of the remainder protein dispersion

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) of the free liquid remainder after gel formation was performed to analyze the protein species associated with the formation of the gel network. Slab SDS–PAGE was carried out by the Laemmli discontinuous buffer system (Laemmli 1970*)* in a Mini Protean II Dual Slab Cell (BIO-RAD). A 3% stacking gel and a resolving gel prepared by a gradient from 3 to 15% acrilamide (1 mm thickness) were used. Protein concentration was determined by the biuret method previously mentioned. Samples were treated with buffer 8 M urea, 0.3 % SDS, pH 9.0 with and without β–mercaptoethanol (ME), and volumes containing 30 μg of protein were loaded onto each lane. Gels were stained with Coomassie brilliant blue R-250 (0.2 % p/v), captured by a GEL DOC 1000 densitometer controlled through the Molecular Analyst Software (BIO-RAD).

Statistical analysis

Rheological parameters (G′ and η*), texture profile parameters (H, S_{ins}, S_{ret}, and C) and WHC data were analyzed using analysis of variance (ANOVA) according to the General Linear Model Procedure (factors: MDA concentration, time; levels: 0 and 60 mM and 0, 4, and 8 h respectively; random design). When differences were significant ($P < 0.05$), mean values were evaluated by least significant differences (LSD) (Fisher test) using a SYSTAT statistical package (Wilkinson 1990).

Results and Discussion

In order to induce possible deterioration reactions, a temperature of 27 °C, corresponding to inadequate fish processing conditions, was selected for the incubation of myofibrillar proteins of sea salmon with MDA (an accelerated test). In a previous study, denaturation temperatures of these proteins, mainly myosin and actin, were determined by differential scanning calorimetry (DSC) (Tironi and others 2001). Scans showed denaturation temperatures of 43.4 and 52.6 °C (myosin) and 68.7 °C (actin). Taking into account this information and preliminary assays, 80 °C was chosen for gel formation to ensure a complete unfolding of the proteins.

Rheological analysis

The results obtained for the control systems, and for systems treated with MDA, showed constant G′ values and a linear decrease of η* as function of the frequencies tested (Figure 1). In all cases, G″ (loss modulus) values were lower than corresponding G′ values (data not shown). These facts would indicate a gel-like behavior for the systems studied. In the case of the control systems, parameters G′ and η* did not present significant differences ($P > 0.05$) among t = 0, 4, and 8 h (Figure 1-A and C). On the other hand, gels treated with MDA showed significant differences ($P < 0.05$) between t = 0 and those systems with 4 and 8 h of reaction time at 27 °C (Figure 1B and D). Systems with MP + MDA at t = 0 had lower values of G´ and η* than did systems with MP only (t = 0). Nevertheless, after 4 and 8 h of incubation, gels with MDA reached similar values ($P > 0.05$) to those obtained for the corresponding control systems. These results are in agreement with the macroscopic observation, MP + MDA – t = 0 gels were weaker than those from 4 and 8 h of incubation as well as from MP – t = 0, presenting less solid characteristics than others.

In a previous study, myofibrillar proteins in the presence of MDA (t = 0) did not show changes in their thermal behavior examined by DSC. Besides, electrophoretic patterns of this system indicated a

little alteration of the proteins, with the presence of myosin heavy chain (MHC) in the soluble fraction, suggesting that the formation of the protein agreggates has not occurred yet (Tironi and others 2001). These facts show that in case of nonincubated systems, MDA would produce negative effects on the period of gelation, impairing the correct formation of the gel network. Changes in the gel matrix would correlate with the decrease in G′ values observed. Tironi and others (2001) demonstrated previously that, along the incubation time (4 and 8 h), MDA reacts with proteins, inducing their denaturation and polymerization. Then, these protein polymers would participate in the gel network, althought rheological parameters did not present important changes.

Texture analysis

Texture profile analysis. Figure 2 shows a typical force / time curve of double cycle for sea salmon myofibrillar protein gels. Texture parameters such as H, S_{ins}, S_{ret}, and C were obtained in the way indicated, and the results are shown in Table 1. It could be observed that incubation with MDA did not produce any significant change in the gel hardness. S_{ins} values, related to the elastic component of the gel, increased as the incubation time with MDA progressed, being statistically different from control ($P < 0.05$) at t = 8 h. This behavior would suggest a higher degree of elasticity, due to the protein reaction with MDA. S_{ret} includes the initial recovery plus the recovery achieved by the sample during the time between the 2

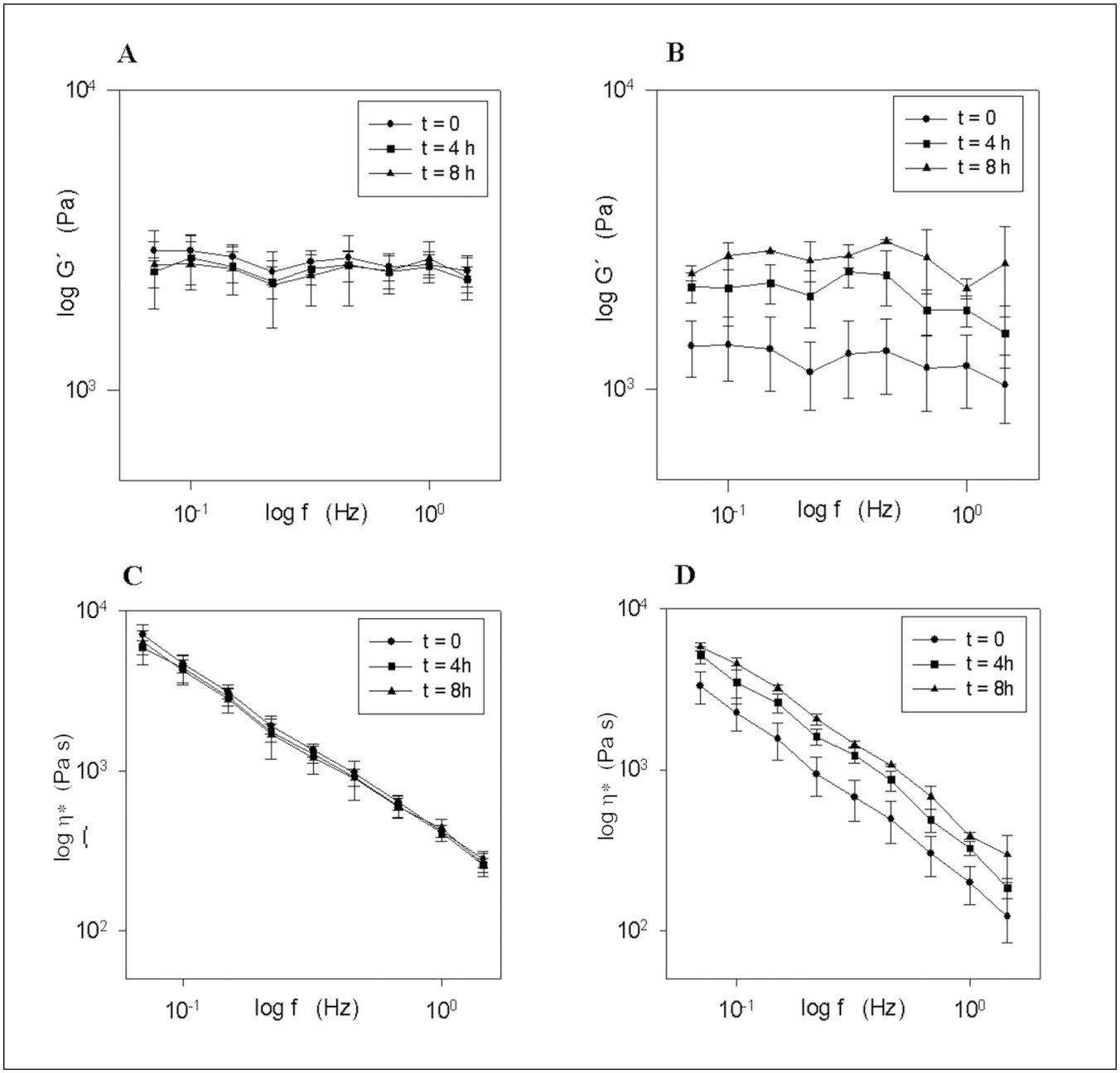

Figure1–Frequency sweeps of gels obtained from myofibrillar proteins (MP) previously incubated with and without MDA. A and C: control systems; B and D: MP + MDA systems.

Table 1–TPA parameters of myofibrillar protein (MP) gels previously incubated with and without MDA for different periods of time.

Incubation time (h)	Model system	H (N)	S_{ins}	S_{ret}	S_{ret} - S_{ins}	C
0	MP	0.20 ± 0.03	0.55 ± 0.04	0.86 ± 0.02	0.31 ± 0.05	0.77 ± 0.02
	MP + MDA	0.16 ± 0.04	0.67 ± 0.05	0.96 ± 0.03*	0.28 ± 0.06	0.84 ± 0.03*
4	MP	0.22 ± 0.02	0.64 ± 0.03	0.88 ± 0.02	0.23 ± 0.04	0.78 ± 0.02
	MP + MDA	0.22 ± 0.02	0.69 ± 0.03	0.96 ± 0.02*	0.27 ± 0.04	0.88 ± 0.02*
8	MP	0.19 ± 0.03	0.58 ± 0.03	0.92 ± 0.02	0.34 ± 0.04	0.79 ± 0.02
	MP + MDA	0.21 ± 0.03	0.74 ± 0.03*	0.96 ± 0.02	0.22 ± 0.04*	0.88 ± 0.02*

Each value is represented by the mean ± SD of at least 3 determinations on samples from 2 independent experiments. An asterisk indicates that there are significant differences ($P < 0.05$) with respect to the corresponding control system

compression cycles, corresponding to a possible viscous element (Fiszman and others 1998). The results obtained for this parameter showed a significant increase ($P < 0.05$) due to the treatment with MDA for t = 0 and 4 h. At t = 8 h, S_{ret} did not present significant differences with respect to the control system, but a decrease of the difference ($S_{ret} - S_{ins}$) for these systems revealed a minor viscous component associated with the effect of the MDA after 8 h of incubation ($P < 0.05$). It is important to remark the increase observed in the S_{ret} values for the control systems as a function of the incubation time, suggesting some effect of the temperature (27 °C) on the gel formation.

Additionally, gels made with MDA had higher cohesiveness values ($P < 0.05$) than control systems for all incubation times, probably due to stronger internal forces among the molecules than only protein gels.

For all systems, an absence of adhesiveness, which is seen as a negative force area during the 1st cycle, was noticed.

Relaxation test. Figure 3 shows normalized curves for gels cooked after 4 h of incubation at 27 °C treated or not treated with MDA, evidencing differences in the relaxation response of these systems. The value of the difference ($100 - F_{15}$) could be considered as an index of gel elasticity (Roussel and Cheftel 1988). In this way, it was possible to see that MP + MDA at t = 4 h gels had a larger elasticity index than the respective control system. Similar behavior was observed for systems corresponding to t = 0 and 8 h of incubation at 27 °C (data not shown). The elasticity index presented an increase from 1 % for control systems to 23 % associated with MP + MDA systems for all times of incubation. These results are in agreement with those obtained by the texture profile analysis, evidencing a higher degree of solidity as a result of the incubation with MDA.

Water holding capacity (WHC)

Figure 4 shows the results obtained for WHC determinations. Statistical analysis exhibited significant differences ($P < 0.05$) for control systems as a function of the incubation time, probably related to the "suwari" phenomenon (gel setting) observed in some other fish species (Roussel and Cheftel 1990). This process would be produced by protein–protein interactions, due to a partial unfolding of the myosin during incubation at temperatures between 0 and 40 °C along different times. Also, it could be associated with the small increase observed in S_{ret} for control systems as a function of the incubation time (Table 1).

In the case of gels obtained from myofibrillar proteins treated with MDA, the results showed significant differences ($P < 0.05$) for

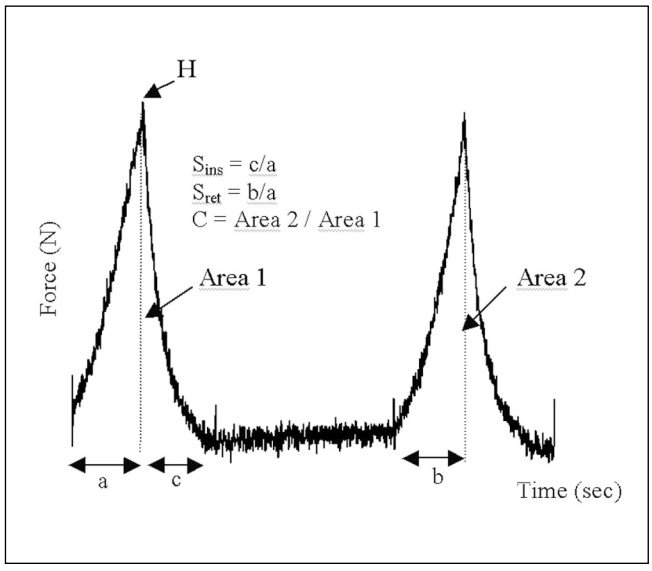

Figure 2–Characteristic texture profile curve obtained for sea salmon myofibrillar proteins, showing the corresponding parameters.

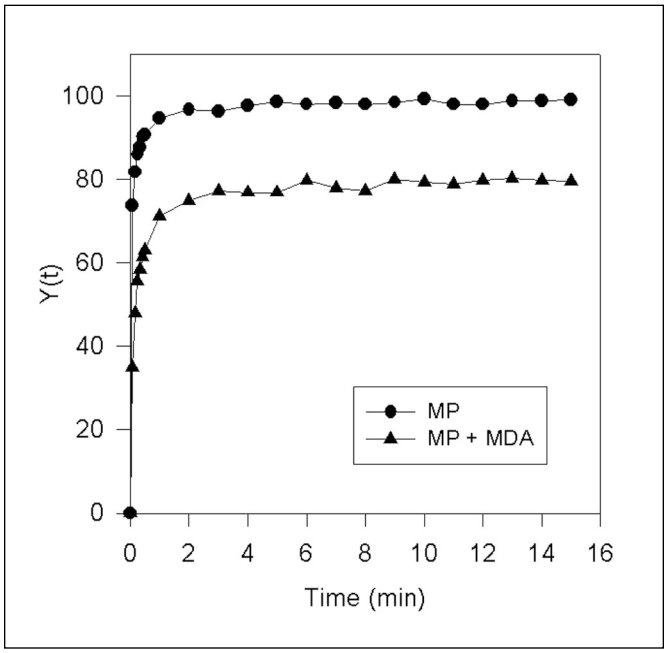

Figure 3–Normalized curves of relaxation (Y(t) = (Fo – F(t)) / FoT× 100) for gels obtained from myofibrillar proteins (MP) previously incubated with and without MDA (t = 4 h) and then heat set at 80 °C.

t = 0 and 8 h of incubation with respect to their corresponding control systems. MDA produce negative effects on the capacity of gels to retain water, probably due to its reaction with ϵ-NH_2 groups of proteins (Buttkus 1967). In this way, less groups capable to form hydrogen bonds with the molecules of water would be present in the MP + MDA gels.

Electrophoresis of the remainder protein dispersion

Figure 5 shows the electrophoretic pattern of residual liquids obtained by SDS–PAGE without ME (patterns with ME were similar). In all cases, the band corresponding to the myosin heavy chain (MHC) (205 kDa) did not appear, since it is the main protein constituent of the gel matrix. Also, actin (40 kDa) was included in the gel

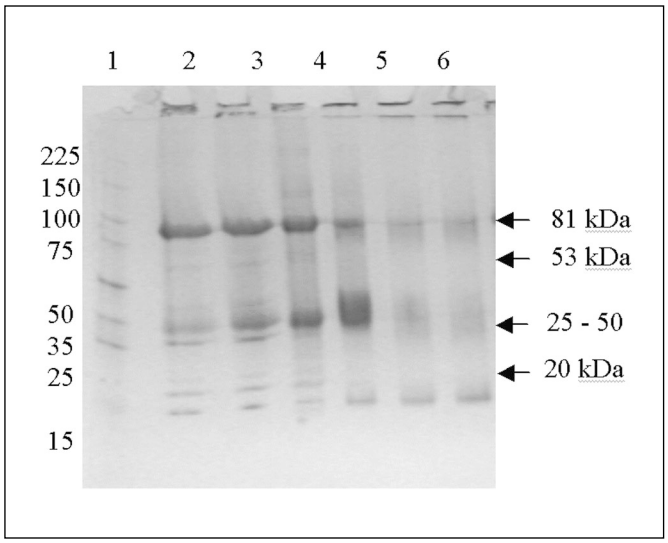

Figure 5–SDS–PAGE (in absence of ME) of the remainder protein dispersions of myofibrillar protein (MP) gels previously incubated with and without MDA for different periods of time: (1) molecular weight standards, (2), (3), and (4) control systems MP, t = 0, 4, 8 h, respectively; (5), (6), and (7) MP + MDA, t = 0, 4, 8 h, respectively.

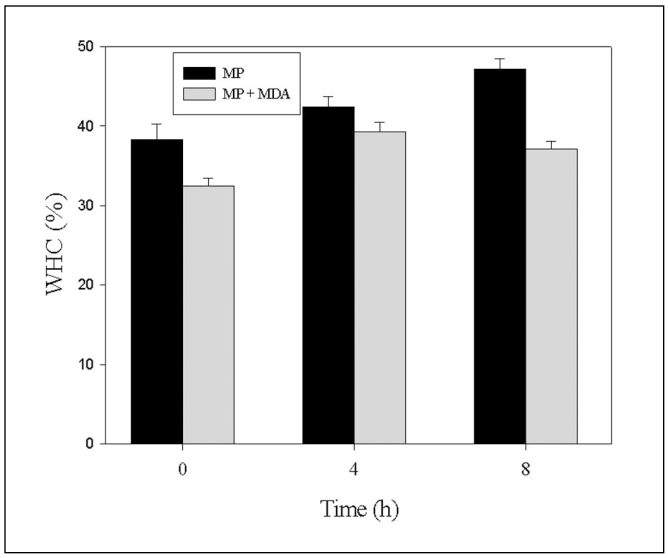

Figure 4–WHC of myofibrillar protein (MP) gels as a function of the incubation time with and without MDA. Error bars represent the SD of at least 3 determinations on samples from 2 independent experiments.

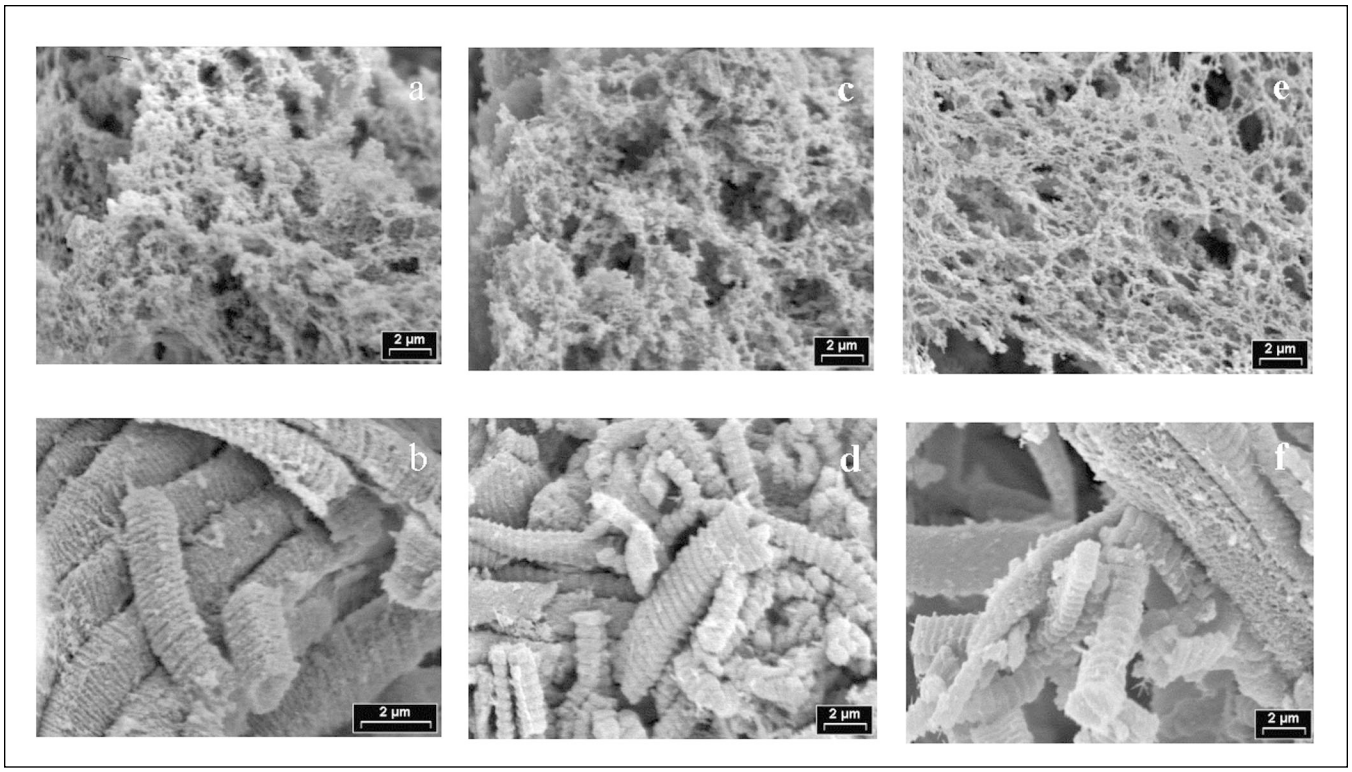

Figure 6–Scanning electron micrographs of myofibrillar protein (MP) gels previously incubated with and without MDA for different periods of time: (a), (c), and (e) control systems MP, t = 0, 4, and 8 h, respectively; (b), (d), and (f) MP + MDA, t = 0, 4, and 8 h, respectively.

network, as demonstrated by the very low intensity of the corresponding band. It is important to consider the decrease in the intensity of bands corresponding to 81, 53, 25 to 50, and 20 kDa, which could be observed in the presence of MDA, indicating that these protein species would remain in the gel matrix. These results are in agreement with those obtained in a previous paper (Tironi and others 2001), where it was possible to detect the formation of aggregates involving MHC, myosin light chains (MLC) (13 to 20 kDa) and 81 and 53 kDa species.

Scanning electron microscopy (SEM)

Gels obtained from nontreated myofibrillar protein (t = 0, 4 and 8 h) showed the formation of a typical 3-dimensional network with a continuous matrix (Figure 6) (Samejima and others 1981; Smith 1987; Lefèvre and others 1998). In the case of proteins treated with MDA, the appearance of big "filaments" agreggated in a disorganized way, which would replace the characteristic gel structure, was noted. The presence of these filaments would demonstrate the aggregation of the myofibrillar proteins by reaction with MDA, correlating with the decrease in WHC observed. In this way, the formation of aggregates would produce a reduction in groups available to form hydrogen bonds with water molecules. It is important to take into account that the changes described before could be observed at all times of incubation (t = 0, 4, and 8 h). Nevertheless, at t = 0, the matrix presented some different characteristics, though they are less compact than the ones observed at other times. This fact could be related to the hypothesis about the occurrence of the protein–MDA interaction during or prior to the gelation process, as stated previously.

Conclusions

THE EXPERIMENTAL RESULTS DEMONSTRATED IMPORTANT MODIFICATIONS in the microstructure of gels due to the myofibrillar proteins of sea salmon–MDA interaction. These changes are related to protein polymerization and the participation of these polymers in the gel matrix. It was possible to elucidate that new protein species appeared in the structures studied, which correlate with the protein modifications produced by the initial deteriorative process. Textural analysis showed gels with more solid characteristics, probably due to the presence of new bonds between the protein molecules. MDA caused a decrease in the WHC of gels, with possible negative consequences for further technological purposes. It is important to state that the presence of MDA during the gelation process, still without previous interaction with the proteins, had negative effects on the gel properties, producing a decrease of G′. Overall, protein–MDA interaction causes marked changes in functional and structural aspects in these model systems. Further experiments are necessary to extrapolate this information and understand the possible negative impact of lipid oxidation products on the whole muscle under inappropriate storage conditions.

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