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The effects of an iron-catalyzed oxidation system on lipids and proteins of dark muscle fish

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Abstract

The effects of a hydroxyl radical-generating system induced by iron catalyzed oxidation (Fe^{2+}/H_2O_2) on lipids and proteins of sardine, Atlantic bonito, anchovy and bluefish were investigated. Thiobarbituric acid-reactive substances (TBARs, mg *malonaldehyde*/kg fish muscle) formation was used to evaluate oxidative damage of the lipid in dark muscle fish *due to* iron catalyzed oxidation. *The amount of TBARs was observed to increase significantly* in sardine and anchovy *as the incubation time increased*, while Atlantic bonito and bluefish reached their maximum values of TBARs within the first 3 h of incubation, and after that did not change ($p < 0.05$). Carbonyl contents (nmol carbonyl/mg protein) of all fish samples *measured as an index of protein oxidation* were affected differently by the iron-catalyzed oxidation system during incubation. However, significant increases in the carbonyl groups were detected in sardine, Atlantic bonito and bluefish, but not in anchovy as a result of the long incubation time (5 h) ($p < 0.05$). When comparing to increases Atlantic bonito showed the maximum increase protein carbonyl. The electrophoretic patterns in the presence and absence of β-mercaptoethanol showed that a loss of proteins generally occurred in all fish at the end of incubation, and the greatest alteration in protein bands was observed in anchovy during Fe^{2+}-catalyzed oxidation. The bands above 50 kDa disappeared within the first 1 h of incubation. The loss of protein (both high and low molecular weight) may involve disulfide and non-disulfide covalent linking during the iron catalyzed incubation. These data suggest that an increase in TBARs and fragmentation, and a loss of proteins exposed to iron-catalyzed oxidation may explain the oxidative damage of lipids and proteins which causes quality loss and limits the storage life of dark muscle fish.

Keywords: Oxidation; TBARs; Protein carbonyl; SDS–PAGE; Iron catalyzed oxidation system; Dark muscle fish

1. Introduction

Reactive oxygen species (ROS), such as superoxide ($O_2^{\cdot -}$), hydroxyl ($OH^{\cdot}$), peroxyl ($RO_2^{\cdot}$), alkoxyl ($RO^{\cdot}$) and hydrogen peroxide (H_2O_2), in food and biological systems, are naturally generated and can react with biological molecules. However, an excessive production of ROS may damage complex cellular molecules, such as fats, proteins, or DNA. Convincing scientific evidence shows that ROS play a crucial role in many diseases, and are even considered to be a component of nearly every disease process (Dalle-Done, Rossi, Giustarini, Milzani, & Colombo, 2003). In living organisms, they create anti-oxidative defence mechanisms which neutralize the production and adverse effects of reactive oxygen species, but unfortunately these mechanisms do not work after death. This can cause accumulation of ROS in muscles, which leads to lipid and protein modifications. There is general agreement that oxidation of lipids in fatty fish is a major problem during storage and processing, particularly in dark muscles of pelagic species. A possible reason behind this vulnerability is the amounts of pro-oxidants, such as heme proteins (Hb), myoglobin, low molecular weight (LMW) transition metal complexes, and lipoxygenases, in their dark muscles

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(Hultin, 1992; Richards, Østdal, & Andersen, 2002; Undeland, Kristinsson, & Hultin, 2004; Pazos, Medina, & Hultin, 2005). The oxidizing of proteins and their damage in biological systems has been studied over the past two decades. Results of these studies showed that ROS lead to oxidation of amino acid side chains, formation of protein–protein cross-linkages, and oxidation of protein backbone, resulting in protein fragmentation (Berlett & Stadtman, 1997; Soyer & Hultin, 2000; Stadtman & Levine, 2003). There is no doubt that free radical-mediated oxidation of proteins, as much as that of lipids, is unavoidable in the aqueous phase where many of the free radicals are produced in fish muscles (Srinivasan & Hultin, 1997; Soyer & Hultin, 2000).

Metal-catalyzed oxidation systems (MCO) have been extensively used as a model for oxidative damage in biological samples (Madurawe & Lumpkin, 1997; Kato, Kıtamoto, Kawai, & Osawa, 2001). Recently, it has become evident that oxidation of specific proteins, especially that due to metal-catalyzed oxidation systems, leads to loss of function, structure change, and altered susceptibility to degradation (Stadtman & Oliver, 1991; Requena, Levine, & Stadtman, 2003; Biridgewater, Lim, & Vachet, 2006). Decker, Xiong, Calvert, Crum, and Blanchard (1993) observed, in turkey muscles, that oxidizing of muscle proteins, by both iron and copper in the presence of ascorbate, resulted in alteration of protein conformation, increase of carbonyl content, loss of protein solubility and reduction in myofibrillar gel strength and gel water-holding capacity. They also found that non-specific protein fragmentation or polymerization of low molecular weight proteins occurred in iron-oxidized proteins. Martinaud et al. (1997) found that myosin and troponine T were the most sensitive proteins to oxidation, and that Fe^{2+}/H_2O_2 oxidation, among the metal catalyzed oxidation systems was the most efficient system, giving the highest carbonyl content. Similar observations where also found by Mercier, Gateller, and Renerre (2004). Srinivasan and Hultin (1997) observed a decrease in protein solubility, an increase in protein carbonyl, and some loss in sulfhydryl groups and myosin heavy chain in washed minced cod muscle when the protein was oxidized by a non-enzymatic free radical-generating system. Saeed, Fawthrop, and Howell (1999) found that free radical transfer from the oxidized lipids to protein caused protein denaturation and the formation of insoluble aggregates in Atlantic mackerel. Stadtman and Oliver (1991) reported that protein and lipid oxidation appeared to begin simultaneously, and the primary role of oxidized protein in cod sarcoplasmic reticulum might be in competition for free radicals for membrane lipids. Saeed and Howell (2004) reported that the formation of protein with lipid oxidation products, such as free radicals, in frozen Atlantic mackerel, was partly caused by production of aggregates and protein cross-linking.

Among the ROS, the hydroxyl radical is one of the most damaging radicals, and it can be generated by the interaction of $O_2^{\cdot-}$ and H_2O_2, especially in the presence of catalytic iron salts (Gutteridge, 1981; Gutteridge, Rowley, & Halliwell, 1981; Halliwell & Gutteridge, 1999), and its catalyzing effects on muscle foods were also demonstrated (Decker et al., 1993; Huang, Hultin, & Jafar, 1993; Ahn & Kim, 1998; Mei, Decker, & McClements, 1998; Baron & Andersen, 2002; Soyer & Hultin, 2000). It is well known that iron has strong pro-oxidative activity in dark muscle fish (Richards et al. (2002); Richards & Hultin, 2003; Undeland et al., 2004). From this point of view, the iron-catalyzed oxidation system may be a good indicator of oxidative damage in dark muscle fish. Until now, the studies on oxidative damage of proteins by metal-catalyzed oxidation systems are very limited in fish muscle and are especially concentrated on mackerel and cod. However, sardine, anchovy, Atlantic bonito and bluefish are also important fish species for many countries, and oxidative damage can also cause important loss of quality. The aim of this work was to determine the effects of iron-catalyzed oxidation systems on proteins and lipids of dark muscle fish.

2. Material and methods

2.1. Materials

The sardine (*Sardine pilchardus*), anchovy (*Engrailus engrasichalus*), Atlantic bonito (*Sarda sarda*) and bluefish (*Pomatomus saltator*) were purchased from a local fish market in Adana. The fish were transported to the laboratory on ice and were gutted, washed, filleted and homogenized. Some of the samples were processed for oxidation procedures immediately, while others were stored at a low temperature vial at −80 °C for one month.

2.2. Methods

2.2.1. Oxidation procedure

One gramme of fish muscle tissue was homogenized in 10 ml of 100 mM sodium phosphate buffer (pH 7.0) using a homogeniser (Ultra-turrax, Ika T8) for 1 min at high speed. Ferrous sulphate (0.5 mM) and hydrogen peroxide (1 mM) were added to the homogenate. A Fe^{2+}/H_2O_2 ratio of 1:2 was used to obtain maximum effect of the oxidative system as recommended by Mercier et al. (2004). The mixtures were incubated for 0, 3 and 5 h at 37 °C in a water bath, and the reaction was stopped by adding butylated hydroxytolune (BHT, to 0.02% final concentration) to aliquots of 2 ml of homogenate. Some of the mixture was analyzed for lipid oxidation immediately, while some was stored at −80 °C for 8 days.

2.2.2. Determination of lipid oxidation

Lipid oxidation was evaluated by production of thiobarbutiric acid-reactive substances (TBARs), according to the method of Lynch and Frei (1993). 0.25 ml of 1% 2-thiobarbutiric acid (w/v) in 0.05 M NaOH and 0.25 ml of

2.8% trichloracetic acid (w/v) were added to 0.5 ml of incubated samples. The samples were then incubated in a boiling water bath for 10 min, cooled and extracted with 2 ml of *n*-butanol. After clarifying the pink chromagen, the colour intensity of the *n*-butanol layer was measured at 535 nm against a blank of *n*-butanol. The concentrations of TBARs in samples were calculated by reference to a standard curve by using 1,1,3,3,-tetramethoxypropane. The results were expressed as mg malonaldehyde (MDA) per kg fish muscle.

2.2.3. The determination of protein oxidation

2.2.3.1. Protein carbonyl content. The protein carbonyl contents were detected by reactivity with 2,4-dinitrophenylhyrazine (DNPH) as described by Levine, Williams, Stadtman, and Shacter, 1994. Two equal aliquots of incubated samples (for 0, 1, 3 and 5 h) were precipitated with 10% trichloracetic acid (TCA; w/v, final concentration) and then centrifuged at 12,000 rpm for 3 min. One pellet was treated with 2 M HCl, and the other was treated with 10 mM DNPH in 2 M HCl. Samples were placed in a sample holder and shaken continuously for 1 h at room temperature. The two fractions were then precipitated with 10% TCA (final concentration) and centrifuged (12,000 rpm/3 min). The pellets were washed with 1 ml of ethanol:ethyl acetate (1:1 v/v) to remove free DNPH reagent. The samples were centrifuged at 12,000 rpm for 3 min, and the supernatants were discarded. The washing procedure was repeated three times in total. The proteins were finally dissolved in 1 ml of 6 M guanidine–HCl in 20 mM potassium phosphate buffer (pH 2.3, adjusted with trifluoroacetic acid). All samples were then centrifuged to remove any insoluble material. The concentration of DNPH was determined at 360 nm against the blank of 1 ml guanidine solution, and a molar absorption coefficient of 22,000 M^{-1} cm^{-1} was used to quantify the levels of protein carbonyls. Protein concentration was determined at 280 nm (absorbance) in the samples using BSA in 6 M guanidine as standard. The results were expressed as nmol carbonyl/mg protein.

2.2.3.2. Electrophoresis of oxidized samples. Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis, in the absence and presence of β-mercaptoethanol, was used to monitor polymerization or fragmentation of oxidized proteins using a BioRad Mini vertical gel electrophoresis. SDS–PAGE was performed in a 10% resolving gel and 4% stacking gel according to the method of Laemmli (1970).

2.3. Statistical analyses

All experiments were performed in duplicate or triplicate. Data were analyzed by one-way analysis of variance (ANOVA) using the SPSS 10.0 for Windows. Duncan's multiple range test was used to find significant differences between the incubation times.

3. Result and discussion

3.1. Lipid oxidation

The results of TBARs values of dark muscle fish induced by iron catalyzed oxidation systems are shown in Table 1.

Lipid oxidation, measured by TBARs value in sardine and anchovy, was significantly increased by the iron catalyzed oxidation system with increasing incubation time. However, the maximum value of TBARs in Atlantic bonito and bluefish was obtained within 3 h of incubation and did not change thereafter ($p < 0.05$). The iron-induced lipid oxidations of sardine, atlantic bonito and bluefish were greater than those of anchovy. This suggested that the ferrous state of iron accelerated the lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via Fenton-type reactions (Dunford, 1987). Saeed et al. (1999) found that the TBARs value of cod sarcoplasmic reticulum lipids, induced by a non-enzymatic iron ascorbate oxidation system, increased rapidly and then built up only a little with incubation time course. They suggested that the lipid oxidation built up more rapidly and quickly reached its maximum value with increasing iron concentration. Mei et al. (1998) observed that increasing Fe^{2+}, (coming from $FeSO_4$) concentration increased TBA formation in SDS-emulsified salmon oil. Huang et al. (1993) also estimated that the extent of sarcoplasmic reticular lipid oxidation, as indicated by TBARs, was proportional to the iron levels added, using Fe^{2+}-ADP as a catalyst. They suggested that lipid hydroperoxides were limited by the amount of iron present. Many studies have shown that the Fe^{2+}/H_2O_2 mixture was the most efficient among different metal-catalyzed oxidation systems (Mercier et al., 2004; Martinaud et al., 1997; Nagasawa, Yonekura, Nishizawa, and Kitts, 2001). Fomusa, Corredig, and Akoh (2002) reported that Fe^{2+} could decompose hydroperoxides more rapidly than could Cu^{2+}. Iron, as a transition metal, may be the most important lipid oxidation pro-oxidant in dark muscle fish due to its high reactivity and concentration (Mei et al., 1998; Richards et al., 2002; Richards & Hultin, 2003; Undeland et al., 2004). However, the degree of oxidation of these fish by iron-catalyzed oxidation can differ,

Table 1
The results of TBARs values (mg malonaldehyde/kg fish muscle) of dark muscle fish induced by iron-catalyzed oxidation system[A]

Fish	Incubation time (h)		
	0	3	5
Sardine	1.87 ± 0.31^{a}	6.51 ± 0.52^{b}	7.85 ± 0.37^{c}
Anchovy	6.56 ± 0.07^{a}	9.74 ± 0.58^{b}	12.3 ± 0.49^{c}
Atlantic bonito	2.79 ± 0.11^{a}	9.92 ± 0.48^{b}	10.6 ± 0.18^{b}
Bluefish	1.81 ± 0.03^{a}	5.31 ± 0.33^{b}	6.13 ± 0.64^{b}

Means within the same column having different superscripts are significantly different at $p < 0.05$.

[A] Data are expressed as mean ± standard deviation ($n = 2$).

depending on many factors, including the concentrations of unsaturated fatty acids, the type and the amount of iron, other pro-oxidants and antioxidants; and also their balance.

3.2. Protein oxidation

The results of protein carbonyl contents (nmol carbonyl/mg protein) in dark muscle fish oxidized by the iron-catalyzed oxidation system are shown in Table 2.

Carbonyl contents, measured as an index of protein oxidation, were affected differently by the iron-catalyzed oxidation system over the time course of incubation in different fish muscles. A significant increase in the carbonyl groups was detected in sardine, bluefish and Atlantic bonito as a result of the long incubation time (5 h) ($p < 0.05$). Sardine showed a 2.4-fold increase in carbonyl content during the 1 h of incubation, to reach its maximum, level and then decreased ($p < 0.05$). Similar observations have been found in meat exposed to Fe^{2+}/H_2O_2 oxidation systems following a long incubation (Mercier et al., 2004) and different oxidation systems (Batifoulier, Mercier, Gateller, & Renerre, 2002). The increasing rate of bluefish was slower than sardine, and reached maximum levels of protein carbonyl at the end of the incubation time with a 1.4-fold increase. Atlantic bonito reached its maximum level of carbonyl by a 5-h of incubation time with a 5-fold increase. Atlantic bonito showed the highest rate of increase in protein carbonyl among the other fish species. Lin and Lin (2005) observed that there was no significant change in carbonyl content in bonito fillets at the end of the 16 weeks of frozen storage. In our study, the carbonyl content, within 1 h, did not increase either. These data show that protein oxidation in Atlantic bonito may occur for long time periods when they are exposed to free radical-generating systems. Srinavasan and Hultin (1995) estimated that the carbonyl content in mackerel fillets were increased by a free radical-generating system between day 0 and day 8. Similarly, Srinivasan and Hultin (1997) found that a significant increase of protein carbonyl content occurred in washed minced cod muscle stored at 5 °C for 2 and 24 h, when they generated the free radical system. Contrary to other fish, anchovy showed no significant increase in carbonyl contents at the end of the incubation time with respect to the initial level (0 h), and even a decrease was found in protein carbonyl content during the 1st hour of incubation.

Table 2
The protein carbonyl content (nmol carbonyl/mg protein) of dark muscle fish oxidized by iron catalyzed oxidation system[A]

Incubation time (h)	Sardine	Anchovy	Atlantic bonito	Bluefish
0	0.49 ± 0.06^{a}	1.25 ± 0.02^{ac}	0.38 ± 0.05^{a}	0.60 ± 0.02^{a}
1	1.16 ± 0.06^{b}	0.70 ± 0.05^{b}	0.56 ± 0.18^{a}	0.78 ± 0.06^{b}
3	0.96 ± 0.07^{c}	1.19 ± 0.03^{a}	1.13 ± 0.35^{b}	0.69 ± 0.06^{c}
5	0.98 ± 0.01^{c}	1.33 ± 0.09^{c}	1.89 ± 0.16^{c}	0.86 ± 0.02^{b}

Means within the same column having different superscripts are significantly different.
[A] Data are expressed as means ± standard deviation ($n = 2$).

3.3. SDS–PAGE

SDS–PAGE patterns of Atlantic bonito, bluefish, sardine and anchovy during 0, 1, 3 and 5 h of incubation with the Fe^{2+}/H_2O_2 mixture are shown in Fig. 1.

The electrophoretic patterns of every fish showed their own responses when exposed to MCO systems. Loss of proteins generally occurred in anchovy, sardine, and Atlantic bonito at the end of incubation time in the presence of β-mercaptoethanol (Fig. 1). The most notable alteration in protein bands was observed in anchovy during Fe^{2+}-catalyzed oxidation, both in the absence and presence of β-mercaptoethanol. The bands above 50 kDa disappeared in anchovy after 3 h of incubation. The disappearance of polypeptides in the presence and absence of reducing agent suggested that the polymers could be formed by non-disulfide covalent bands, because the polymers with higher molecular weight were not seen in the gels obtained and

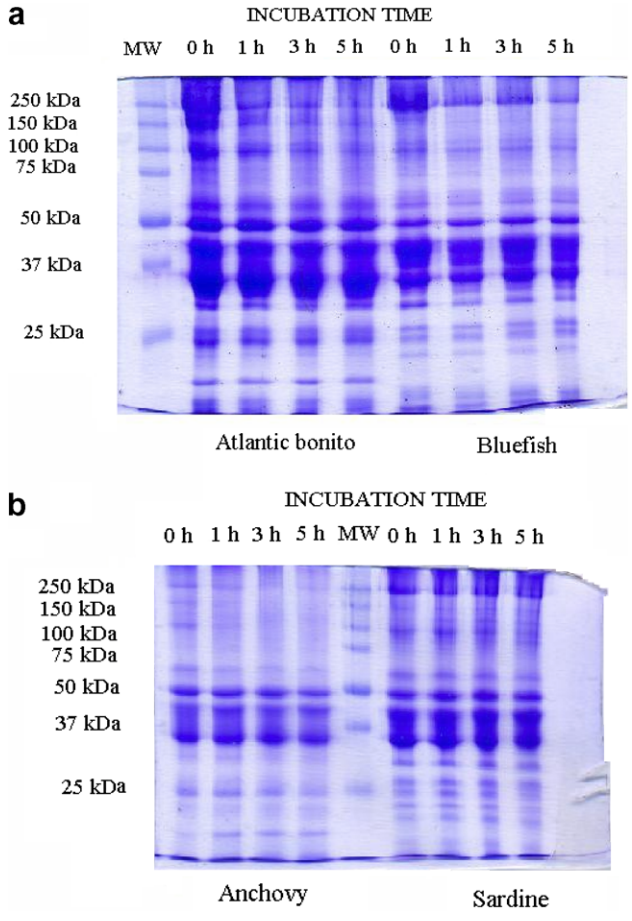

Fig. 1. Electrophoretic patterns of proteins extracted from Atlantic bonito, bluefish, sardine and anchovy during 0, 1, 3 and 5 h of incubation with Fe^{2+}/H_2O_2 mixture. Electrophoresis of samples was performed in the absence (a–b) and presence of β-mercaptoethanol (c–d).

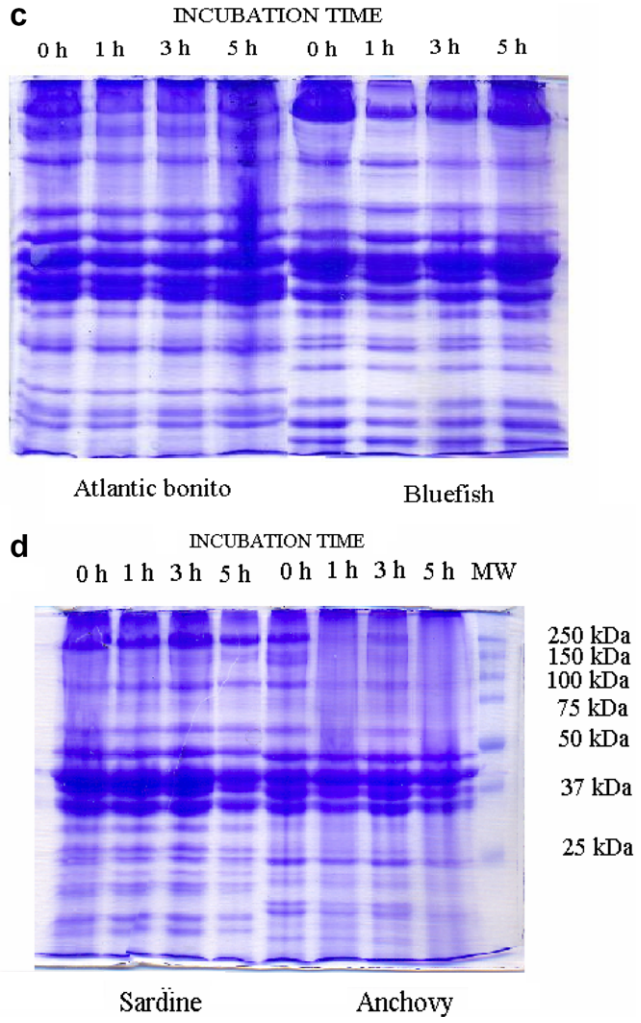

Fig. 1 (*continued*)

were dissociable in the presence of reducing agent (Mathews, Park & Anderson, 1980; Decker et al., 1993; Saeed & Howell, 2002). Srinivasan and Hultin (1997) found that no observable effects of free radical-generating systems were seen in electrophoretic bands of cod proteins in the presence of the disulfide splitting reagent when stored for 2 h at 5 °C, but a decrease in the MHC band was found as a result of an increase in ascorbate concentration in the absence of the DTT. However, they found several faint bands of high molecular weight material above 200 kDa at high concentrations of pro-oxidant and at longer storage times (24 h at 5 °C) in the presence of disulfide reducing agent, which could be due to non-disulfide linkages. Interestingly, the disappearing bands above 50 kDa during the 1st hour of incubation reappeared by the 3rd hour of incubation and then disappeared again in the presence of β-mercaptoethanol. The reappearance of the disappeared bands, with and without β-mercaptoethanol, and appearance of new bands in the presence of β-mercaptoethanol, suggested that the high molecular weight polymers could be formed via disulfide cross-linking. Similarly, many studies have shown that the fragmentation and polymerization of oxidized proteins with MCO systems occur via disulfide cross-linking (Liu & Xiong, 2000; Ooizumi & Xiong, 2004; Thanonkaew, Benjakul, Visessanguan, & Decker, 2006). The decrease of intensity in the band near 100 kDa without reducing agent in Atlantic bonito and sardine and the increase of intensity in this band, and the appearance of new bands with reducing agent, may have resulted in fragmentation of polymerized higher molecular weight proteins via disulfide linkages during incubation. The intensity of protein bands in bluefish also decreased without β-mercaptoethanol and new bands appeared with β-mercaptoethanol during incubation.

Our findings indicate that the protein aggregation or polymerization may generally occur via disulfide linking in all fish. Non-disulfide covalent linking can also occur depending on species, during the metal-catalyzed oxidation. Many studies have shown that several mechanisms could affect the oxidative modification of proteins as a result of intra- or inter-protein cross-linked derivatives (Jiang & Lee, 1985; Stadtman, 1990; Berlett & Stadtman, 1997; Dalle-Done et al., 2001; Stadtman & Levine, 2003). Stadtman and Levine (2003) reported that protein oxidation could cause cleavage of the polypeptide chain and formation of cross-linked protein aggregates. It was also suggested that functional groups of proteins could react with oxidation products of polyunsaturated fatty acids to produce inactive derivatives and lead to aggregation and protein cross-linking (Saeed et al., 1999; Refsgaard, Tsai, & Stadtman, 2000; Stadtman & Levine, 2003; Saeed & Howell, 2004). In this study, it is not clear whether the protein–protein or the protein–lipid interaction, or both of them, cause the loss of proteins in the electrophoretic patterns of anchovy.

The accumulation of oxidized proteins is often measured by the content of reactive carbonyls. Fe^{2+} can be bound to a metal-binding side on the protein and lead to conversion of some amino acid residues to carbonyl derivatives. The level of protein carbonyl groups can be used as a measure of protein damaged by metal-catalyzed oxidation under various physiological conditions (Stadtman, 1990). Many assays are available for detection of protein carbonyl. Among them, the fastest, most convenient and inexpensive procedure is detection of protein carbonyl using spectrophotometer, after reaction with 2,4-dinitrophenylhydrazine (DNPH). Moreover, protein carbonyls can be measured by DNPH-derived proteins (Adams et al., 2001; Berlett & Stadtman, 1997; Dalle-Done et al., 2003). However, the carbonyl contents may not fully describe the protein oxidation in muscles, as suggested by Chang, Chou, and Chang (2000) and Dalle-Done et al. (2001). It provides information only about carbonyl derivatives, but not others such as oxidation products of histidine (Uchida & Stadtman, 1992) and the loss of thiol groups (Takenaka, Miki, Yasuda, & Mino, 1991). The electrophoretic results in this study showed that oxidation of protein occurred in anchovy, although these oxidative changes were not detected by the carbonyl method. Similar observations

were reported by Liu and Xiong (1996) and Mercier et al. (2004).

The results demonstrated that iron-catalyzed oxidation systems were involved in oxidation of lipids and proteins in dark muscle fish, which may implicate functional and sensory quality changes of processed dark muscle fish. The electrophoretic patterns show that the oxidation of proteins occurs, not only via disulfide linking but also by non-disulfide covalent linking. As a result of this investigation it appears that, the response of the fish to oxidation of protein, by an iron-catalyzed oxidation system, can vary, depending on fish species. However, detailed investigations, based on species-specific tests, should be carried out to determine the effect of free radical-generating systems on protein in future research.

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