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Chemical oxidation decreases proteolytic susceptibility of skeletal muscle myofibrillar proteins

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Abstract

The objective of this study was to investigate the effect of chemical oxidation on proteolysis susceptibility of myofibrillar proteins. Myofibrils were prepared from pig *M. longissimus dorsi* and oxidised by a hydroxyl radical generating system. Protein oxidation level was measured by the carbonyl content, free thiol group content and bityrosine formation. Oxidised or non-oxidised myofibrillar proteins were exposed to papain and proteolysis was estimated by fluorescence using fluorescamine. Oxidation of myofibrillar proteins was dependent upon the oxidising agent concentration. Disulfide bridge and bityrosine formation indicated that oxidation by OH° can induce protein polymerization. Electrophoretic study showed that myosin was the protein most sensitive to oxidation. Results showed a direct and quantitative relationship between protein damages by hydroxyl radical and decreased proteolytic susceptibility. Electrophoretic observations suggest that polymerization and aggregation may explain in part decreased susceptibility of myofibrillar proteins to proteolysis.

Keywords: Myofibrils; Protein oxidation; Carbonyl; Free thiol; Bityrosine; Electrophoresis; Proteolysis

1. Introduction

Among eating qualities of meat, texture is rated as most important by the consumer and inconsistent tenderness is a major quality problem in the meat industry. Tenderisation of meat is dependent on several factors, including hydrolysis of structural proteins by endogenous proteolytic enzymes. Proteolysis in meat and meat products is mainly effected by cathepsins (Molly et al., 1997; Roncalés et al., 1995) and calpains (Goll, Thompson, Li, Wei, & Cong, 2003; Koohmaraie, 1992) but also by proteasome (Taylor et al., 1995).

Postmortem changes in muscle also include a decrease of the antioxidant defense system (Renerre, Dumont, & Gatellier, 1996; Renerre, Poncet, Mercier, Gatellier, & Metro, 1999) and an increase in the degree of lipid and protein oxidation (Martinaud et al., 1997; Mercier, Gatellier, Viau, Remignon, & Renerre, 1998; Renerre et al., 1999) under the action of free radicals. Formation of carbonyl groups, thiol oxidation and aromatic hydroxylation are the main known chemical modifications of amino acids during oxidation (Davies, 1987; Davies, Delsignore, & Lin, 1987; Stadtman, 1990, 1993). Oxidation may alter the secondary and tertiary structure of proteins, thereby increasing surface hydrophobicity (Grune, Jung, Merker, & Davies, 2004; Pacifi, Kono, & Davies, 1993). It can also lead to aggregate formation through cross links formed between cysteines (disulfide bond) or tyrosines (bityrosine bonds). In oxidative conditions interaction of proteins with other biomolecules can also lead to cross-linking/polymerization. Thus, carbohydrates or aldehydic lipid oxidation products (malondialdehyde or 4-hydroxynonenal) can react with amino groups of proteins to form fluorescent aggregates termed lipofuscin or ceroid (Friguet, Stadtman, & Szewda, 1994; Grune et al., 2004; Grune, Reinheckel, & Davies, 1997). Finally, severe oxidation can induce frag-

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mentation through direct attack of peptide bonds by free radicals (Davies, 1987; Stadtman, 1990, 1993).

The interaction between proteolysis and oxidation of proteins has for years been extensively studied in biomedical sciences. Contrasting effects of oxidation on proteolysis (enhancement or impairment) have been reported depending on the type of chemical modification. For example, increase of surface hydrophobicity can enhance protein degradation by proteases (Cervera & Levine, 1988; Davies, 2001; Davies & Goldberg, 1987; Levine, Mosoni, Berlett, & Stadtman, 1996). On the contrary, intermolecular cross-links and formation of aggregates in highly oxidative conditions can make proteins less susceptible to enzymatic proteolysis. Formation of lipofuscins has also been described to inhibit proteases (Friguet et al., 1994; Grune et al., 2004; Sitte et al., 2000). In eucaryotic cells, the proteasome system has been shown to recognize and degrade mildly oxidized proteins thus minimizing their toxicity, but more severe oxidative stress causes dramatic oxidation of proteins which become resistant to the proteasome system (Davies, 2001).

In meat, the link between enzymatic proteolysis and oxidative processes is rather poorly documented. However, Rowe, Maddock, Lonergan, and Huff-Lonergan (2004a, 2004b) have demonstrated that oxidation of muscle proteins has a negative effect on beef tenderness and attributed this effect to inactivation of μ-calpain and subsequent decrease in proteolysis. The present study was designed to explore the effects of oxidative conditions on proteolysis of myofibrillar proteins from pork muscle. Myofibrils were exposed to different concentrations of hydroxyl radical ($OH^{\bullet}$) generating system and subsequently hydrolysed by papain. Proteolytic susceptibility of oxidised myofibrillar proteins is presented and discussed in relationship to protein modifications such as carbonyl formation, loss of free SH groups, bityrosine formation, and aggregation.

2. Materials and methods

2.1. Animals and samples

Three Large White crossed Landrace pigs were slaughtered at 6 months of age. About 800 g of *M. longissimus dorsi* was removed immediately after bleeding. Fat was trimmed away and muscle was cut into cubes and frozen at −80 °C until use.

2.2. Isolation of myofibrils

Myofibrils were prepared according to the method of Ouali and Talmant (1990) with some modifications as outlined by Martinaud et al. (1997). Ten grams of frozen muscle were homogenised with a Waring blender in 100 ml of a solution at pH 6.5 containing 150 mM NaCl, 25 mM KCl, 3 mM $MgCl_2$, 4 mM EDTA, to which two protease inhibitors (PMSF 1 mM and E64 1 μM) had been added. The homogenate was ground with Polytron for 30 s, and collagen was eliminated by filtration on gauze. After 30 min of stirring in ice, the extract was centrifuged at 2000*g* for 15 min at 4 °C. The pellet was washed twice with 100 ml of a 50 mM KCl solution at pH 6.4 and once with 100 ml of 20 mM phosphate buffer at pH 6. The pellet was finally resuspended in the same phosphate buffer, and the protein concentration was adjusted to 14 mg/ml by the Biuret method (Gornall, Bardawill, & David, 1949). Two myofibril preparations were performed on three different pigs ($n = 6$).

2.3. Chemical induction of myofibrils oxidation

Myofibrils (10 mg/ml final concentration) were incubated for 3 h at 37 °C in 20 mM phosphate buffer at pH 6 with $FeSO_4$/diethylenetriaminepentaacetic acid (DETAPAC)/H_2O_2 (Fenton's reagent) at various concentrations (from 0 to 20 mM). H_2O_2 concentration was adjusted by absorbance at 240 nm. Phosphate was used in this study because, contrarily to other buffers commonly used for protein studies, it has no marked effects on protein oxidation (Davies et al., 1987). After 3 h no residual H_2O_2 could be detected by absorbance at 240 nm ($\varepsilon = 43.6\ M^{-1}\ cm^{-1}$) but to prevent subsequent oxidation, butylated hydroxy toluene (BHT 1 mM final concentration) was added to the incubation medium. For thiol groups, bityrosine and proteolysis measurements, oxidising agent must be removed. For this reason, samples were centrifuged at 4000*g* for 10 min and pellets were washed with 5 ml of 20 mM phosphate buffer at pH 6 and centrifuged (4000*g*/10 min).

2.4. Determination of carbonyl content

Carbonyl groups were estimated using the method of Oliver, Alin, Moerman, Goldstein, and Stadtman (1987) with slight modifications. Carbonyl groups were detected by reactivity with 2,4-dinitrophenylhydrazine (DNPH) to form protein hydrazones. Two aliquots of 400 μl of myofibrillar suspension were centrifuged at 4000*g* for 10 min. One pellet was treated with 1 ml of 2 N HCl and the other with an equal volume of 0.2% (w/v) DNPH in 2 N HCl. Both samples were incubated for 1 h at room temperature under agitation. The samples were centrifuged at 4000*g* for 10 min. The pellets were then washed three times with 1 ml of ethanol:ethyl acetate (1:1) to eliminate free DNPH. Myofibrillar proteins were then dissolved in 2 ml of 6 M guanidine HCl with 20 mM sodium phosphate buffer at pH 6.5. To remove insoluble material, samples were centrifuged 10 min at 4000*g*. Protein concentration in the supernatant was calculated at 280 nm in the HCl control using BSA in 6 M guanidine as standard. Carbonyl concentration was measured on the treated sample by measuring DNPH incorporated on the basis of an absorption of $21.0\ mM^{-1}\ cm^{-1}$ at 370 nm for protein hydrazones. The results were expressed as nanomoles of DNPH fixed per milligram of protein.

2.5. Determination of free thiol (SH) content

Thiol oxidation was measured by a modification of Ellman's method using 2,2′-dithiobis(5-nitropyridine) DTNP (Winterbourn, 1990). Washed pellets (containing 4 mg of myofibrillar proteins) were dissolved in 3 ml of 100 mM phosphate buffer at pH 8 containing 8 M urea. About 30 μl of 10 mM DTNP (stock solution in ethanol) was added and, after an incubation for 1 h at room temperature, the absorbance at 386 nm was measured against a blank of protein at the same concentration without DTNP. The absorbance of diluted DTNP was subtracted, and thiol concentration was calculated using an absorption coefficient of $14\,mM^{-1}\,cm^{-1}$. The results were expressed as nanomoles of free thiol per milligram of protein.

2.6. Determination of bityrosine formation

Bityrosine formation was estimated by the method of Davies et al. (1987). Washed pellets (containing 10 mg of myofibrillar protein) were dissolved in 3 ml of high ionic buffer (20 mM phosphate buffer at pH 6 containing 0.6 M KCl). Solutions were filtered through filter paper to eliminate residual lipids and insoluble material. Soluble protein concentration was estimated by the Biuret method. Bityrosine content was estimated by fluorescent measurement at 420 nm (band width = 10 nm) after excitation at 325 nm (band width = 10 nm), using a Shimadzu RF-1501 spectrofluorophotometer fitted with a 150 W Xenon lamp. Corrected fluorescence was obtained by dividing measured fluorescence by protein concentration. The results were expressed in arbitrary units.

2.7. SDS-PAGE gel electrophoresis

Myofibrils were incubated for 10 min at 90 °C in a buffer containing 30% (v:v) glycerol, 5% (v/v) mercaptoethanol, 2.3% (w/v) SDS, 62.5 mM Tris–HCl (pH 6.8) and 0.05% (w/v) bromophenol blue. SDS-PAGE was performed according to the method of Laemmli (1970) using 12% (17 × 17 cm, 0.8 mm thick) polyacrylamide gels. The protein load was adjusted to 20 μg per lane. Gels were stained with Coomassie blue. Myosin heavy chain and actin band intensities were measured by Quantity one (Biorad).

2.8. Measurement of proteolysis rates

Washed pellets (containing 10 mg of myofibrillar proteins) were suspended in 1 ml of 20 mM phosphate buffer at pH 6. 0.1 mg (100 U) of lyophilised papain (Sigma) was added and suspensions were incubated at 25 °C under agitation. Papain digestion was terminated by addition at various times (0, 5, 10, 15, 20, 25, 30, 40, 60 min) of 10% (final concentration) trichloroacetic acid (TCA). In practice, 100 μl aliquots of reaction mixture were removed and diluted with 400 μl of 12.5% TCA. After centrifugation for 10 min at 4000*g*, the concentration of peptides and aminoacids in the supernatant was measured as described by Friguet et al. (1994) with modifications. Supernatant was first neutralised with 500 μl of 2 M potassium borate, pH 10. 300 μl of fluorescamine (Sigma) at a concentration of 0.3 mg/ml in acetone were added. Reactive volume was finally adjusted to 3 ml with distilled water. Fluorescamine reacts quantitatively with primary amines, forming a fluorescent product which was quantified by fluorescence measurement (excitation at 375 nm; band width = 10 nm and emission at 475 nm; band width = 10 nm). Glycine was used in parallel as amino acid standard. The concentration of primary amines in TCA-soluble fractions reflects the rate of myofibrillar protein proteolysis by papain, expressed in nanomoles of free amino groups per minute per milligram of papain.

3. Results and discussion

3.1. Carbonyl content

Amino acids with NH or NH_2 groups on their side chains are very reactive to $OH^{\cdot}$. These groups are transformed to carbonyl groups during protein oxidation (Stadtman, 1990, 1993). In our experiment, carbonyl content in fresh myofibrils (control) was 1.7 nmol/mg protein (Fig. 1), a value close to those reported by Martinaud et al. (1997) on bovine myofibrils and Liu and Xiong (2000a) on chicken myosin. Incubation for 3 h at 37 °C without oxidising agent induced a slight increase (+12%) of carbonyl content when compared with fresh myofibrils. Addition of oxidising agent produced an increase in carbonyl content in a concentration dependent manner: between 0 and 3 mM, carbonyl content increased linearly following the equation: [carbonyls] = 8.46[Fenton's reagent] + 1.14 ($R^2 = 0.991$). From 3 to 10 mM, carbonyls further increased to a maximum of 34 nmol/mg protein, but more slowly, and a lower value was observed at 20 mM. Values obtained are fairly consistent with previous experiments on beef myofibrils (Martinaud et al., 1997), while a lower value of 9 nmol/mg protein was observed by Liu and Xiong (2000a) on purified myosin treated for 1 h with ferric iron/H_2O_2/ascorbate, suggesting that the oxidative system used has an influence on oxidation levels. Protein carbonyl content increases during meat ageing but generally remains below levels obtained after chemical oxidation. Antioxidant protection of muscle remaining active for a few days after animal death (Renerre et al., 1999) certainly contributes to limit carbonyl formation. For example, after 10 days of meat ageing, Martinaud et al. (1997) measured carbonyl values of 5.1 nmol/mg and 6.9 nmol/mg protein in myofibrils from *M. longissimus lumborum* and *M. diaphragma pedialis*, respectively. In our experiment, such levels were reached at approximately 0.5–1 mM oxidising agent.

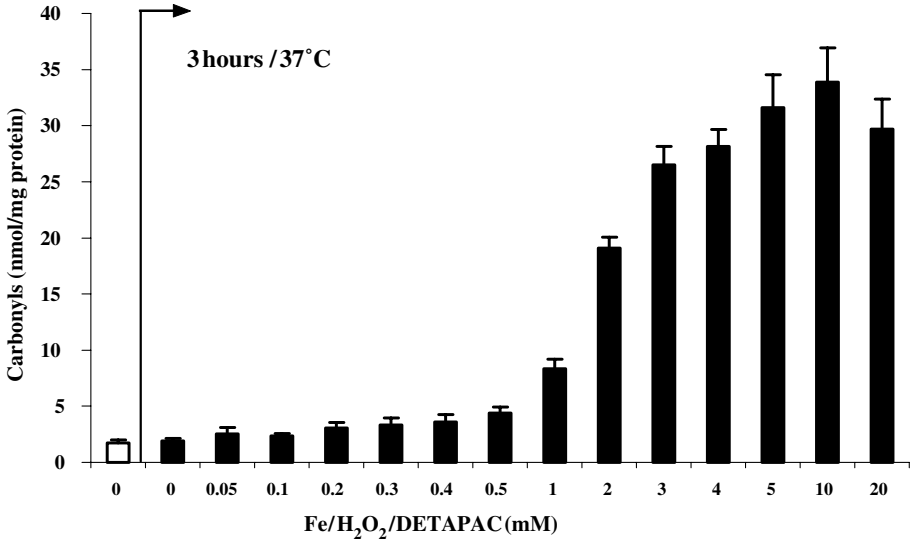

Fig. 1. Effect of different concentrations of free radical generating system on carbonyl content of myofibrillar proteins. White bar corresponds to fresh myofibrils. Values are means ± SEM of six independent determinations.

3.2. Free thiol content

The free thiol content of fresh myofibrils was 33 nmol/mg protein (Fig. 2); i.e. twofold lower than those measured on bovine muscle myofibrils (Martinaud et al., 1997) and rat heart myofibrils (Suzuki, Kaneko, Chapman, & Dhalla, 1991). Incubation for 3 h at 37 °C without oxidising agent induced only a slight decrease (−4%) of free thiol content when compared with fresh myofibrils. The level of free thiols was not significantly altered from 0 to 0.2 mM oxidising agent but 80% of free SH groups were lost from 0.2 to 20 mM oxidising agent. With exposure of rat myofibrils to xanthine/xanthine oxidase, which is known to generate superoxide radicals, Suzuki et al. (1991) measured a 20% decrease in SH content but incubating conditions were milder (30 min at 30 °C). Contrary to Liu and Xiong (1996) who indicated that loss of thiol groups in chicken meat could occur without formation of carbonyl derivatives, our results clearly show a correlation between the two phenomena (Table 1) translating the broad spectrum of targets of $OH^{\bullet}$.

SH loss observed during meat ageing, for example 10–17% after 10 days storage, (Martinaud et al., 1997) corresponded in our experiment to 0.3–0.4 mM oxidising agent.

3.3. Bityrosine formation

Bityrosine production has been described to be a useful marker for protein modification by $OH^{\bullet}$ (Davies et al., 1987). An increase in bityrosine has also been evidenced after exposure of myosin to H_2O_2-activated metmyoglobin (Hanan & Shaklai, 1995; Kristensen et al., 1997). In our

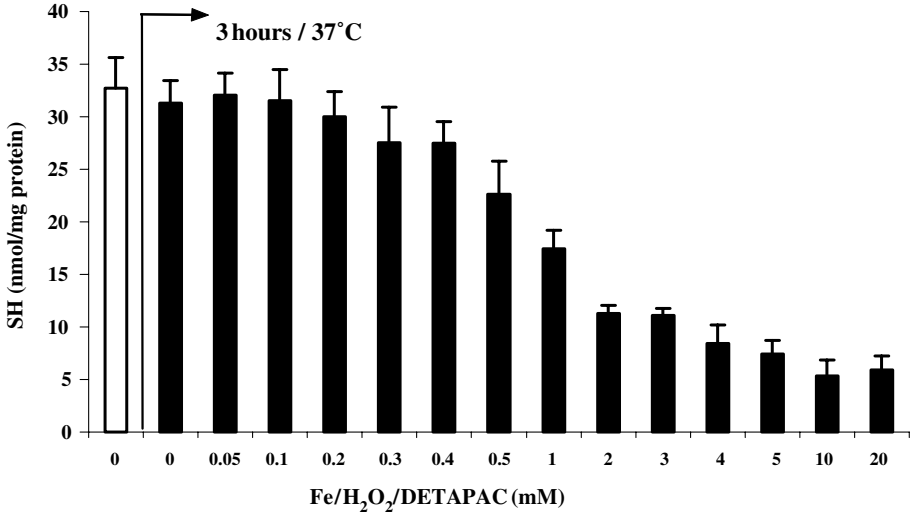

Fig. 2. Effect of different concentrations of free radical generating system on free thiol content of myofibrillar proteins. White bar corresponds to fresh myofibrils. Values are means ± SEM of six independent determinations.

Table 1
Correlation matrix between oxidation parameters (carbonyl, free SH and bityrosine), electrophoretic band intensities (myosin HC and actin) and proteolysis rate

	Carbonyl	Free SH	Bityrosine	Myosin HC	Actin	Proteolysis
Carbonyl	1					
Free SH	−0.959	1				
Bityrosine	0.935	−0.904	1			
Myosin HC	−0.868	0.934	−0.834	1		
Actin	−0.837	0.845	−0.836	0.860	1	
Proteolysis	−0.942	0.979	−0.930	0.919	0.871	1

Fresh myofibrils were excluded from correlation measurements. All correlations are highly significant ($p < 0.01$).

experiment (Fig. 3), we observed an important and sudden increase of bityrosine formation when myofibrils were exposed to 1 mM oxidising agent. Table 1 shows good correlations between bityrosine formation and other oxidation parameters.

3.4. Electrophoretic studies

Electrophoresis was performed in order to observe modifications induced by chemical oxidation of myofibrillar proteins. For example, carbonyls may react with the free amino groups of non oxidised amino acids to form amide bond (Liu & Xiong, 2000a). If this reaction occurs between different proteins, it can lead to protein polymerisation and aggregate formation. Similarly, thiol oxidation leading to disulfide bonds may generate intermolecular cross-links. Thus, during metal-catalysed oxidation of turkey myofibrillar proteins, Decker, Xiong, Calvert, Crum, and Blanchard (1993) observed high molecular weight polymers, produced by disulfide linkages and mainly derived from myosin and actin. It should be noted, however, that electrophoresis in denaturing conditions breaks up disulfide bonds and aggregates formed through this mechanism can not be visualized. Finally, bityrosine formation between protein molecules can also lead to polymerisation and aggregation (Davies, 1987). Such intermolecular bonding has been observed in oxidised myosin (Hanan & Shaklai, 1995; Kristensen et al., 1997).

SDS-PAGE patterns showed the decrease of bands corresponding to myosin heavy and light chains after exposure to oxidising agent (Fig. 4). This decrease was especially pronounced between 0.5 and 1 mM oxidising agent, which coincides well with the sharp increase in bityrosine formation. In highly oxidative conditions, high molecular weight compounds, probably aggregates, accumulated at the top of the gel. We therefore propose that polymerisation of myosin intervened mainly via bityrosine formation, in accordance with Kristensen et al. (1997), although aggregation indirectly due to carbonyl formation is also possibly involved. The actin band was relatively more stable and was affected only at higher oxidant concentrations (3 mM), oppositely to Dalle-Donne et al. (2001) who demonstrated that purified actin is particularly prone to chemical oxidation by hypochlorous acid. The difference between these findings are probably explained by accessibility of oxidation sites: in purified actin, such sites are free while in myofibrillar suspensions, interaction of actin with myosin chains may mask oxidation sites.

No fragmentation of myofibrils has been detected in our experiment even in the higher oxidative conditions. This

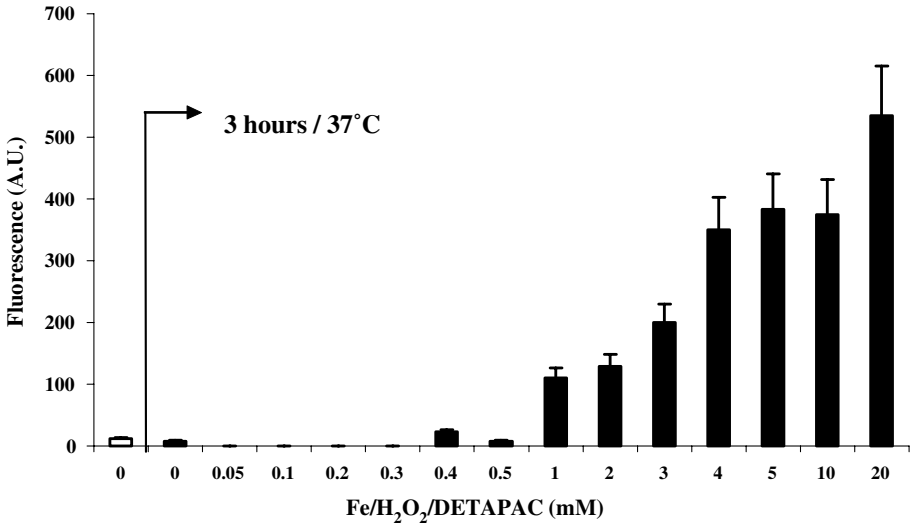

Fig. 3. Effect of different concentrations of free radical generating system on bityrosine formation in myofibrillar proteins measured by fluorescence. White bar corresponds to fresh myofibrils. Values are means ± SEM of six independent determinations.

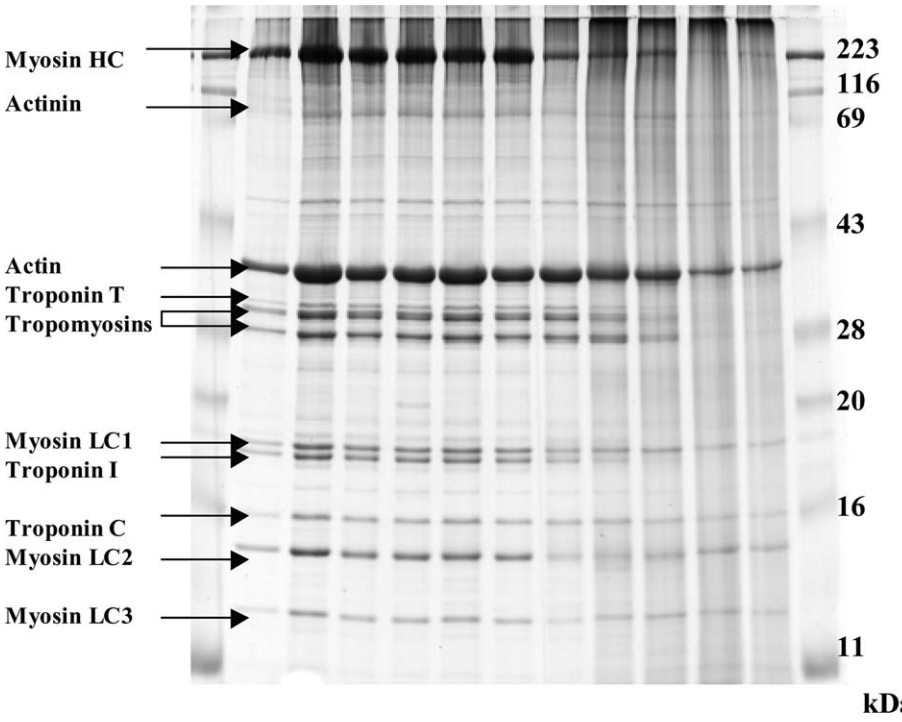

Fig. 4. Gel electrophoresis patterns of myofibrillar proteins oxidised by different concentrations of free radical generating system. Different lanes are from the left to the right; molecular weights, fresh myofibril, oxidised myofibrils (with 0, 0.05, 0.1, 0.3, 0.5, 1, 3, 5, 10, 20 mM Fenton's reagent) and molecular weights.

result was confirmed by fluorescamine amino acids detection (data not shown) showing no proteolysis during oxidation by Fenton's reagent. Ooizumi and Xiong (2004) also failed to observe myosin fragmentation when chicken myofibrils were exposed to ferric iron/ascorbate/H_2O_2 oxidative system even with a concentration up to 5 mM in H_2O_2.

3.5. Effect of oxidation of myofibrils on proteolytic susceptibility to papain

Papain is a plant cysteine proteinase. It presents a high homology in amino acids sequence with lysosomal cysteine proteinases (cathepsin L, B and H), especially in the catalytic site (Dufour, 1988). Moreover, papain has an optimum of activity at pH close to the ultimate pH in meat. Papain was therefore chosen in this study as a relevant model of a muscle proteolytic system.

Fig. 5 shows the effect of prior oxidative treatments of myofibrils on proteolysis rate by papain. Incubation of myofibrils for 3 h at 37 °C without oxidising agent already produced a 24% decline in the rate of proteolysis by papain when compared with fresh myofibrils. Rate of proteolysis remained stable between 0 and 0.3 mM oxidising agent but further declined at higher concentrations. At 20 mM oxidising agent, a residual proteolysis rate of only 2% (of the initial rate) was measured. Some authors have recently reported a biphasic response when proteolysis was measured on oxidised proteins (Davies, 2001; Grune et al., 2004): proteolytic susceptibility initially increases before declining at higher oxidant concentration, due to the formation of protein aggregates changing both chemical and physical recognition sites. This contrasts with our findings but may be explained by the use of insoluble myofibrillar proteins, spontaneously prone to aggregation, while earlier studies were performed on soluble proteins. On the other hand, our results are in good accordance with those obtained by Kristensen et al. (1997) who showed that oxidative modification of myosin produces high molecular weight aggregates and, at the same time, decreases its susceptibility to proteolytic degradation by cathepsin B. Similarly, Liu and Xiong (2000b) showed that, under non reducing conditions, oxidation decreases the susceptibility of myosin to pepsin, trypsin and chymotrypsin.

To establish links between oxidation and proteolysis, the relationships between indicators of protein oxidation and proteolysis rate were assessed by a correlation study. High correlations were obtained between proteolysis rate and free SH content as between proteolysis rate and bityrosine formation (Table 1). These results suggest that intermolecular cross-links via disulfide bridges and bityrosine, formed during oxidation, are an important cause of the decrease in proteolysis susceptibility. Importance of polymerisation/aggregation phenomena was confirmed by correlation measurements between decrease in proteolysis rate and decrease in protein band intensities, due to aggregation, as measured by electrophoresis (Table 1). Furthermore, good correlation was also obtained between carbonyl content and proteolysis rate (Table 1), which was expected since papain is a cysteine endopeptidase hydrolysing proteins at bonds involving Arg, Lys, His, Gly and Leu and

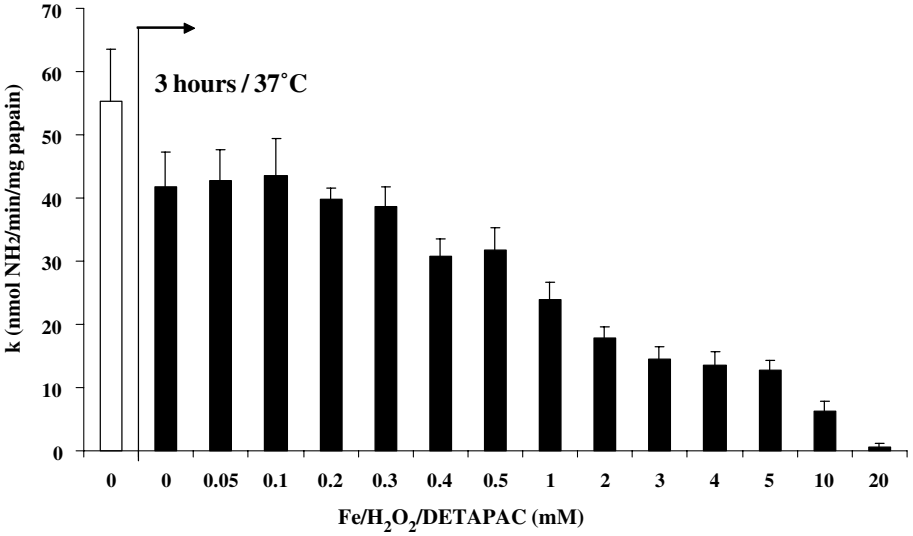

Fig. 5. Effect of prior oxidative treatment by different concentrations of free radical generating system on proteolysis rate (k) of myofibrils by papain. White bar corresponds to fresh myofibrils. Values are means ± SEM of six independent determinations.

many of these amino acids have their side chains converted to carbonyl groups during oxidation. Our results indicate that oxidation of myofibrillar proteins cause changes, polymerisation (via bityrosine and disulfide bridges) and specific chemical modifications of some amino acids, which can lead directly or indirectly to a decrease in proteolytic susceptibility. Polymerisation with other biomolecules (carbohydrates or lipid oxidation products) has not been studied here but this would be an obvious area to continue to get a further understanding on oxidative modifications in relation to meat quality development.

Even if levels of myofibril oxidation obtained in this model are higher than those produced in situ, this negative effect of oxidation on proteolysis should apply to some extent to meat. This is all the more likely because during meat ageing, proteases are also the target of free radical attacks, especially cysteine proteases that can be oxidized at their active site, which decreases their activity (Rowe et al., 2004b). Therefore, concomitant oxidation of proteases and of their myofibrillar substrates can act in synergy to decrease the extent of muscle tenderisation, especially in conditions generating high levels of lipid and protein oxidation (irradiation, freezing/unfreezing cycles or feeding animals with high unsaturated oils). This was used as an argument by Kristensen et al. (1997) to explain differences between oxidative and glycolytic muscles, the latter being less prone to postmortem lipid and protein oxidation and tenderising faster than oxidative muscle. Similarly, it has been demonstrated recently in beef that high oxygen packaging, a favourable medium for free radical production, has a negative effect on tenderness (Sorheim, Wahlgren, Nilsen, & Lea, 2004).

Improved antioxidant protection, provided for example by dietary treatment such as vitamin E supplementation (Mercier et al., 1998) and already used to prevent lipid and myoglobin oxidation in meat, could also have a beneficial effect on tenderness. This effect needs to be tested before giving recommendations to professionals in the meat industry.

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