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Detection and Localization of Oxidized Proteins in Muscle Cells by Fluorescence Microscopy

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In meat, no detailed studies on the intracellular distribution of oxidized proteins during oxidative stress have been performed, to our knowledge. Therefore, we used fluorescence microscopy to detect and locate protein carbonyls, oxidation products of basic amino acids, generated in bovine M. *Rectus abdominis* during either exposition to a chemical free radical generating system, or refrigerated storage, or cooking. The technique consisted of an immunohistochemical detection of carbonyls by reaction with the specific probe DNPH (2,4-dinitrophenylhydrazine) followed by the sequential addition of a first antibody against DNPH-carbonylated proteins and a CY3-labeled secondary antibody. The fluorescence of the CY3 probe increased regularly with level of free radical generating system and storage time. Moreover, an important heterogeneity of carbonyl distribution was observed, with a higher oxidation level at the periphery than inside the muscle cells. Cooking induced fluorescence increase only at the periphery of cells. Specific coloration of collagen by Sirius red showed that collagen was not involved in fluorescence. We can deduce that accumulation of oxidized proteins observed in the cell periphery was linked to membrane protein oxidation and not to connective tissue oxidation. Biochemical assays were performed in parallel on membrane and myofibrillar proteins to provide complementary quantitative data on level of oxidized proteins.

KEYWORDS: Meat; fluorescence microscopy; protein oxidation; carbonyl; DNPH

INTRODUCTION

Oxidation is a leading cause for quality deterioration of meat. The implications of lipid oxidation in meat quality have been extensively described for a long time while interest in protein oxidation has appeared more recently. There is now some evidence that, under the action of free radicals and due to loss in antioxidant protection *postmortem*, oxidized proteins accumulate in cells and tissues during meat aging and processing (*1–5*).

Formation of carbonyl groups, thiol oxidation, and aromatic hydroxylation are the main oxidative modifications affecting amino-acids (*6–9*). Alterations of the secondary and the tertiary structures of proteins have also been described, leading to changes in physical properties of proteins such as solubility, viscosity, hydrophobicity, and water holding capacity with consequences on technological properties such as gelation (*10*) and emulsification (*11*). By generating intermolecular bridges, such as disulfide or dityrosine bridges, oxidation can initiate protein aggregation, a consequence of which is a decrease in the proteolytic susceptibility of proteins (*12–14*), impairing the meat tenderness (*4*, *5*). Protein aggregation can also decrease protein digestibility (*15–17*) and therefore can affect the nutritional value of muscle food.

Biochemical assays, using specific probes of amino acid oxidation, have been carried out to provide quantitative data on the level of oxidatively damaged meat proteins (*1*). Carbonyls formed by oxidation of amino groups (Lys, Arg, His) are good tracers of protein oxidation in meat, and their determination by DNPH (2,4-dinitrophenylhydrazine) is the most popular chemical test to evaluate protein oxidation. However, little is known about the cellular localization of oxidized proteins in muscle cells. Therefore, the present study was designed to explore the intracellular distribution of oxidized proteins by immunohistochemistry during exposition to a chemical free radical generating system, refrigerated storage, and cooking. To accomplish this goal, we employed a fluorescence microscopic technique using a specific antibody against DNPH-carbonylated proteins and a CY3-labeled secondary antibody to reveal the acute oxidative damage caused to meat proteins. Myofibrillar and membrane protein oxidations were measured in parallel with the carbonyl chemical test using the same DNPH probe.

MATERIALS AND METHODS

Meat Sampling and Storage. The experiment was carried out with bovine M. *Rectus abdominis*. Four animals (Charolais heifers) were

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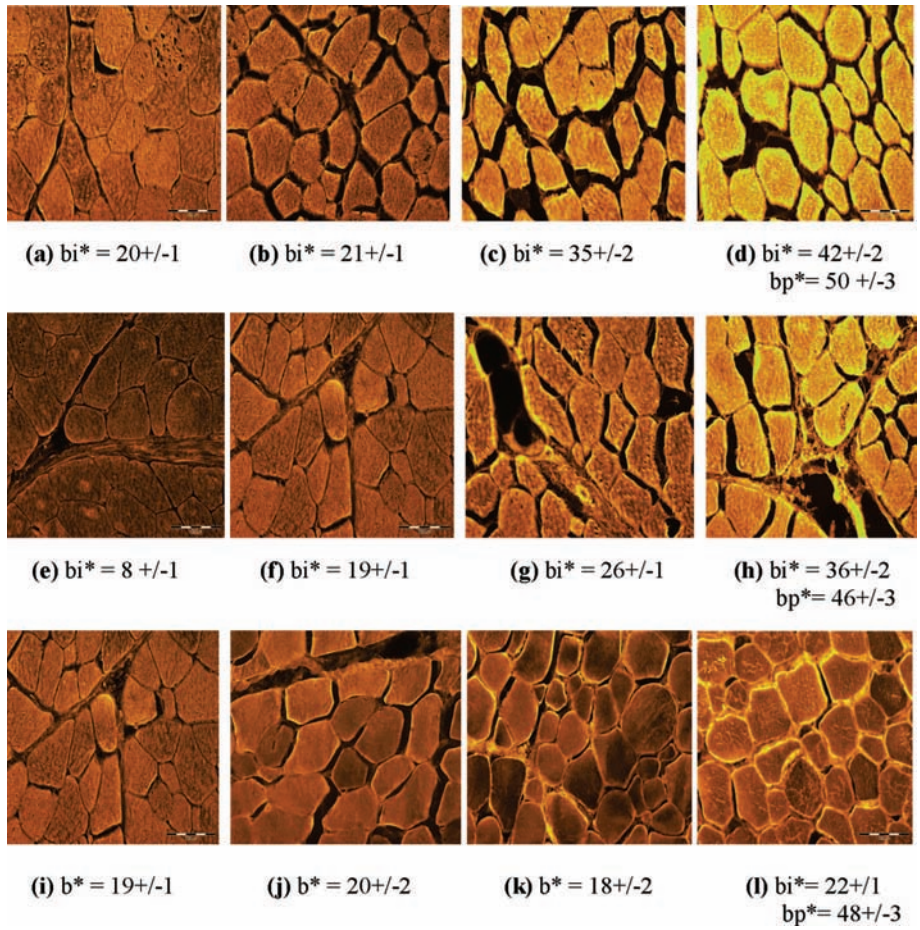

Figure 1. Microscopic images of protein carbonyl distribution achieved with the CY3 fluorescent probe (λ_{ex} = 550 nm; λ_{em} = 565 nm; exposure time = 2.7 s; magnification = 200). bi* is the yellowness measured inside the cells, and bp* is the yellowness measured at the periphery of the cells. Values were the means +/− SD of six determinations on different cells. (**a**–**d**) Effect of oxidation by Fe^{2+}/H_2O_2: 0 mM (**a**), 0.1 mM (**b**), 0.5 mM (**c**), and 1 mM (**d**). (**e**–**h**) Effect of a refrigerated storage of 0 days (**e**), 1 day (**f**), 7 days (**g**), and 10 days (**h**). (**i**–**k**) Effect of a 100 °C cooking during 0 min (**i**), 15 min (**j**), and 30 min (**k**). (**l**) Effect of a 270 °C cooking during 1 min.

killed in the experimental slaugtherhouse of INRA Theix Research Centre. Muscles were immediately removed from carcass and placed on a fiber board tray, wrapped in air-permeable film, and stored in darkness at 4 °C for 10 days to mimic commercial conditions. The biochemical results presented here were the mean of four determinations on different animals while, for practical reasons, the microscopy photographs shown corresponded to only one animal, representative of the observations.

Chemical Induction of Protein Oxidation by a Free Radical Generating System. Hydroxyl radicals ($OH^{\bullet}$) were produced by decomposition of hydrogen peroxide in the presence of ferrous iron. After 24 h of refrigerated storage, when the ultimate pH was reached (pH_{24h} measured on five animals = 5.65 +/− 0.05), small samples of muscle were cut in parallelepipeds (0.5 cm × 0.5 cm × 2 cm) in parallel with muscle fibers and incubated for 48 h at 4 °C in 20 mM phosphate buffer at pH 6 + 0.1 M NaCl with a $FeSO_4$ and H_2O_2 mixture at various concentrations (from 0 to 1 mM). The H_2O_2 concentration was adjusted by absorbance at 240 nm (ϵ = 43.6 M^{-1} cm^{-1}). Samples were then washed twice with a large excess of the same phosphate buffer to remove oxidizing agent on the meat surface and immediately frozen in isopentane cooled by liquid nitrogen.

Meat Cooking. After 24 h of refrigerated storage, small samples of muscle were cut in parallelepipeds (0.5 cm × 0.5 cm × 2 cm). Two cooking processes were performed. Some samples were shut up in polypropylene test tubes and heated at 100 °C in a temperature-controlled dry bath (Block-heater, Stuart-Scientific) during 0, 15, and 30 min. Other samples were shut up in aluminum tubes and heated at 270 °C in an oil bath during 1 min. Samples were then cooled at room temperature during 15 min and frozen in isopentane cooled by liquid nitrogen.

Immunostaining of Muscle Samples. Protein carbonyls were detected by the method developed on neuronal HT22 cells by Jung et al. (*18*) with modifications suited to the muscle tissue. The main difference was in cell fixation, which was performed before immunochemical treatment in the Jung method while, in this study, tissues were fixed after the immunochemical treatment. Thin transverse sections (10 μm) of meat samples were sliced with a microtome and mounted on microscopic glass slides. Samples were exposed for 16 h to 0.02% DNPH in 20 mM phosphate buffer at pH 6 + 0.1 M NaCl. Because of its low solubility in water, DNPH was used at a lower level than in the Jung method (0.3% in acidified ethanol). DNPH generates adducts by reacting with carbonyl groups that are detectable using anti-DNP antibody. After removal of the DNPH solution, samples were washed gently five times with PBS with 0.1% Tween at pH 6.75. Afterward, samples were incubated 10 min in PBS containing 10% of fetal goat serum in order to block unspecific binding sites. After serum removal, the primary DNP-adduct specific antibody (purchased from Chemicon International) was applied for 1 h with a 1:100 dilution suspended in PBS. After removal of the primary antibody and five times washing with PBS with 0.1% Tween, the CY3-labeled secondary antibody (purchased from Jackson Immuno Research) was added in a dilution of 1:600 in PBS for 1 h at room temperature under dark conditions. Samples were washed again five times with PBS in order to remove unbound secondary antibody and immediately fixed with a crystal mount.

Microscopic Detection of Protein Carbonyls. Fluorescence microscopy was performed using an Olympus BX61 transmission fluorescence microscope with the excitation and emission wavelengths fixed respectively at 550 and 565 nm. In order to make comparisons between treatments, the observations were all performed at the same

Table 1. Effect of a Chemical Free Radical Generating System, Refrigerated Storage, and Cooking on the Carbonyl Content of Myofibrillar and Membrane Proteins. Values are expressed in nanomoles of DNPH fixed per milligram of protein[a]

	chemical oxidation (Fe^{2+}/H_2O_2)				storage time				cooking			
									100 °C			270 °C
	0 mM	0.1 mM	0.5 mM	1 mM	D0	D1	D7	D10	0 min	15 min	30 min	1 min
myofibrillar proteins	2.2 +/− 0.2	5.0 +/− 1.5	5.7 +/− 1.4	5.8 +/− 1.5	1.5 +/− 0.2	2.1 +/− 0.2	2.2 +/− 0.2	2.8 +/− 0.1	2.1 +/− 0.2	4.3 +/− 0.8	5.5 +/− 0.8	5.9 +/− 0.5
membrane proteins	4.6 +/− 0.9	5.8 +/− 0.9	5.8 +/− 0.8	5.2 +/− 0.9	1.6 +/− 0.6	4.6 +/− 0.8	4.3 +/− 0.6	4.3 +/− 0.4	4.6 +/− 0.8	11.6 +/− 2.3	25.6 +/− 2.6	77.3 +/− 15.0
S	*	NS	NS	NS	NS	*	*	*	*	*	**	**

[a] Values are means +/− SEM of four independent determinations. The unpaired Student t-test was used to determine the level of statistical significance (S) between myofibrillar and membrane protein oxidation ($p > 0.05$, NS; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***).

magnification (×200) and the same lighting conditions (exposure time = 2.7 s). Controls with only DNPH or with DNPH and the first antibody were performed to test the possibility of an autofluorescence of these products. All these controls were negative; no fluorescence was detected under these conditions (data not shown). Controls without primary antibody treatment but in thepresence of DNPH and secondary antibody were also performed to rule out the hypothesis of a nonspecific fixation of the secondary antibody. These controls were also negative (data not shown).

In order to estimate the level of carbonyl proteins, we developed a semiquantitative method based on color measurement of microscopy photographs. Because of its high sensitivity, this method was preferred to the method commonly used by conversion of images to grayscale. Images achieved via fluorescence microscopy were converted into color photography using Adobe Photoshop Album Starter (Edition 3.0) and printed with an optimal quality on a matt photographic paper. Color coordinates of images were then determined in the CIE L*a*b* (1976) system with an Uvikon 933 (Kontron) spectrophotometer, and yellowness (b*) was used in this study as a tracer of the yellow fluorescence. Color measurements were performed on photographic enlargements (15 cm × 15 cm) while paying attention to an exact positioning of the beam of light. b* was measured inside the cell (bi*), and when it was possible, b* was also determined at the periphery of the cell (bp*). To be representative of the whole tissue, each b* value is the mean +/− SD of six determinations on different cells.

Sirius Red Collagen Staining. Collagen was stained with Sirius red according to the method of Flint and Pickering (*19*) modified by Liu et al. (*20*).

Biochemical Determination of Protein Oxidation. Myofibrillar proteins were prepared according to the method of Ouali and Talmant (*21*) with some modifications as outlined by Martinaud et al. (*1*), and membrane proteins were prepared according to Anton et al. (*22*). The membrane fraction used in this study corresponds essentially to the cellular membrane. The purity of the different fractions was controlled. The purity of myofibrillar proteins was assessed by SDS-PAGE gel electrophoresis as previously described by Santé-Lhoutellier et al. (*17*), and no contamination by sarcoplasmic proteins was detected (result not shown). Cytochrome C oxidase activity was measured to detect contamination of membrane fractions (extracted from raw meat) by mitochondria. A very low level of contamination (<5%) was observed, which corresponded to results previously reported (*22*).

To determine the level of carbonyl group formation, proteins were treated with DNPH using the method of Oliver et al. (*23*) with slight modifications (*1*). The results were expressed as nanomoles of DNPH fixed per milligram of protein.

Statistical Analysis. Data of the biochemical study were analyzed under the SAS system. To assess the effect of the different treatments, data were analyzed by a one-way analysis of variance (ANOVA). The unpaired Student *t*-test was used, to determine the levels of statistical significance between groups: $p > 0.05$, NS; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***.

RESULTS AND DISCUSSION

Effect of a Free Radical Generating System on the Distribution of Oxidized Proteins in Meat. The $Fe^{2+} + H_2O_2$ chemical system has been largely used in model systems to generate hydroxyl radical ($OH^{\bullet}$), and the doses used in this study have been described to induce oxidation levels which mimic those found in meat during storage (*1*, *14*). **Figure 1a–d** shows that fluorescence, estimated by bi*, was dose dependent. Without chemical treatment (**Figure 1a**), fluorescence was already observed, which corresponded to a basal level of protein oxidation. The yellow fluorescence increased progressively with increasing level of oxidizing agent, and a 2-fold increase was observed between 0 and 1 mM of oxidizing agent. For the highest levels of oxidizing agent (0.5 and 1 mM), protein oxidation was not equally distributed in muscle cells. Visually, we can distinguish two different cell areas: a peripheral area, corresponding to the cell membrane and to a region in close

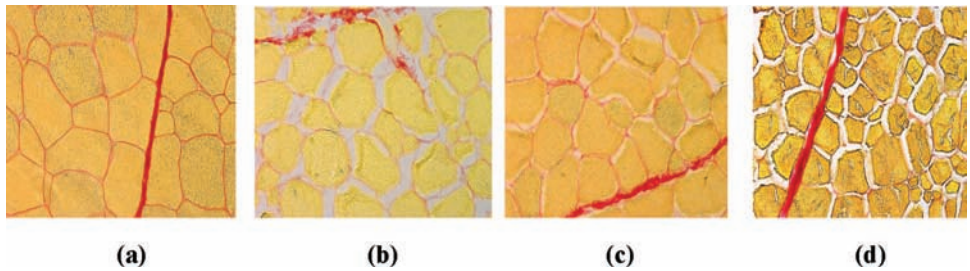

Figure 2. Microscopic images of muscle cells after Sirius red collagen staining. (**a**) Control performed 24 h postmortem. (**b**) Effect of oxidation by 1 mM Fe^{2+}/H_2O_2. (**c**) Effect of a refrigerated storage of 10 days. (**d**) Effect of cooking for 30 min at 100 °C.

contact with the cell membrane, and an inner area containing myofibrils and sarcoplasmic proteins, with a yellow fluorescence intensity more pronounced in the periphery than inside the cells (bp* = 1.19bi*). To explain this heterogeneity in the distribution of oxidized proteins, we can hypothesize that oxidative species initially induced oxidation in the cell membrane where proteins are in close contact with phospholipids, especially polyunsaturated fatty acids, which are known to initiate and propagate free radical reactions. Diffusion of oxidative species from the membrane to the interior could subsequently oxidize sarcoplasmic and myofibrillar proteins. Another hypothesis would be that, in cell membranes, aldehydes formed during lipid peroxidation, such as malondialdehyde and hydroxynonenal, react with amino side chains of proteins to give Schiff bases, introducing in that way carbonyl groups to proteins (*12*). We have recently described the formation of such products in chicken meat during a refrigerated storage (*25*).

The measurement of protein oxidation by the biochemical test (**Table 1**) showed slightly different results. In the control sample, a higher level of carbonyl groups was detected in membrane proteins than in myofibrillar proteins while no difference was observed between the two kinds of protein after exposure to a $Fe^{2+} + H_2O_2$ mixture. Moreover, **Table 1** shows that the maximum level of oxidation was reached from 0.1 mM oxidizing agent while fluorescence increased regularly from 0 to 1 mM. To explain these conflicting results, we must take into account that myofibrils and membranes were prepared from muscles impregnated with oxidizing agent which could generate protein oxidation during the successive steps of extraction. This artifact could erase differences due to protein location or level of oxidizing agent.

Between cells we found less protein oxidation. The space between cells is filled with connective tissue with essentially collagen fibers. A distinctive feature of collagen is the regular arrangement of amino acids in each of the three chains of the collagen subunits. The sequence often follows the pattern Gly-X-Pro or Gly-X-Hyp, where X may be any of various other amino acid residues. The three predominant amino acids of collagen cannot form carbonyl groups. Hydroxylysine, derived from lysine, is sometime present, but disaccharides are often attached to hydroxylysine, which can prevent the conversion of amino groups to carbonyls. Finally, we can also notice an important increase in the extracellular space when tissues were exposed to oxidizing agent. This phenomenon can be due to the disintegration, under the action of free radical generating system, of perimysium and endomysium collagen fibers observed by Sirius red collagen staining (**Figure 2b**). Comparison of **Figures 1d** and **2b** clearly demonstrated that the increase of fluorescence in the peripheral area of cells, induced by free radicals, was due to membrane protein oxidation and not to collagen oxidation.

Effect of Refrigerated Storage on the Distribution of Oxidized Proteins in Meat. Various radical species (hydroxyl, superoxide, peroxyl, tyrosyl, and nitric oxide radicals) can be generated during meat storage, depending on the pro- and antioxidant status of the muscle. An important increase of protein carbonyls was observed during refrigerated storage with a 4 times increase of bi* between day 0 and day 10 (**Figure 1e–h**). The refrigerated storage produced fluorescence images similar to those obtained after chemical oxidation, and an important heterogeneity of fluorescence was also observed, especially after 10 days of storage, between the periphery and the inside of the cells (bp* = 1.28 bi*). Biochemical determination of carbonyls (**Table 1**) confirms this heterogeneity of distribution of oxidized proteins induced by refrigerated storage with carbonyl levels in membrane proteins which were twice that observed in myofibrillar proteins. Fluorescence intensities and biochemical measurements show that the oxidation levels reached during storage are lower than those obtained by chemical oxidation.

An increase of the extracellular space was also noticed during the refrigerated storage, and **Figure 2c** shows an important disintegration of collagen fibers comparable to that induced by the chemical oxidative treatment.

Effect of Cooking on the Distribution of Oxidized Proteins in Meat. With cooking (**Figure 1i–l**) the difference between the peripheral and the internal areas of cells was considerably more pronounced than was the case with the two precedent oxidative conditions. After 30 min of cooking at 100 °C or after 1 min of cooking at 270 °C, the periphery of the cells showed an intense yellow fluorescence while the interior produced the same fluorescence as that of the uncooked sample. Since diffusion of oxidative species from the membrane to the muscle cell inside is a slow phenomenon, we could hypothesize that this process does not have sufficient time to take place during heating. But microscopic observation was in contradiction with biochemical measurements of carbonyl groups (**Table 1**), showing an important increase of both myofibrillar and membrane protein oxidation with cooking time. These conflicting results could be explained by myofibrillar protein denaturation and aggregation, induced by heating, restricting the diffusion of DNPH and antibodies in the interior of the muscle cell during immunostaining. However, biochemical analysis confirmed the heterogeneity of the oxidized protein distribution, with the level of carbonylation considerably higher in membrane proteins than in myofibrillar proteins, especially for 30 min of cooking at 100 °C and 1 min of cooking at 270 °C.

The increase of extracellular space observed after cooking was less pronounced than those observed after chemical treatment or after storage. Nevertheless, an important disintegration of collagen fibers was also observed (**Figure 2d**), confirming that oxidation observed around cells was linked to membrane proteins and not to collagen.

Statistical Analysis. The effect of the different treatments, measured by the biochemical test of carbonyls, was evaluated by analysis of variance. Cooking time had a significant effect

on myofibrillar protein oxidation ($p < 0.01$) and membrane protein oxidation ($p < 0.01$). In myofibrillar proteins, a significant increase ($p < 0.01$) of protein oxidation was already measured between 0 and 15 min of cooking. The concentration of oxidizing agent had a significant effect on myofibrillar protein oxidation ($p < 0.05$) with a significant increase ($p < 0.05$) between 0 and 0.1 mM. The effect of the refrigerated storage time was never significant ($p > 0.05$). This analysis supports the conclusion that cooking is an important free radical generating process and, in an attempt to minimize nutritional loss in meat products, it would be of great importance to control protein oxidation during this process.

Perspectives in the Study of Meat Protein Oxidation by Microscopy. Our results show that immunocytochemistry is a powerful tool to detect and locate protein oxidation in meat. This method supplements other recent approaches, which are able to identify specific protein oxidation in muscle/meat tissue by a proteomic technique (*26*) or by a combined immunologic and proteomic technique (*27*). This approach provides more information on the mechanism of protein oxidation during meat processes than the global estimation of oxidized proteins commonly performed by biochemical assay. Knowledge of the intracellular distribution of protein carbonyls could be useful in the choice of an antioxidant strategy. Using this technique, we have clearly demonstrated the heterogeneity of protein oxidation in muscle cells with an initiation localization site in a membrane cell. So, in order to limit protein oxidation during meat processes, lipophilic antioxidants, such as vitamin E, would be of great interest. Microscopic detection and localization of other amino acid oxidations such as thiol oxidation and aromatic hydroxylation are theoretically feasible; chemical probes and specific spectroscopic detection of these oxidations are also still being used (*1*, *14*, *17*). Their determination could allow us to understand the oxidative process of meat proteins in full. Determination of protein physicochemical changes induced by oxidation as hydrophobicity or aggregation at the ultrastructural level would also be an obvious area to continue to research to obtain further understanding of oxidative modifications during meat storage or processing.

LITERATURE CITED

(1) Martinaud, A.; Mercier, Y.; Marinova, P.; Tassy, C.; Gatellier, P.; Renerre, M. Comparison of oxidative processes on myofibrillar proteins from beef during maturation and by different model oxidation systems. *J. Agric. Food Chem.* **1997**, *45*, 2481–2487.

(2) Mercier, Y.; Gatellier, P.; Viau, M.; Remignon, H.; Renerre, M. Effect of dietary fat and vitamin E on lipid and protein oxidation in turkey meat during storage. *Meat Sci.* **1998**, *48*, 301–317.

(3) Renerre, M.; Poncet, K.; Mercier, Y.; Gatellier, P.; Metro, B. Influence of dietary fat and vitamin E on antioxidant status of muscles of turkey. *J. Agric. Food Chem.* **1999**, *47*, 237–244.

(4) Rowe, L. J.; Maddock, K. R.; Lonergan, S. M.; Huff-Lonergan, E. Influence of early post mortem protein oxidation on beef quality. *J. Anim. Sci.* 2004a*82*, 785–793.

(5) Rowe, L. J.; Maddock, K. R.; Lonergan, S. M.; Huff-Lonergan, E. Oxidative environments decrease tenderization of beef steaks through inactivation of μ-calpain. *J. Anim. Sci.* 2004b*82*, 3254–3266.

(6) Davies, K. J. A. Protein damage and degradation by oxygen radicals. I: General aspects. *J. Biol. Chem.* **1987**, *262*, 9895–9901.

(7) Davies, K. J. A.; Delsignore, M. E.; Lin, S. W. Protein damage and degradation by oxygen radicals. II. Modification of amino acids. *J. Biol. Chem.* **1987**, *262*, 9902–9907.

(8) Stadtman, E. R. Metal ion-catalysed oxidation of proteins: biochemical mechanism and biological consequences. *Free Radical Biol. Med.* **1990**, *8*, 315–325.

(9) Stadtman, E. R. Oxidation of free amino acids and amino acids residues in proteins by radiolysis and by metal-catalyzed reactions. *Annu. Rev. Biochem.* **1993**, *62*, 797–821.

(10) Srinivasan, S.; Xiong, Y. L. Gelation of beef heart surimi as affected by antioxidants. *J. Food Sci.* **1996**, *61*, 707–711.

(11) Srinivasan, S.; Hultin, H. O. Chemical, physical, and functional properties of cod proteins modified by a non enzymatic free-radical-generating system. *J. Agric. Food Chem.* **1997**, *45*, 310–320.

(12) Friguet, B.; Stadtman, E. R.; Szewda, L. I. Modification of glucose-6-phosphate dehydrogenase by 4-hydroxy-2-nonenal. Formation of cross-linked protein that inhibits the multicatalytic protease. *J. Biol. Chem.* **1994**, *269*, 21639–21643.

(13) Grune, T.; Jung, T.; Merker, K.; Davies, K. J. A. Decreased proteolysis caused by protein aggregates, inclusion bodies, plaques, lipofuscin, ceroid, and aggresomes during oxidative stress, aging, and disease. *Int. J. Biochem. Cell Biol.* **2004**, *36*, 2519–2530.

(14) Morzel, M.; Gatellier, Ph.; Sayd, T.; Renerre, M.; Laville, E. Chemical oxidation decreases proteolytic susceptibility of skeletal muscle proteins. *Meat Sci.* **2006**, *73*, 536–543.

(15) Kamin-Belsky, N.; Brillon, A. A.; Arav, R.; Shaklai, N. Degradation of myosin by enzymes of the digestive system: Comparison between native and oxidatively cross-linked protein. *J. Agric. Food Chem.* **1996**, *44*, 1641–1646.

(16) Liu, G.; Xiong, Y. L. Electrophoretic pattern, thermal denaturation, and in vitro digestibility of oxidized myosin. *J. Agric. Food Chem.* **2000**, *48*, 624–630.

(17) Santé-Lhoutellier, V.; Aubry, L.; Gatellier, Ph. Effect of Oxidation on In Vitro Digestibility of Skeletal Muscle Myofibrillar Proteins. *J. Agric. Food Chem.* **2007**, *55*, 5343–5348.

(18) Jung, T.; Engels, M.; Klotz, L. O.; Kroncke, K. D.; Grune, T. Nitrotyrosine and protein carbonyls are equally distributed in HT22 cells after nitrosative stress. *Free Radical Biol. Med.* **2007**, *42*, 773–786.

(19) Flint, F. O.; Pickering, K. Demonstration of collagen in meat products by an improved picro-Sirius red polarisation method. *Analyst* **1984**, *109*, 1505–1511.

(20) Liu, A.; Nishimura, T.; Takahashi, K. Structural changes in endomysium and perimysium during post-mortem aging of chicken semitendinosus muscle. Contribution of structural weakening of intramuscular connective tissue to meat tenderization. *Meat Sci.* **1994**, *38*, 315–328.

(21) Ouali, A.; Talmant, A. Calpain and Calpastatin distribution in bovine, porcine and ovine skeletal muscles. *Meat Sci.* **1990**, *28*, 331–348.

(22) Anton, M.; Gatellier, Ph.; Renerre, M. Microsomal lipid peroxidation in relation with oxidation of bovine myoglobin. Proc. Int. Congr. Meat Sci. Technol. (Kulmbach, Germany) 1991, *3*, 320–323.

(23) Oliver, C. N.; Alin, B. W.; Moerman, E. J.; Goldstein, S.; Stadtman, E. R. Age-related changes in oxidized proteins. *J. Biol. Chem.* **1987**, *262*, 5488–5491.

(24) Friguet, B.; Stadtman, E. R.; Szewda, L. I. Modification of glucose-6-phosphate dehydrogenase by 4-hydroxy-2-nonenal. Formation of cross-linked protein that inhibits the multicatalytic protease. *J. Biol. Chem.* **1994**, *269*, 21639–21643.

(25) Gatellier, Ph.; Gomez, S.; Gigaud, V.; Berri, C.; Le Bihan-Duval, E.; Santé-Lhoutellier, V. Use of a fluorescent front face technique for measurement of lipid oxidation during refrigerated storage of chicken meat. *Meat Sci.* **2007**, *76*, 543–547.

(26) Yan, J. X.; Harry, R. A.; Wait, R.; Welson, S. Y.; Emery, P. W.; Preedy, V. R.; Dunn, M. J. Separation and identification of rat skeletal muscle proteins using two-dimensional gel electrophoresis and mass spectrometry. *Proteomics* **2001**, *1*, 424–434.

(27) Stagsted, J.; Bendixen, E.; Andersen, H. J. Identification of specific oxidatively modified proteins in chicken muscles using a combined immunologic and proteomic approach. *J. Agric. Food Chem.* **2004**, *52*, 3967–3974.

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