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Effect of Oxidation on In Vitro Digestibility of Skeletal Muscle Myofibrillar Proteins

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The objective of this study was to investigate the effect of chemical oxidation on myofibrillar protein digestibility. Myofibrils were prepared from pig M. longissimus dorsi and oxidized by a hydroxyl radical generating system. Oxidative modifications of proteins were assessed by the carbonyl content, surface hydrophobicity, electrophoresis, and immunoblotting. Oxidized or nonoxidized myofibrillar proteins were then exposed to proteases of the digestive tract (pepsin, trypsin, and α-chymotrypsin). Results showed a direct and quantitative relationship between protein damages by hydroxyl radical and loss of protein digestibility.

KEYWORDS: Myofibrils; carbonyl; hydrophobicity; electrophoresis; immunoblotting; proteolysis; digestibility

INTRODUCTION

Meat proteins serve as an important source of energy and essential amino acids for humans. If in the general population the basic needs in amino acids are properly provided by diet, the elderly and children often present some nutritional deficiency, especially in essential amino acids. So, digestibility of meat and meat products remains an important problem for these populations. To enter the bloodstream, proteins must be broken down into amino acids or small peptides by proteases within the digestive tract and pass through the small intestine wall. This digestive process depends on the physicochemical state of proteins. The efficiency of this process is essential to ensure the nutritional value of meat and also from the point of view of human health because accumulation of nonhydrolyzed or only partially hydrolyzed proteins in large intestine could be a cause of colon cancer. Proteins that escape assimilation in the small intestine are extensively fermented, leading to formation of altered products by colonic flora (*1*, *2*). Phenol and *p*-cresol, which are potentially mutagenic products, are specific bacterial metabolites of tyrosine.

During meat storage or processing (mincing, cooking, salting, and irradiation), free oxygenated radical production, accompanied with a decrease of antioxidant defense systems, leads to the accumulation of oxidative damage in proteins (*3*−*6*). Different groups of aminoacid are sensitive to oxidation (*7*−*10*). Basic amino acids are oxidized in carbonyls. Thiol groups of cystein can be oxidized with the formation of disulfide bridges. Tyrosine can give dityrosine bridges. These bridges can originate the polymerization and aggregation of proteins. Interaction of free NH_2 groups of lysine with aldehydic products of lipid oxidation can also induce protein aggregation by the formation of Schiff bases (*11*−*13*). Moreover, oxidation can also alter the secondary and tertiary structure of proteins, leading to changes in physical properties of proteins as solubility and hydrophobicity.

The interaction between proteolysis and oxidation of proteins has been extensively studied in medical sciences with contrasting effects; enhancement or impairment of proteolysis have been reported depending on the type of chemical modification. In meat, the link between protein oxidation and digestibility is poorly documented. However, it has been shown that myosin oxidation can affect its proteolytic degradation by enzymes of the digestive tract (*14*, *15*).

The present study was designed to explore the effect of oxidative conditions on in vitro digestibility of myofibrillar proteins from pork muscle. It is undoubtedly true that model systems using myofibrils are more representative of meat proteins than those with purified myosin. The myofibrillar structure is a more complex system than myosin with many protein−protein interactions and even protein−lipid interactions, which can affect protein susceptibility to proteolysis. Myofibrils were exposed to different concentrations of hydroxyl radical ($OH^{\bullet}$) generating systems and subsequently hydrolyzed by proteases of the digestive tract in conditions of pH and temperature, which mimic digestive conditions. Proteolytic susceptibility of myofibrillar proteins is presented and discussed in relationship to their oxidative modifications such as carbonylation, hydrophobicity, and polymerization/aggregation.

MATERIALS AND METHODS

Animals and Samples. Four Large White crossed Landrace pigs were slaughtered at 6 months of age. An 800 g amount 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.

Isolation of Myofibrils. Myofibrils were prepared according to the method of Ouali and Talmant (*16*) with some modifications as outlined by Martinaud et al. (*17*). Ten grams of frozen muscle was homogenized

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with a Waring blender in 100 mL of a solution at pH 6.5 containing 150 mM NaCl, 25 mM KCl, 3 mM $MgCl_2$, and 4 mM EDTA, to which two protease inhibitors (1 mM PMSF and 1 μM E64) 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 (*18*).

Chemical Induction of Myofibril 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 at various concentrations (from 0 to 5 mM for each constituent). The H_2O_2 concentration was adjusted by absorbance at 240 nm ($\epsilon = 43.6\ M^{-1}\ cm^{-1}$). Phosphate was used in this study because, contrarily to other buffers commonly used for protein studies, it has no marked effects on protein oxidation (*8*). After 3 h, no residual H_2O_2 could be detected by absorbance at 240 nm, but to prevent subsequent oxidation, butylated hydroxytoluene (1 mM final concentration) was added to the incubation medium. For carbonyl determination, oxidized samples were used without previous treatments. For electrophoresis, hydrophobicity, and digestibility measurements, the oxidizing agent was removed by centrifugation (4000*g* for 10 min), and the pellets were washed extensively with corresponding buffers.

Determination of Carbonyl Content. Carbonyl groups were estimated using the method of Oliver et al. (*19*) 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 for 10 min at 4000*g*. The protein concentration in the supernatant was calculated at 280 nm in the HCl control using BSA in 6 M guanidine as standard. The carbonyl concentration was measured on the treated sample by measuring the 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.

Determination of Protein Surface Hydrophobicity. After oxidation, myofibrillar proteins were washed twice in 20 mM phosphate buffer at pH 6, and the final concentration was adjusted at 10 mg/mL in this same buffer. The hydrophobicity of myofibrillar myofibrils was determined using the hydrophobic chromophore bromophenol blue (BPB) according to Chelh et al. (*20*). This method has been developed in our laboratory especially for the determination of surface hydrophobicity of nonsolubilized proteins. To 1 mL of myofibril suspension, 400 μL of 1 mg/mL BPB (in distilled water) was added and mixed well. A control, without myofibrils, was done by addition of 400 μL of 1 mg/mL BPB (in distilled water) to 1 mL of 20 mM phosphate buffer at pH 6. Samples and control were kept under agitation, at room temperature, for 10 min and then centrifuged for 15 min at 2000*g*. The absorbance of supernatant was measured at 595 nm against a blank of phosphate buffer. The amount of BPB bound, given by the following formula, is used as an index of hydrophobicity:

$$\text{BPB bound } (\mu g) = 400\ \mu g \times (\text{OD control} - \text{OD sample})/\text{OD control}$$

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) Gel Electrophoresis. Myofibrils were incubated for 5 min at 100 °C in a buffer containing 7.5% (v:v) glycerol, 1% (v/v) mercaptoethanol, 6% (w/v) SDS, and 50 mM Tris-HCl (pH 6.8). SDS-PAGE was performed according to the method of Laemmli (*21*) using 12.5% (6 cm × 8 cm, 0.75 mm thick) polyacrylamide gels. The protein load was adjusted to 10 μg per lane. Two gels were run in parallel: One was Coomassie blue stained, and the other was used for immunobloting. Gels were scanned with a GS-800 calibrated densitometer (UMAX) controlled by a PDQuest application program, and the protein band intensity was evaluated with Sigma gel (Sigma).

Immunoblotting. Specific protein oxidation was evaluated by labeling protein carbonyls with DNPH followed by immunobloting (Oxyblot kit from Chemicon International) of proteins separated by 12.5% SDS-PAGE.

In Vitro Digestibility. After oxidation, myofibrillar proteins were washed twice in 33 mM glycine buffer at pH 1.8 (gastric pH). Myofibrillar proteins are poorly soluble in glycine buffer even at pH 1.8. This was confirmed by the estimation of protein concentrations before and after washings. Loss of myofibrillar proteins during washings did not exceed 2−3%. The final concentration was adjusted at 0.8 mg/mL in this same buffer. Proteins were digested first by gastric pepsin (20 U/mg myofibrillar proteins) for 1 h at 37 °C. Pepsin, from porcine gastric mucosa, was purchased from Sigma. Digestion was terminated by addition at various times (0, 10, 20, 30, 40, and 60 min) of 15% (final concentration) trichloroacetic acid (TCA). After centrifugation for 10 min at 4000*g*, the content of hydrolyzed peptides in the soluble fraction was estimated by absorbance at 280 nm, and the rate of proteolysis was expressed in optical density units by hour (ΔOD/h).

Methods using fluorescamine or o-phthaldialdehyde acid generally used to measure peptides were not considered since these methods rely on the determination of NH_2 group content and we used a glycine buffer. Moreover these methods can be criticized because amino groups can be destroyed by oxidation. Methods based on peptidic bond determination cannot be used at all because of loss of these bonds during proteolysis. Our method relies on tryptophan absorption. Therefore, like every aromatic amino acid, it is prone to oxidize, its oxidized form, hydroxytryptophan, has similar UV spectrum and then will not interfere with the optical density at 280 nm.

The non soluble fractions of the 60 min pepsin hydrolyzate was washed twice in 33 mM glycine buffer at pH 8 (duodenal pH) and final concentration was adjusted at 0.8 mg/mL in this same buffer. Proteins were then hydrolyzed 30 min at 37° by a mixture of trypsin and α-chymotrypsin (6.6 U and 0.33 U/mg myofibrillar proteins). Trypsin and α-chymotrypsin from porcine pancreas were purchased from Sigma. Digestion was terminated by addition at various times (0, 5, 10, 20, 30 min) of 15% (final concentration) TCA and the rate of proteolysis was determined as previously described.

Statistical Analysis. Statistical analysis was performed on a SAS system. All values are reported as the mean ± standard error of the mean of four independent determinations. The unpaired Student *t* test was used to determine the levels of statistical significance between groups, with $p > 0.05$, NS; $p < 0.05$, *; $p < 0.01$, **; and $p < 0.001$, ***. The relationships between the different parameters were assessed by calculation of Pearson correlation coefficients.

RESULTS AND DISCUSSION

Carbonyl Content of Oxidized Myofibrillar Proteins. Amino acids with NH or NH_2 groups on their side chains are very reactive to $OH^{\bullet}$, and these groups are transformed in carbonyls groups during protein oxidation. In our experiment, the carbonyl level of nonoxidized myofibrils (control) was 1.4 nmol/mg protein (**Figure 1**), a value close to those reported by Liu and Xiong (*22*) on chicken myosin and Martinaud et al. (*17*) and Morzel et al. (*23*), respectively, on beef and pork myofibrils. The addition of the oxidizing 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] = 5.2 [oxidizing agent] + 2.0 ($R^2 = 0.90$) and reached a maximum of 16.3 nmol/mg protein. The addition of 5 mM oxidizing agent did not generate a higher carbonyl level. From 0.1 mM oxidizing agent, carbonyl levels were significantly higher than the nonoxidized control. The effect of oxidation on carbonyl group content

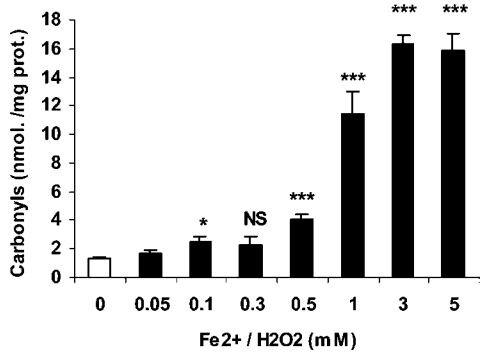

Figure 1. Effect of different concentrations of free radical generating systems on the carbonyl content of myofibrillar proteins. Levels of oxidation were statistically evaluated by comparison with that of nonoxidized myofibrils (white bar). Significance is noticed as follows: $p > 0.05$, NS; $p < 0.05$, *; $p < 0.01$, **; and $p < 0.001$, ***.

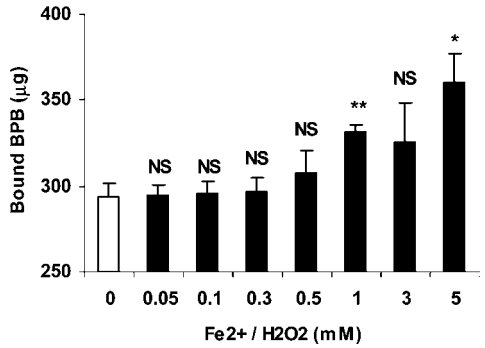

Figure 2. Effect of different concentrations of free radical generating systems on myofibrillar protein surface hydrophobicity. Hydrophobicity values were statistically evaluated by comparison with that of nonoxidized myofibrils (white bar). Significance is noticed as follows: $p > 0.05$, NS; $p < 0.05$, *; $p < 0.01$, **; and $p < 0.001$, ***.

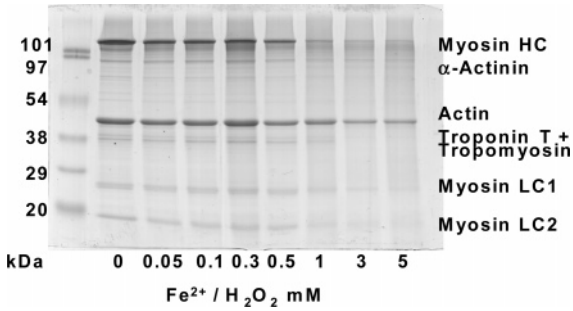

Figure 3. Gel electrophoresis patterns of myofibrillar proteins oxidized by different concentrations of free radical generating systems (HC, heavy chain; LC, light chain).

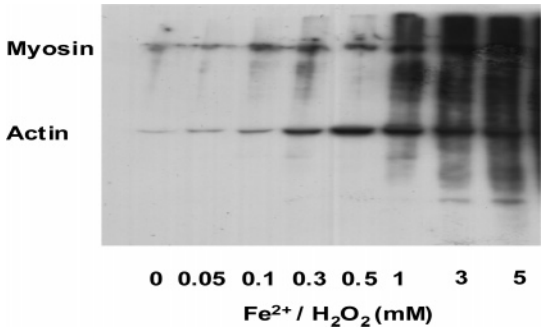

Figure 4. Immunobloting of myofibrillar proteins oxidized by different concentrations of free radical generating systems.

measured in the present study was fairly consistent with our previous experiments on beef (*17*) and pork (*23*) myofibrils.

In this study, myofibrils were extracted just after slaughter to prevent endogenous proteolysis, but in practice, meat is stored several days before eating. Protein oxidation increases during meat aging, and with the view of comparison, the carbonyl content was measured on myofibrillar proteins extracted after 10 days of meat refrigerated storage. A value of 4.3 nmol/mg protein was observed at that time, which corresponded to a carbonyl content obtained in vitro in the presence of 0.5 mM oxidizing agent.

Surface Hydrophobicity of Oxidized Myofibrillar Proteins. In view of its capacity to monitor subtle changes in chemical and physical states of protein, hydrophobicity can be a suitable parameter to estimate protein denaturation. Protein hydrophobicity has been the object of numerous studies in relation to oxidation, and it is generally accepted that oxidation enhances protein surface hydrophobicity by exposition of hydrophobic amino acids, which are buried in protein inside in native conditions (*13*, *24*). **Figure 2** shows the effect of increased oxidizing agent on protein surface hydrophobicity of myofibrillar proteins. Until 0.3 mM oxidizing agent, we did not observe any change in hydrophobicity. From 0.5 mM, hydrophobicity continually increased, and the value obtained at 5 mM was 22% higher than that of the control.

For comparison with the aging process, protein surface hydrophobicity was also measured on myofibrillar proteins extracted after 10 days of meat refrigerated storage. A 35% increase was observed between fresh and 10 day myofibrillar proteins. With a heating treatment, we have previously observed a considerably higher hydrophobicity increase (*20*). For example, a 300% increase in hydrophobicity was measured after heating myofibrillar proteins for 60 min at 70 °C.

Electrophoretic Studies of Oxidized Myofibrillar Proteins. Electrophoresis was performed to observe modifications induced by chemical oxidation of myofibrillar proteins. SDS-PAGE patterns (**Figure 3**) showed the decrease of bands corresponding to myosin heavy and light chains after exposure to oxidizing agent. This decrease was especially pronounced between 0.5 and 1 mM oxidizing agent, which coincides well with the sharp increase in carbonyl formation (**Figure 1**). No fragmentation of myofibrils has been detected in our experiment even in the higher oxidative conditions, so this disappearance of myosin bands can be due to high molecular weight compounds, probably aggregates, which cannot penetrate the gel network and accumulate at the top of the gel. Aggregation cannot be due to disulfide bridge formation, as electrophoresis was performed in reducing conditions, but many other aminoacid oxidative modifications can lead to protein aggregation. Carbonyls may react with free amino groups of nonoxidized amino acids to form amide bonds (*22*). Bityrosine formation between protein molecules can also lead to polymerization and aggregation (*7*), and we have previously detected such products in oxidized myofibrils (*23*). Schiff bases are also formed in oxidized myofibrils (*25*), which can generate aggregates.

The actin band was relatively more stable and was affected only at higher oxidant concentrations, oppositely to Dalle-Donne et al. (*26*), who demonstrated that purified actin is particularly prone to chemical oxidation by hypochlorous acid.

Immunoblotting of Oxidized Myofibrillar Proteins. Immunoblotting using antibody against DNPH-carbonylated pro-

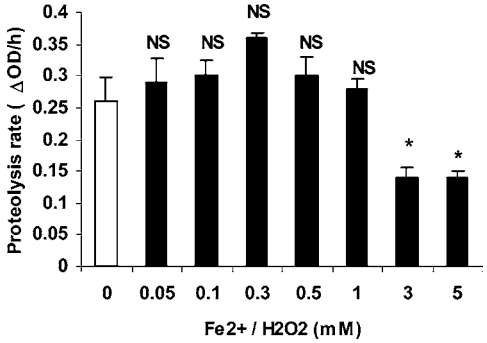

Figure 5. Effect of prior oxidative treatment by different concentrations of free radical generating systems on the proteolysis rate of myofibrillar proteins by gastric pepsin. Rates were statistically evaluated by comparison with that of nonoxidized myofibrils (white bar). Significance is noticed as follows: $p > 0.05$, NS; $p < 0.05$, *; $p < 0.01$, **; and $p < 0.001$, ***.

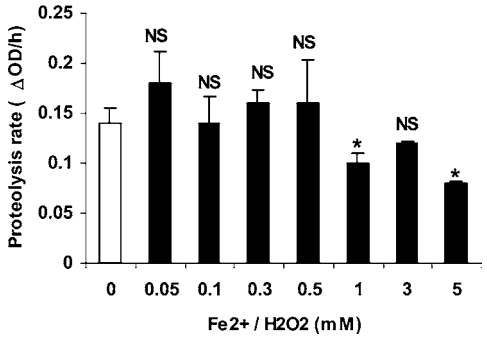

Figure 6. Effect of prior oxidative treatment by different concentrations of free radical generating systems on proteolysis rate of myofibrillar proteins by pancreatic trypsin + α-chymotrypsin. Rates were statistically evaluated by comparison with that of nonoxidized myofibrils (white bar). Significance is noticed as follows: $p > 0.05$, NS; $p < 0.05$, *; $p < 0.01$, **; and $p < 0.001$, ***.

teins is a very sensitive and specific method (*27*). **Figure 4** confirms the formation of carbonyl groups on myofibrillar proteins. Carbonylated myosin heavy chains and actin were already detectable on the nonoxidized myofibrils, and then, their level of carbonylation regularly increased with increased oxidizing agent. Until 0.5 mM oxidizing agent, only myosin heavy chains and actin oxidation were detectable. The intensity of coloration was more pronounced in actin than in myosin. This difference can be explained by the loss of myosin, observed by Coomassie blue staining, due to aggregation. From 1 mM oxidizing agent, the formation of smears on gel above myosin-HC band indicated protein aggregation. Other myofibrillar proteins (α-actinin, troponin, tropomyosin, and myosin-LC) were less sensitive to oxidation. Their carbonylation was observable only from 1 mM, and their bands were poorly resolved.

In Vitro Digestibility of Oxidized Myofibrillar Proteins. **Figures 5** and **6** show the effect of a prior oxidative treatment on myofibrillar protein degradation by the digestive tract proteases. With pepsin, a protease of stomach, a biphasic curve was observed. Low levels (from 0 to 3 mM) of oxidizing agent increased slightly and not significantly proteolysis rate while above 3 mM an important decreased rate was observed for which aggregation occurred on gel electrophoresis. The maximum decrease of proteolysis rate measured between control and the highest oxidation was 46%. With pancreatic proteases (trypsin + α-chymotrypsin), a decrease in proteolysis rate was observed especially from 1 mM Fe^{2+}/H_2O_2. The maximum decrease of proteolysis rate measured between control and the highest oxidation was 43%. With the two digestive systems, the decrease observed was significant only for the high levels of oxidizing agent (>1 mM).

These results are in good accordance with those of Davies (*28*) and Grune et al. (*13*), showing a biphasic response when proteolysis was measured on oxidized proteins. For these authors, proteolytic susceptibility initially increases before declining at a higher oxidant concentration, due to the formation of protein aggregates changing both chemical and physical recognition sites. Kristensen et al. (*29*) 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 (*15*) showed that, under nonreducing conditions, oxidation decreases the susceptibility of myosin to pepsin, trypsin, and chymotrypsin. In a recent study, we have demonstrated that oxidation of myofibrillar proteins leads to a rapid decline of their susceptibility to proteolysis by papain (*23*).

Even if levels of myofibril oxidation obtained in this model are probably higher than those observed in raw meat, this negative effect of oxidation on protein digestibility should apply to some extent to meat in conditions generating high levels of free radicals (cooking, irradiation, freezing/unfreezing cycles, high oxygen packaging, or feeding animals with high unsaturated oils). This would be an obvious area to continue within to get a further understanding on oxidative modifications of meat proteins in relation to meat processes. Development of new processes or improvement of existing processes with the aim to generate lower level of free radicals should be of great interest to preserve the nutritional quality of meat. In cooking, which is the most usual meat process, we are going to study the influence of time/temperature parameters on protein oxidation with the aim to give recommendations to consumers. Improved antioxidant protection of meat product, provided, for example, by animal dietary supplementation with vitamin E or vegetable antioxidants, could also have a beneficial effect on meat protein digestibility and will soon be studied.

Correlations between Oxidation and Digestibility Measurements. A correlation study was performed to establish links between all parameters measured in this study. **Table 1** is the

Table 1. Correlation Matrix between Parameters of Oxidative Modification (Carbonyl Groups, Hydrophobicity, and Electrophoretic Band Intensities of Myosin HC and Actin) and Protease Activities[a]

	carbonyls	hydrophobicity	myosin HC band	actin band	pepsin activity	trypsin + chymotrypsin activity
carbonyls	1					
hydrophobicity	0.91**	1				
myosin HC band	–0.93**	–0.88**	1			
actin band	–0.91**	–0.86**	0.87**	1		
pepsin activity	–0.84**	–0.75*	0.85**	0.88**	1	
trypsin + chymotrypsin activity	–0.85**	–0.91**	0.86**	0.71*	0.71*	1

[a] Significance is noticed as: $p > 0.05$, NS; $p < 0.05$, *; $p < 0.01$, **; and $p < 0.001$, ***.

correlation matrix between parameters of oxidative modification (carbonyl, hydrophobicity, and electrophoretic band intensities) and proteolysis rates. All parameters of oxidative modification were highly correlated among themselves. Myosin HC and actin electrophoretic band intensities were negatively correlated with carbonyl levels showing the possible implication of carbonyl groups in polymerization by formation of amide bonds with residual amino groups. Proteolysis rates were highly and negatively correlated with carbonyl group level. These negative correlations can also be explained by formation of amide bonds. Moreover, trypsin, a serine endopeptidase that hydrolyzed specifically proteins at the carboxy side of the basic aminoacids (Arg and Lys), can be disrupted by the conversion of NH/NH_2 groups into carbonyls. The proteolysis rate was also negatively correlated with surface hydrophobicity. This result is rather surprising, as many studies have shown an increase of proteolysis with hydrophobicty at least during the early stage of oxidation (*28*, *30*−*32*). 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. We can hypothesize that, in myofibrillar proteins, an increase of hydrophobicity favors protein aggregation with a negative effect on proteolysis susceptibility. Moreover, pepsin, an aspartic endopeptidase, cleaves bonds preferentially at the carboxy side of Phe and Trp, and α-chymotrypsin, a serine endopeptidase, specifically hydrolyzes peptide bonds at the C termini of Tyr, Phe, and Trp; these aromatic aminoacids are particularly sensitive to free radical attack when they are exposed to protein surface. The importance of polymerization/aggregation in protein digestibility was confirmed by the significant correlations measured between myosin HC and actin electrophoretic band intensities and protease activities. This result suggests that polymerization and aggregation may in part explain the decrease of digestibility observed with high levels of oxidizing agent.

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