

MEAT PROTEIN AGGREGATION: FIRST INVESTIGATIONS ON THE ROLE OF THE LIPID OXIDATION PRODUCT, 4-HYDROXY-2-NONENAL

M. Gobert, T. Sayd, P. Gatellier and V. Santé-Lhoutellier

INRA, UR370 Qualité des Produits Animaux, F-63122 Saint Genès Champanelle, France

Abstract – This paper describes for the first time how 4-hydroxy-2-nonenal (HNE) promotes protein aggregation in meat products. A whole meat extract was incubated with increasing concentrations of HNE during 30min, 1h and 2h at 25°C. In the studied conditions, HNE-protein adducts were detected. In presence of HNE, protein solubility decreased that stressed a possible involvement of HNE in the aggregation process. Different analytical methods were investigated to detect aggregates formation. The aggregation phenomenon remained low. Employed methods give us information on the implicated bonds. Electrophoresis with or without using a reducing agent coupled with western-blotting analyses revealed that HNE attacks preferentially the cysteine residues (by thiols function) and then lysine and histidine.

Key Words – protein, aggregation, 4-hydroxy-2-nonenal, meat, cross-link, oxidation.

I. INTRODUCTION

Meat content in n-6 polyunsaturated fatty acids (PUFA) could be increased by adapted nutritional supplementations given to animal during the finishing period [1, 2]. PUFA are very sensitive to oxidation, which is the major cause for quality deterioration of meat and meat products during processing and storage. N-6 PUFA peroxidation yields a specific lipid oxidation product, the 4-hydroxy-2-nonenal (HNE), which is particularly reactive. HNE is a possible agent involved in cross-linking of oxidized proteins [3], which could negatively impact on nutrition and sensory values of food [4]. Bifunctional HNE product is a possible agent involved in cross-linking and aggregation of proteins, *via* Michael adducts formation on lysine (Lys), histidine (His) or cysteine (Cys) residues, followed by Schiff base formation [3]. The objective of this research was to investigate the formation of protein aggregates in presence of 4-hydroxy-2-nonenal and to identify the

involved cross-links in a whole bovine meat extract.

II. MATERIALS AND METHODS

Samples preparation

Semi membranosus muscle of Charolais heifer was stored under air during 4 days, which correspond to commercial practices. Meat was homogenized at 15000 rpm during 20 s with a polytron in a 0.06 M, pH 6 KCl solution (10% w/v). A one hour sedimentation step allowed the elimination of most connective tissues. Care was taken to minimize oxidation during the extraction process; all manipulations were achieved at 4°C. Meat extract, containing 4,1 mg of protein, was combined with increasing concentrations of HNE (10, 33 and 166µM) and placed at 25°C under agitation during 30 min, 1h and 2h, with 4 repetitions by group. Control samples were HNE-free. After incubation, butylated hydroxytoluene solution (0.1 mM final concentration) was added and samples were stored at -80°C until analyses.

Protein solubility

Protein concentrations in samples from the different conditions were adjusted on the less concentrated samples with the homogenization KCl solution. In order to solubilize proteins, which are not implicated in the insoluble aggregates, a concentrated KCl solution (4.5M) was added to achieve 0.9M final concentration in the samples. After mixing and centrifugation (1500 rpm, 15 min) protein concentrations of supernatant were determined by the Bradford method.

Electrophoresis and Western blotting

Samples with or without β-mercaptoethanol were separated on 10% SDS-PAGE (Sodium Dodecyl Sulfate - Polyacrylamide Gel

Electrophoresis). Gels were stained with Coomassie Blue R250 or for western blotting, transferred to polyvinylidene difluoride membranes using a trans-blot apparatus. Membranes were blocked with 5% w/v skim milk in TBS with 0.05% v/v Tween 20 and incubated overnight in appropriate dilutions of the anti-HNE polyclonal antibody. After extensive washing, membranes were incubated with anti-rabbit IgG horseradish peroxidase for 1 hr, and washed before detection by ECL-Prime chemiluminescence detection reagent.

III. RESULTS AND DISCUSSION

Proteins recovery after solubilization

Statistical analysis (linear model, SAS) showed no time or HNE concentration effect on protein concentration recovery (Figure 1). Regarding only the HNE concentration effect, the protein concentration tended to decrease with increasing HNE concentrations ($P=0.06$).

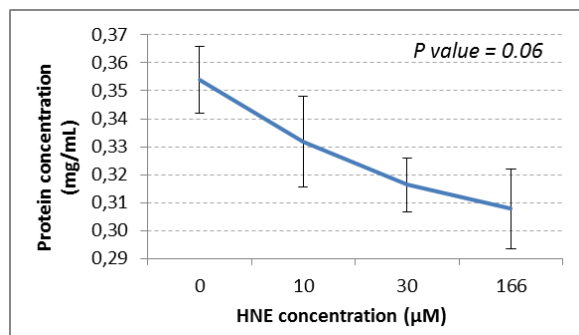


Figure 1: Protein concentrations in supernatant of HNE treated meat extract after addition of 0.9 M KCl.

The hypothesis is that proteins solubilized in a high ionic strength solution are not implicated in aggregates. This observation led to the previous hypothesis in favor of aggregates formation with increasing HNE concentrations. It would be interesting to investigate deeper the solubility of proteins (determination of salting-in and -out effects) by using different ionic strength solutions. To continue the investigations on the processes of aggregation, we carried out gel of electrophoresis.

Electrophoresis

Electrophoresis in denaturing conditions was performed in order to observe possible protein pattern changes induced by HNE. On the one

hand, samples were treated with a reducing agent β -mercaptoethanol (+ β ME) before loading on the SDS-PAGE. Gel shows the evolution of bands from the meat proteins after exposure to HNE with different duration (Figure 2) implicating bi-tyrosine cross-links and HNE-adducts on Lys and His (not destroyed by β ME). On the other hand, HNE is known to react readily on the thiol function of Cys residue to form a Michael adduct [3, 5]. To study this specific bond, we used gel without reducing agent (β ME) to visualize individual proteins and their cross-links *via* HNE-adducts on Lys, His and Cys, but also the disulfide bridges. The SDS-PAGE electrophoresis done without reducing agent (- β ME) did not largely differ from figure 2. Bands were slightly less numerous and less clear in - β ME than in + β ME, since disulfides bridges limit progression of proteins in the acrylamide gel (not shown).

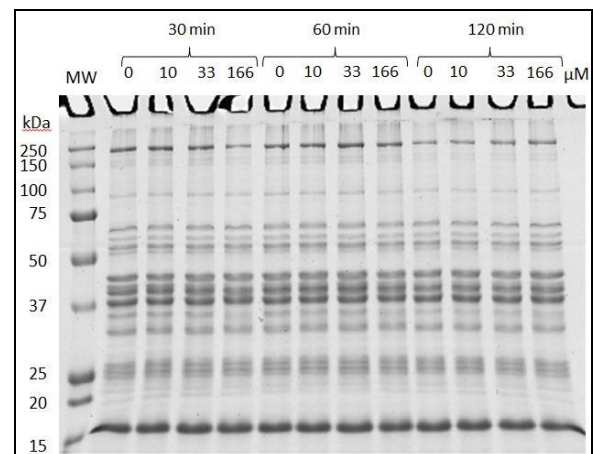


Figure 2: Gel electrophoresis patterns of meat extract proteins (+ β ME) incubated without (Control: 0 μ M) or with different HNE concentrations (10, 33 or 166 μ M) and different incubation times (30, 60 and 120 min). MW: molecular weight.

In the +/- β ME gels, protein profiles of samples did not change largely according to the different conditions tested. Protein quantifications need to be done in order to precise these observations. Only one difference was noted: the band of 250 kDa, which corresponds to myosin heavy chain (MHC) band, decreased slightly after 120 min whatever the HNE concentration (Figure 2). However, no low molecular weight bands appeared which indicated that the disappearance of MHC could rather be due to aggregation than

degradation. It is possible that after 2 hours, aggregates implicating MHC have been formed and could not enter the gel as suggested by Morzel et al. (2006) [6]. In the latter study, oxidants conditions were drastic and aggregation was clearly visible. In the present study, protein aggregation could be implicated by HNE, but intensity is weak and not really demonstrated by electrophoresis. Moreover, the possible implication of HNE-adduct on Cys was not demonstrated with the +/- β ME method.

Western-blotting

On the + β ME gel, a western-blot was performed with anti-HNE antibody (specific of Michael adducts on Lys, His and Cys residues) to detect HNE-protein adducts formed *via* Michael adducts on Lys and His residues (HNE-adduct on Cys was broken by β ME [7]). After transfer on membrane, the corresponding western blot is presented in figure 3.

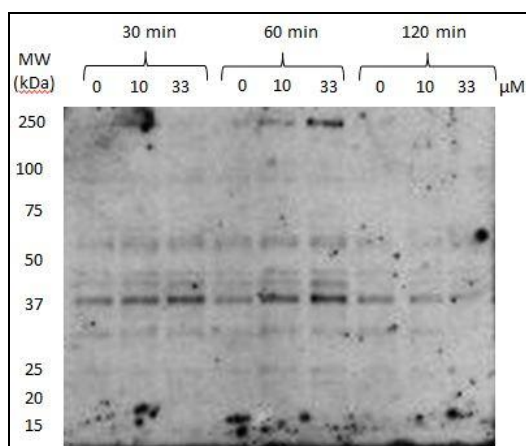


Figure 3: Western-blotting patterns of meat extract proteins (with anti-HNE antibodies on + β ME gel) incubated without (Control: 0 μ M) or with different HNE concentrations (10 or 33 μ M) and different incubation times (30, 60 and 120 min).

In the control samples, low intensity HNE-protein adducts were already detected, demonstrating their formation on Lys and/or His at a basal level during a 4 d. under air meat storage, which fit with previous work on HNE-protein quantification on bovine meat stored under air [8]. HNE-proteins were detected between 20 and 75 kDa with a higher detection above 37 kDa; actin (42 kDa) or β -enolase (47 kDa) would be major concerned. The same low

intensity of HNE protein adducts was monitored with different incubation times (30, 60 and 120 min), independently of duration.

In the HNE supplemented conditions, HNE-protein adducts intensity increased with 10 and 33 μ M of HNE at 30min. This effect was more pronounced at 60min. As compared with control samples, HNE linked with additional proteins, such as high molecular weight proteins. MHC (250kDa), most abundant protein in muscle, seemed to be a good candidate. Increasing HNE concentration increased HNE-protein adducts formation, which made sense. But surprisingly, with 120min incubation, HNE-protein adducts intensity decreased with increasing HNE concentrations. The loss of HNE antibody recognition with increasing HNE concentrations cannot be explained by a rupture of HNE-protein bonds in the incubation tubes, since Michael adducts are not reversible in physiological conditions [5]. It could be due to protein aggregates unable to enter the gel. Aggregates could come from the formation of a bridge implicating a HNE, a Michael adduct and a Schiff base with two MHC, then the antibody could not recognize HNE Michael adduct. Another hypothesis is a possible imprisonment of HNE in a protein aggregate; due to mechanisms linked to protein and/or lipid oxidation after 2h in meat extract.

On the samples without reducing agent (- β ME), the western-blot with the same anti-HNE antibody (figure 4) allows detection of HNE-protein adducts concerned not only Lys and His, but also Cys residues.

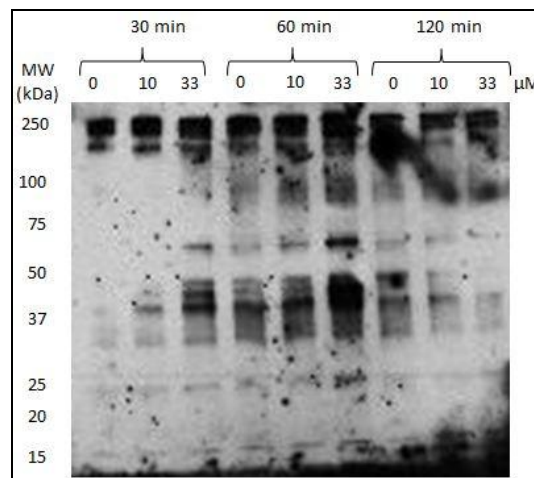


Figure 4: Western-blotting patterns (with anti-HNE antibodies on β ME gel) of total meat extract proteins incubated without (Control: 0 μ M) or with different HNE concentrations (10 or 33 μ M) and different incubation times (30, 60 and 120 min).

First of all, comparison of the two western-blotting patterns (+ vs β ME) showed clearly the affinity of HNE to Cys (vs His and Lys residues) to form Michael adducts in meat proteins, which fit with previous works on *in vitro* peptides model [3]. These observations are consolidated by the composition of myosin heavy chain 1, containing 1939 amino acids, with only 16 Cys towards 37 His and 209 Lys.

In the control samples, we observed high intensity HNE-protein adducts and the same evolutions than those of figure 2. These observations confirmed i) the basal intensity of HNE adduction on meat proteins during 4 d. under air storage; ii) the possible involvement of not only actin or β -enolase, but also numerous other proteins among them MHC. Nevertheless, in contrast with the control samples implicating Lys and/or His (figure 2), incubation duration increased HNE-protein detection (in figure 3), which supposes a time effect on HNE-protein adducts implicating Cys. It is possible that time had a detectable effect only on the higher adducts level, in that case HNE-protein adduct on Cys.

In the HNE supplemented conditions, HNE-protein adducts intensity increased with HNE concentrations at 30 min. As for the western-blot with β ME, signals were more pronounced at 60 min and decreased at 120 min. Similar schemes of aggregation than those described earlier seemed to be implicated. HNE-proteins adducts on Cys seemed to be major implicated. Thus, in proteins, thiol oxidation leading to disulfide bonds may generate intermolecular cross-links [3]. Double bond of HNE can react quickly with the free SH functions [3, 5]. However, electrophoresis, using β -mercaptoethanol (β ME) as reducing agent, breaks disulfides and HNE-protein adducts on Cys [7]. Then, aggregates formed through these mechanisms cannot be detected with electrophoresis, but western-blotting allows visualization of HNE-protein adducts on the different amino acid residues.

IV. CONCLUSION

These results are the first investigations on aggregating power of HNE in total meat proteins. We showed that numerous HNE-proteins adducts were formed. As a result the proteins solubility tended to decrease with increasing HNE concentrations, which reinforce the possible role of HNE in aggregation process. Electrophoresis revealed its limitation in detection of aggregation. However, we characterized the bonds implicated in HNE protein aggregation and demonstrated that HNE reacted preferentially on cysteine residue to form protein aggregates. Further investigations are needed to identify by mass spectrometry the target protein involved in aggregation.

REFERENCES

1. Bauchart, D., Gladine, C., Gruffat, D. (2005). Indicators of milk and beef quality. European Association for Animal Production Publication.
2. Kouba, M., & Mourot, J. (2011). A review of nutritional effects on fat composition of animal products with special emphasis on n-3 polyunsaturated fatty acids. *Biochimie*. 93: 13-17
3. Doorn, J.A., Petersen, D.R. (2002). Covalent adduction of nucleophilic amino acids by 4-hydroxynonenal and 4-oxononenal. *Chemical-biological interactions*. 143: 93-100
4. Pokorny, J. (1977). Interactions of oxidized lipids with protein. *Rivista Italiana delle Sostanze Grasse*, 54 : 389-393
5. Esterbauer, H., Schaur, R. J. & Zollner, H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malondialdehyde and related aldehydes. *Free Radical Biology and Medicine*. 11: 81-128
6. Morzel, M., Gatellier, P., Sayd, T., Renerre, M., & Laville, E. (2006). Chemical oxidation decreases proteolytic susceptibility of skeletal muscle myofibrillar proteins. *Meat Science* 73: 536-543
7. Siems, W.G., Hapner, S.J., VanKuijk, F.J. (1996). 4-hydroxynonenal inhibits Na⁺-K⁺-ATPase. *Free Radical Biology and Medicine*. 20: 215-223
8. Gobert, M., Cossou, C., Comte, B., Pujos-Guillot, E., Gladine, C., Joly, C., Bauchart, D., Durand, D. (2010). Are hydroxyalkenals bound to proteins discriminant markers of n-6 and n-3 PUFA oxidation in bovine meat? In *Proceedings 13th Muscle and Meat Science and Technology* (pp.67-68), 19-20 October 2010, Clermont-Ferrand, France.