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EFFECT OF PROTEIN OXIDATION ON PARTICLE SIZE OF MYOFIBRILS

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Abstract – In order to study the effect of protein oxidation on the structure of myofibrils, extracted myofibrils from porcine *longissimus dorsi* muscle were incubated with different concentrations of the oxidant NaClO (0, 5, 10, 20 and 40 mM) at 5 ºC for 16 h. The myofibrils were homogenized after the incubation. Increasing concentrations of NaClO led to a greater loss of free thiols and a decrease in small particles together with an increase in larger particles. Generally, all the statistical parameters (D(v, 0.1), D(v, 0.5), D(v, 0.9), D(3, 2) and D(4, 3)) for the particle size distribution had greater values with increasing concentrations of NaClO. Light microscope imaging showed that the myofibrils oxidized in 40 mM NaClO were less broken following the homogenization as compared to the non-oxidized group (0 mM NaClO). The increased structural integrity of myofibrils is likely caused by oxidation-induced protein cross-links, which resulted in a larger particle size when the myofibrils were subjected to homogenization.

Key Words – Thiol groups, protein cross-links, pork, myofibril structure

I. INTRODUCTION

Protein oxidation in meat has been associated with increased toughness [1, 2]. The mechanism of increased toughness in oxidized meat appears to be through increased protein cross-linking rather than reduced proteolysis [2]. Protein cross-linking can be speculated to lead to increased structural integrity and therefore increased toughness. The structural properties of meat can be evaluated by the particle size distribution of meat homogenates. For instance, it has been reported that particle size analysis is a simple and fast estimation of myofibril fragmentation [3, 4]. Particle size measurement is increasingly used as a basic measurement in meat science, and may also reflect oxidation-induced changes in the meat structure.

The aim of the present study was to investigate the effect of protein oxidation on structural changes of myofibrils through particle size analysis in combination with microscope imaging. Myofibrils extracted from porcine *longissimus dorsi* muscle were incubated with different concentrations of the oxidant NaClO (0, 5, 10, 20 and 40 mM). Free thiol groups, particle size distribution and micrographs of oxidized myofibrils were evaluated.

II. MATERIALS AND METHODS

Extraction of myofibrils

Porcine M. *longissimus dorsi* was used in the present study. The meat was frozen 24 h postmortem and stored at -20 ºC until extraction of myofibrils (used within one month). Myofibrils were extracted as described by Bertram et al. [5] with slight modifications. Briefly, Five grams of minced meat was homogenized in 20 mL cold extraction buffer (100 mM KCl, 10 mM Tris (pH 6.8), 2 mM EDTA, 2 mM $MgCl_2$, and 1 mM DTT) for 15 s with a speed of 13500 rpm using an IKA Ultra Turrax T25 homogenizer (Labortechnik, Staufen, Germany). The homogenate was centrifuged at 1500 g for 15 min at 4 ºC. The pellet was washed with 20 mL of the cold extraction buffer and centrifuged at 1500 g for 15 min at 4 ºC. Subsequently, the pellet was washed twice with MES buffer (100 mM KCl, 100 mM MES (2-(N-Morpholino) ethanesulfonic acid hydrate, 4-Morpholineethanesulfonic acid) hydrate, 2mM $MgCl_2$, 2 mM EGTA (ethylene glycol tetraacetic acid), pH 5.6) using the same centrifugation conditions to bring down pH of the myofibril pellets close to 5.6.

Oxidation of the myofibrils

Two batches of oxidation and subsequent analysis were performed. Oxidation of the extracted

myofibrils was initiated by adding NaClO. The concentration of NaClO was determined and adjusted to 100 mM by measurement of the absorbance at 292 nm using an extinction coefficient of 350 M^{-1} cm^{-1} [6] and the pH of the 100 mM NaClO solution was adjusted to pH 8.0 with acetic acid.

The reaction mixtures contained 2.5 g of the extracted myofibrils and different amounts of NaClO (100 mM, pH 8.0) was added to give a final concentration of 0, 5, 10, 20 and 40 mM, respectively. In addition, distilled water was added to keep the volume of the reaction system the same among different oxidation levels (illustrated in Table 1).

Table 1. The composition of the reaction mixtures of different oxidation levels.

Components	NaClO (mM)				
	0	5	10	20	40
NaClO, 100 mM, pH 8.0 (mL)	0	0.25	0.5	1	2
Extracted Myofirbils (g)	2.5	2.5	2.5	2.5	2.5
Distilled water (mL)	2.5	2.25	2	1.5	0.5

The reaction mixture was incubated overnight (about 16 h) at 5 ºC and then the myofibrils were washed with the MES buffer (see above) followed by centrifugation at 1500 g for 5 min at 4 ºC. The washing step was repeated and the resulting myofibril pellets were used for free thiol groups and particle size analysis.

Determination of free thiol groups

The determination of free thiol groups was carried out as described by Bao & Ertbjerg [2] with some modification. One gram of myofibrils was homogenized with 15 mL 5% (w/v) SDS in 0.1 M Tris-HCl (pH 8.0) at 8000 rpm for 15 s. The homogenates were heated in water bath at 80 ºC for 1 h. After cooling, the homogenates were filtered through filter paper (Whatman 4, GE Healthcare). Protein concentration of the filtrate was determined by absorbance at 280 nm. Thiol groups were measured by mixing 0.1 mL filtrate, 0.9 mL of 0.1 M Tris-HCl (pH 8.0) and 0.05 mL 10 mM DTNB in 0.1 M Tris-HCl (pH 8.0). The mixture was incubated in dark at room temperature for 30 min. Absorbance at 412 nm was recorded and the content of thiol groups were calculated using an extinction coefficient of 13600 M^{-1} cm^{-1}. The results were expressed as nmol/ mg protein.

Particle size analysis

Particle size distribution of the meat homogenate was determined by a Mastersizer 3000 (Malvern Instruments Ltd., Malvern, UK) according to Lametsch et al. [3] with slight modification. Briefly, one gram of myofibrils was homogenized in 10 mL MES buffer (see above) at 8000 rpm for 15 s using an Ultra-Turrax T25. Every homogenate was analyzed five times using tap water as dispersant. The refractive index was set to 1.46 and the absorption coefficient to 0.01, and the particles were considered as non-spherical. Volume weighted distribution and the following statistical parameters were reported:

D(v, 0.1) – the size of the particle for which 10% of the sample is below this size;

D(v, 0.5) – the size of the particle for which 50% of the sample is below this size;

D(v, 0.9) – the size of the particle for which 90% of the sample is below this size;

D(3, 2) – the surface area moment mean diameter, $D(3, 2) = \sum n_i d_i^3 / \sum n_i d_i^2$, where n_i is the number of particles with diameter d_i and was calculated from the size distribution;

D(4, 3) – the volume moment mean diameter, $D(4, 3) = \sum n_i d_i^4 / \sum n_i d_i^3$, where n_i is the number of particles with diameter d_i and was calculated from the size distribution.

Light microscopy

The suspensions of myofibrils for particle size analysis were examined using a light microscope (Axio Lab.A1, Carl Zeiss AG, Oberkochen, Germany) fitted with a 100 × oil immersion objective.

Data analysis

Data were analyzed by the IBM SPSS Statistics 23 software using general linear model. Concentration of NaClO was included as fixed factor and batch number as random factor. Tukey HSD test was used to find significant differences at a level of $P < 0.05$.

III. RESULTS AND DISCUSSION

Protein oxidation

Hypochlorous acid (HClO) is a highly reactive oxidant that can mediate protein oxidation. Addition of the sodium salt, NaClO, to meat has been reported to promote protein oxidation [7] as an alternative to the more widely used Fenton reaction.

Thiol oxidation by HClO and chloramines occurs readily, and chloramines are the products when HClO reacts with amines [8]. In the present study, the content of free thiol groups in the oxidized myofibrils decreased with increasing concentration of NaClO (Fig. 1). In agreement, packaging of pork in modified atmosphere with high oxygen have shown a decrease in the content of free thiol groups [1, 2].

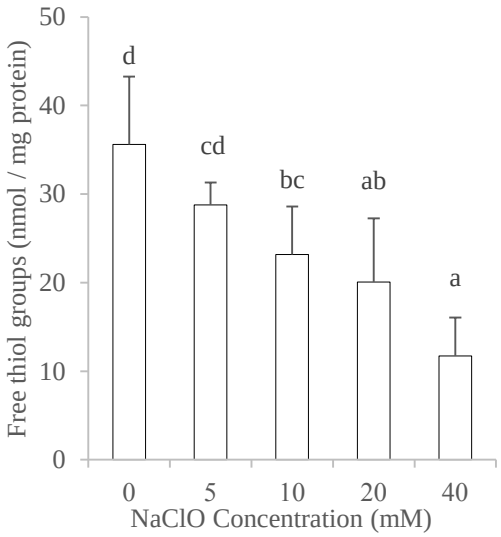

Fig. 1. Free thiol groups of myofibril proteins after incubation (5 ºC, 16 h) with different concentrations of NaClO. Means with standard deviations (n=6) are shown. [a-d]Means with the same letter do not differ (P > 0.05).

Particle size analysis and micrograph of myofibrils

A particle size distribution is often visualized by plotting the particle size versus the intensity. Parameters based on the maximum particle size for a given percentage of the sample are often reported, such as D(v, 0.1), D(v, 0.5) and D(v, 0.9). These three parameters can indicate if there are significant changes in the average particle size and at the extremes (below 10 and more than 90%) of the distribution. The changes at extremes could be due to the presence of very small or very large particles. With increasing concentrations of NaClO, the particle size distribution curves clearly show a decrease in the small particles together with an increase in the larger particles (Fig. 2).

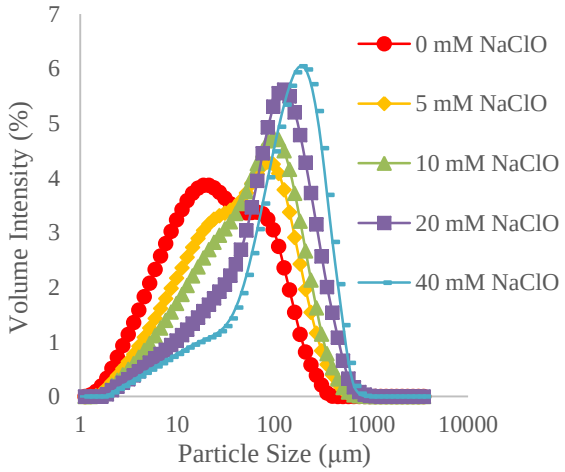

Fig. 2. Average (n = 6) particle size distribution of myofibrils after incubation (5 ºC, 16 h) with different concentrations of NaClO (0, 5, 10, 20 and 40 mM).

The different parameters of particle size distribution are summarized in Table 2. Generally, addition of the oxidant NaClO led to an increase in particle size and the particle size was greater in higher concentrations of NaClO.

Table 2. Effect of different concentrations of NaClO on particle size.

Traits	NaClO (mM)					SEM	Main effect
	0	5	10	20	40		
D(v, 0.1)	6^{a}	9^{a}	11^{ab}	16^{b}	24^{c}	2	***
D(v, 0.5)	31^{a}	50^{ab}	66^{b}	105^{c}	154^{d}	10	**
D(v, 0.9)	118^{a}	183^{b}	206^{b}	288^{c}	345^{d}	18	**
D(3, 2)	15^{a}	22^{ab}	26^{b}	38^{c}	50^{d}	3	***
D(4, 3)	48^{a}	76^{b}	90^{b}	132^{c}	172^{d}	10	**

Means with different superscripts are different (P < 0.05).
SEM: Standard error of the mean.
The significance of the main effect of NaClO is indicated as: ** (P < 0.01) and *** (P < 0.001).

The shift of the distribution curve toward larger particles with increasing concentrations of NaClO (Fig. 2) is likely caused by increased structural integrity of myofibrils after oxidation. Upon homogenization, stronger myofibrils would be more resistant to fragmentation and hence result in larger particles. Decreased fragmentation is supported by microscope images of the oxidized myofibrils (Fig.3). The structural integrity of myofibrils were clearly greater in 40 mM NaClO as compared to 0 mM NaClO.

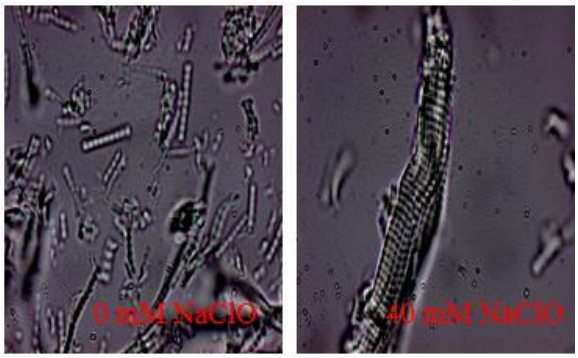

Fig.3. Light microscope images taken at magnification ×1000, of extracted myofibrils after incubation (5 ºC, 16 h) with (0 mM) or without NaClO (40 mM).

We speculate that the mechanism of increased structural integrity in oxidized myofibrils is through the formation of protein cross-links. Protein oxidation has been reported to cause meat toughening by formation of protein cross-links and the myofibrillar protein myosin was involved in the cross-linking [1, 2]. Protein thiols are reactive to oxidants and they can form disulfide links. In the present study, the loss of free thiol groups (Fig. 1) was likely due to the formation of disulfide links, and hence resulted in stronger myofibrillar structures and consequently larger particle sizes.

IV. CONCLUSION

Addition of the oxidant NaClO to myofibrils led to greater protein oxidation and particle size. Myofibrils oxidized with NaClO were less fragmented during homogenization. This study also shows that particle size analysis is a valuable tool to study oxidation-induced structural changes in myofibrils.

ACKNOWLEDGEMENTS

The authors would like to thank the China Scholarship Council for the financial support.

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