DEGRADATION OF MYOSIN BY CATHEPSIN B. COMPARISON OF NATIVE AND OXIDATIVELY MODIFIED PROTEIN

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INTRODUCTION

Tenderization of meat is known to be dependent on endogenous muscle peptidase activity (calpains and cathepsins) which catalyse postmortem hydrolysis of structural muscle proteins. The concentration of peptidases is higher in slow-twitch (red) muscles than in fast-twitch (white) muscles indicating that the tenderization rate should be higher in red muscles compared to white muscles. However, the tenderization rate is generally found to be higher in white muscles. This paradox has still to be elucidated, as higher concentrations of enzyme inhibitor (calpastatin) in red muscles alone cannot explain lower tenderization rate in these compared to white muscles.
The metabolic events in the conversion of muscle to meat can be compared with those occurring in the cardio-vascular system during an ischemic period which enhance oxidative stress. This is supported by recent results showing increased oxidative stress in postmortem tissue in spite of low oxygen influx (Dudda et al., 1996) corresponding the situation in postmortem muscles. The higher oxidative potential in red muscles should accordingly result in enhanced oxidative stress postmortem compared to white muscles. Recently, oxidative modified myosin have been shown to be less susceptible to proteolytic degradation by enzymes of the mammalian digestive tract supposedly due to inter molecular cross-linking (Kamin-Belsky et al., 1996). As oxidative modification of muscle proteins must be expected to occur in postmortem muscles, we suggest that differences in tenderization rate between red and white muscles can be caused by formation of intermolecular cross-linked muscle proteins reducing their susceptibility to proteolysis by endogenous muscle peptidases.
In the present study, myosin isolated from white muscle and red muscle and the respective oxidized myosins were investigated as substrate for cathepsin B in an aqueous model system. In addition possible formation of intermolecular cross-linking between myosin molecules during exposure to oxidative stress was investigated by analysis of di-tyrosine formation.

MATERIAL AND METHODS

Myosin was isolated from *M.masseter* (red muscle) and *M.longissimus dorsi* (white muscle) according to Tonomura *et al.* (1966), and stored at -18°C in 0.5 M NaCl/50% glycerol. Myosin was dialyzed against water for 16 hours and subsequently in 50 mM PO_4^{3-}-buffer (pH 6.0) for hours before use. Myosin concentrations were determined spectrophotometrically in 0.5 M KCl using $\varepsilon_{279} = 5.60$ (Small *et al.*, 1961).
Myosin was exposed to H_2O_2 activated metmyoglobin (MMb/H_2O_2) or MMb (control) in 50mM PO_4^{3-}-buffer (pH 6.0; I=0.16 adjusted with NaCl) for 1 h at 25°C and stored over night at 4°C. Subsequently, native (control) or oxidized myosin were enzymatically hydrolyzed with cathepsin B in 0.05 mM incubation buffer (Tris-acetate containing 2.0 mM dithiothreitol (DTT) and 2.0 mM EDTA; pH 6.0) at 25°C. The reactions were terminated after 0, 2, 4, 6 and 24 hours using a stop solution of 0.125 M Tris-HCl- buffer (pH 6.8) containing 20% Glycerol, 4% SDS, 0.005% bromphenol blue and 2% DTT.
Cathepsin B activity was determined according to Kirschke *et al.* (1983) with minor modifications. The artificial substrate, Z-Phe-Arg-Nmec was used and 360 mM sodium acetate containing 2.6 mM EDTA, 0.3% Brij 35 and 4.6 mM DTT was used as incubation buffer. One enzyme unit (U) was defined as 1 µmol of product per minutes.
SDS-PAGE (Laemmli, 1970) was performed in pre-cast 4% and 8-16% acrylamide gels from NOVEX and stained with 0.2% Coomassie Brilliant Blue. Proteolysis of native and oxidized myosin were evaluated by densitometric scans of the gels. Analysis of di-tyrosine was performed as described by Østdal *et al.* (1996).

RESULTS

Oxidation of myosin. Figure 1 shows that the amount of myosin heavy chain (MHC) decreased with increasing exposure to H_2O_2 activated MMb (simulating oxidative stress). Moreover, in accordance with results by Bhoite-Solomon *et al.* (1992) a band of high molecular weight aggregates (HMA) emerged suggesting formation of a polymeric form of MHC. Finally, very high molecular weight aggregates (VHMA) failing to enter the stacking gel were also formed at high concentrations of MMb/H_2O_2 exposure (lane 6, 7 & 8).

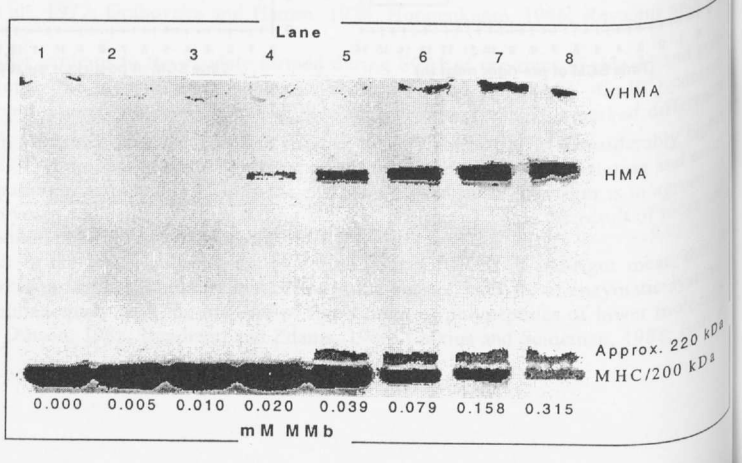

Figure 1. Formation of myosin aggregates with increasing concentration of MMb/H_2O_2, as analyzed in 4 % acrylamide separation gel. Concentration of H_2O_2 was five times the molar concentration of MMb.

To investigate whether formation of VHMA and HMA was caused by intermolecular covalent cross-linking of MHC due to MMb/H_2O_2 exposure as suggested by Hanan & Shaklai, (1995) in a similar model system, the formation of di-tyrosine was analyzed (Table 1). These results clearly indicate that di-tyrosine formation did take place between myosin molecules upon MMb/H_2O_2 exposure. Besides production of HMA and VHMA an oxidation product of approximately 220 kDa was produced (Figure 1). Myosin light chain does not cross-link upon MMb/H_2O_2 exposure (Bhoite-Solomon et al., 1992), why the 220 kDa product most probably is due to cross-linking between myoglobin and MHC.

Table 1. Relative amounts of di-tyrosine in samples of myosin (3.3 μM)/MMb (0.16 mM) mixtures (native) and myosin (3.3 μM) exposed to H_2O_2 (0.80 mM) activated MMb (0.16 mM). All samples are means of three replicates ± standard deviation.

	Myosin from *M. masseter*	Myosin from *M. longissimus dorsi*
Native	100 ± 12.3	100 ± 9.7
Oxidized	420 ± 4.6	379 ± 8.0

Proteolysis of native and oxidized myosin. Proteolysis of native myosin produced light meromyosin as found by SDS-PAGE and subsequent densitometric measurement of the gradient gels (8-16%). Native myosin from *M. longissimus dorsi* had a three fold higher degradation rate than native myosin from *M. masseter* (results not shown) in accordance with data found by Dufour *et al.* (1989). Myosin isolated from *M. masseter* and *M. longissimus dorsi* exposed to H_2O_2 activated MMb (0.80 mM/0.16 mM) were enzymatically hydrolyzed with three different concentrations of cathepsin B, 100, 550 and 1100 U/mg , respectively. Figure 2 presents the relative proportions of myosin heavy chain (MHC) and high molecular weight myosin aggregates (HMA) as a function of time of proteolysis. No significant difference in degradation rates of myosin from *M. masseter,* as measured by decreasing MHC degradation, was registered for the three levels of cathepsin B activity (Figure 2A). In contrast, myosin from *M. longissimus dorsi*, as measured by decreasing MHC degradation, seemed to degrade faster with increasing cathepsin B activity (Figure 2A) compared to myosin from *M. masseter*. Proteolysis of high molecular weight myosin aggregates (HMA) of oxidized myosin from *M. masseter* (Figure 2B) with 100 U and 550 U did not give any detectable degradation, whereas proteolysis with 1100 U seemed to cause limited proteolysis of HMA. Proteolysis of high molecular weight myosin aggregates (HMA) of oxidized myosin from *M.longissimus dorsi* (Figure 2B) showed notable differences between presence of 100 U cathepsin B, which did not catalyze hydrolysis HMA, and presence of 550-1100 U cathepsin B which caused noticeable degradation of HMA.

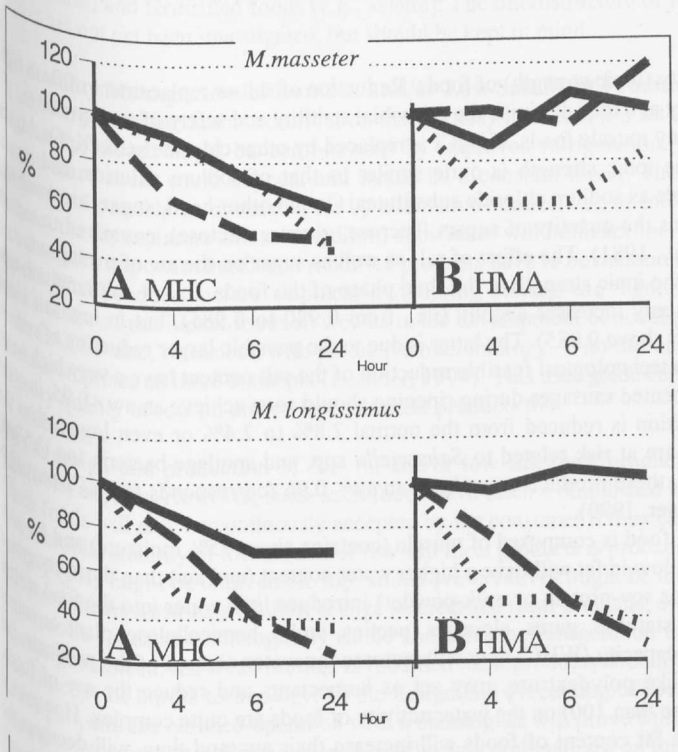

Figure 2. Relative concentrations of native myosin heavy chain (MHC) [A] and high molecular weight aggregates (HMA) [B] from H_2O_2/MMb oxidized myosin during proteolysis by cathepsin B. 100 U mg^{-1} myosin (———), 550 U mg^{-1} myosin (— — —), 1100 U mg^{-1} myosin (- - - - - -).

CONCLUSION

Oxidation of myosin with hydrogen peroxide activated myoglobin formed intermolecular di-tyrosine cross-links between MHC residues. Formation of high molecular aggregates decreased the rate of degradation by cathepsin B. The effect of cross-linking on degradation with cathepsin B was more pronounced in myosin from the oxidative *M. masseter* compared to myosin from the much more glycolytic *M. longissimus dorsi.*

Our results indicates that oxidative modification of muscle proteins decrease their susceptibility to proteolytic degradation. Cathepsin B and myosin have been used as a model for the tenderization process in meat, however, it might be reasonably to conclude that other muscle proteins (e.g. costamere proteins, titin or nebulin) also will form high molecular aggregates upon oxidative exposure by which decrease in susceptibility to proteolytic degradation occur. This may be part of the explanation why white muscle fibers tenderize quicker than red muscle fibers.

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