



Protein oxidation in meat during chill storage

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Protein Oxidation in Meat during Chill Storage

PhD thesis by

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Denmark 2007

Title:

Protein Oxidation in Meat during Chill Storage

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Preface

This PhD thesis is intended to fulfil the requirements for the PhD degree at the Faculty of Life Sciences (formerly the Royal Veterinary and Agricultural University), University of Copenhagen, Denmark. The project has been funded through the Graduate School FOOD with 1/3 funded by the Danish Meat Research Institute, 1/3 by the University of Copenhagen, and 1/3 by the Danish Ministry of Science, Technology and Innovation.

First of all, I would like to sincerely thank my supervisors Professor Leif H. Skibsted from Department of Food Science and Project leader Marchen S. Hviid from the Danish Meat Research Institute, and my co-authors for inspiration, helpful advice and discussions throughout the PhD study.

I would particularly like to thank Professor Mike Davies from the Heart Research Institute, Sydney, Australia for welcoming me into both his research group and home, and for providing invaluable advice to my work. All the employees at HRI are deeply thanked for making my stay in Sydney truly fantastic, especially Ellie, Ojia, Mitch, Gerrit and Annegien, and Cathy for doing the last important dityrosine and amino acid analysis to finish the work (paper IV) after I had to return to Denmark. I would also like to thank the graduate school FOOD for the additional funding I received, which made my trip to Sydney possible.

I also wish to thank Laboratory Technicians Marie Anker and Anders S. Laursen, and all the Laboratory students at Food Chemistry for excellent technical assistance. Associate Professor Søren S. Jørgensen is thanked for advice on analytical chemical issues. MSc. Anne M. Frederiksen is thanked for working hard on establishing an optimal and time-limited procedure for purification of myosin. I am also very grateful to René Lametsch, Marianne K. Thomsen, and Lisbet S. Christensen for valuable comments and proof-reading of this thesis.

I would like to thank all my colleagues at both Food Chemistry and Meat Science at the Department of Food Science for creating a motivating and social environment and to my colleagues at the Danish Meat Research Institute for assistance with storage experiments and discussions of results in relation to storage of meat.

Finally, a very special thank to my husband René Lametsch who has not only supported me throughout the study but has also contributed with fruitful discussions and encouragement, and to Berit, Niels and Jakob Lund for always being there for me.

Marianne Nissen Lund

October 2007

Abstract

Protein oxidation in meat during chill storage in high-oxygen atmospheres (70-80% O₂ and 20-30% CO₂) compared to storage of meat without oxygen was investigated including the consequences of protein oxidation for meat texture. The effect of the fatty acid composition of dietary fat and two antioxidant systems (a rosemary extract and ascorbate/citrate (1:1)) on protein oxidation and lipid oxidation in meat was further investigated. Finally, a detailed mechanistic study of the oxidation of the major myofibrillar protein in meat, myosin, by heme proteins/H₂O₂ was performed in model systems. The pH-dependency of the oxidation of myosin was investigated in the pH interval 5.0-7.8.

Protein oxidation was found to increase significantly in high-oxygen atmospheres compared to storage without presence of oxygen. Tenderness of meat was clearly decreased by the presence of oxygen in the packaging atmosphere. For the first time, it was shown that myosin cross-links through intermolecular disulfide formation in fresh meat due to storage in high-oxygen atmospheres, which cause a reduction in meat tenderness.

Myosin was found to be highly susceptible to oxidation with hypervalent myoglobin species through oxidation of cysteine, tyrosine and other unidentified species on myosin. The generation of thiyl and tyrosyl radicals was consistent with the detection of both disulfide and dityrosine cross-links between myosin molecules. Additionally, a long-lived tyrosine-derived radical was formed on myosin due to oxidation with hypervalent myoglobin species and the significance of the formation of this radical is discussed in relation to meat quality.

Increasing the amounts of unsaturated fat in pork through dietary fat increased lipid oxidation in meat but was not found to have an impact on protein oxidation. Addition of antioxidants capable of inhibiting lipid oxidation was not found to inhibit protein oxidation in meat. These observations indicate that protein and lipid oxidation are not coupled. However, the formation of protein oxidation products in meat was found to be slower compared to the formation of lipid oxidation products.

It is concluded, that protein oxidation in meat caused by packaging in high-oxygen atmospheres influences meat texture negatively, and that cross-linking of myosin is responsible for this decrease in meat texture.

Sammendrag

Proteinoxidation i kød under kølelagring i høj-ilt atmosfærer (70-80% O₂ og 20-30% CO₂) sammenlignet med lagring af kød uden ilt blev undersøgt inklusiv konsekvenserne af proteinoxidation på teksturen af kød. Effekten af fedtsyresammensætningen af foder og to antioxidantsystemer (et rosmarinekstrakt og ascorbat/citrat (1:1)) på proteinoxidation og lipidoxidation i kød blev også undersøgt. Endelig blev der foretaget en mekanistisk undersøgelse af oxidationen af det største myofibrillære protein i kød, myosin, af hæmproteiner og H₂O₂ i modelsystemer. pH-afhængigheden af oxidationen af myosin blev undersøgt i pH-intervallet 5.0-7.8.

Proteinoxidation blev fundet at stige signifikant i høj-ilt atmosfærer sammenlignet med lagring uden tilstedeværelse af ilt. Mørhed af kød blev tydeligt sænket ved tilstedeværelse af ilt i pakkeatmosfæren. For første gang blev det vist, at myosin krydsbinder gennem intermolekylær disulfiddannelse i fersk kød på grund af lagring i høj-ilt atmosfærer, hvilket resulterer i et fald af mørhed af kød.

Myosin var meget sårbar overfor oxidation af hypervalente myoglobinformer gennem oxidation af cysteine-, tyrosin- og andre uidentificerede grupper på myosin. Dannelsen af thiyl- og tyrosylradikaler stemte overens med detektionen af både disulfid- og dityrosinkrydsbindinger mellem myosinmolekyler. Desuden blev et langlivet tyrosin-afledt radikal dannet på myosin ved oxidation med hypervalente myoglobinformer, og betydningen af dannelsen af dette radikal er diskuteret i forhold til kødkvalitet.

En stigning i mængden af umættet fedt i svinekød gennem fodring øgede lipidoxidation, men blev ikke fundet at have nogen betydning for proteinoxidation. Tilsætning af antioxidanter, der er i stand til at hæmme lipidoxidation, blev ikke fundet at hæmme proteinoxidation i kød. Disse observationer indikerer at protein- og lipidoxidation ikke er kobled. Dannelsen af proteinoxidationsprodukter blev imidlertid fundet at være langsommere end dannelsen af lipidoxidationsprodukter.

Det konkluderes, at proteinoxidation i kød forårsaget af pakning i høj-ilt atmosfærer har negativ indflydelse på teksturen af kød, og at krydsbinding af myosin er årsag til dette fald i kødtekstur.

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List of Papers

Paper I

The combined effect of antioxidants and modified atmosphere packaging on protein and lipid oxidation in beef patties during chill storage. **Lund, M.N.**; Hviid, M.S.; Skibsted, L.H. *Meat Science*, **2007**, 76, 226-233.

Paper II

High-oxygen packaging atmosphere influences protein oxidation and tenderness of porcine *longissimus dorsi* during chill storage. **Lund, M.N.**; Lametsch, R.; Hviid, M.S.; Jensen, O.N.; Skibsted, L.H. *Meat Science*, **2007**, 77, 295-303.

Paper III

Effects of dietary soybean oil on lipid and protein oxidation in pork patties during chill storage. **Lund, M.N.**; Hviid, M.S.; Claudi-Magnussen, C.; Skibsted, L.H. *Submitted to Meat Science*, 24/7-07, revised 07/11-07.

Paper IV

Oxidation of myosin by heme proteins generates myosin radicals and protein cross-links. **Lund, M.N.**; Luxford, C.; Skibsted, L.H.; Davies, M.J. *Submitted to Biochemical Journal*, 13/8-07, revised 1/11-07.

Paper V

Oxidation of porcine myosin by hypervalent myoglobin: The role of thiol groups. Frederiksen, A.M.; **Lund, M.N.**; Andersen, M.L.; Skibsted, L.H. *Submitted to Journal of Agricultural and Food Chemistry*, 26/9-07.

Paper VI

Effect of modified atmosphere packaging on mechanical properties of single muscle fibres from bovine and porcine *longissimus dorsi*. **Lund, M.N.**; Christensen, M.; Fregil, L.; Hviid, M.S.; Skibsted, L.H. *Submitted to Meat Science*, 01/10-07

In the text these papers will be referred to by their roman numerals.

Abbreviations and Symbols

AA	ascorbic acid
AAPH	2,2'-azobis(2'-amidinopropane)hydrochloride
AMVN	2,2'-azobis(2,4-dimethylvaleronitrile)
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
CD	conjugated dienes
D(3,2)	surface mean diameter
DETAPAC	diethylenetriaminepentaacetic acid
DNPH	2,4-dinitrophenylhydrazine
DOPA	3,4-dihydroxyphenylalanine
DP	<i>diaphragma pedialis</i>
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTNP	2,2'-dithiobis(5-nitropyridine)
EDTA	ethylenediaminetetraacetic acid
EPR	electron paramagnetic resonance
ESR	electron spin resonance
HI-OX	high-oxygen atmosphere
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
LD	<i>longissimus dorsi</i>
LL	<i>longissimus lumborum</i>
LOOH	lipid hydroperoxide
LTL	<i>longissimus thoracis et lumborum</i>
MbFe(II)	deoxymyoglobin
MbFe(II)O ₂	oxymyoglobin
MbFe(III)	metmyoglobin
MbFe(IV)=O	ferrylmyoglobin
•MbFe(IV)=O	perferrylmyoglobin
MFI	myofibrillar fragmentation index
MHC	myosin heavy chain
MLC	myosin light chain
MS	mass spectrometry
Myosin-S•	myosin-thiyl radical
Myosin-TyrO•	myosin-tyrosyl phenoxyl radical
Myosin-X•	myosin radical (unidentified species)
NADH	nicotinamide adenine dinucleotide
NEM	N-ethylmaleimide
•OH	hydroxyl radical
P	<i>pectoralis</i>
PG	propyl gallate
R	correlation coefficient
RS-	thiolate ion
RS•	thiyl radical

RSH	thiol group
RSOH	sulfenic acid
RSOOH	sulfinic acid
RSSR	disulfide cross-linking
RSSR [•]	disulfide radical
S	<i>sartorius</i>
SDS	sodium dodecyl sulphate
SDS-page	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBARS	thiobarbituric acid reactive substances
TBHQ	<i>tert</i> -butylhydroxyquinone
TPP	tripolyphosphate
WBSF	Warner-Bratzler shear force
X	xanthine
XO	xanthine oxidase

1 Introduction

Lipid oxidation and pigment oxidation are recognised as the most important causes of quality deterioration of both fresh and processed meat during storage (Kanner, 1994; Skibsted *et al.*, 1998). Protein oxidation in meat is only sparsely understood and described contrary to lipid oxidation. The importance of protein oxidation for quality deterioration has only been partially investigated and the coupling between protein oxidation and lipid oxidation is elucidated to an even lesser extent. However, protein oxidation has been investigated extensively in relation to aging and pathology and it is well-known that proteins are modified in human tissue under oxidative stress (Davies & Dean, 2003; Dean *et al.*, 1997; Stadtman, 1992).

In Denmark and in most parts of the Western World retail modified atmosphere packaging with high levels of oxygen is widely used for storage of fresh meat. Packaging in high-oxygen atmospheres with 70-80% O₂/20-30% CO₂ has proven effective to reduce microbial growth in meat and protect the red meat colour for both beef and pork (Asensio *et al.*, 1988; Enfors *et al.*, 1979; Killeffer, 1930). The presence of oxygen causes oxidation of meat lipids and has been found to cause production of unacceptable rancid off-flavours during storage (Asensio *et al.*, 1988; Jackson *et al.*, 1992). In contrast, the extent of protein oxidation in fresh meat stored in high-oxygen atmospheres and the consequences for meat quality have not been studied in any details.

Oxidation of proteins in processed meat products tends to result in reduced water-holding capacity and texture-forming ability (Xiong, 2000). For fresh meat, it has been shown that protein oxidation induced by irradiation leads to reduced tenderness (Rowe *et al.*, 2004b), but the fundamental mechanisms behind this change in protein functionality has not been clarified. In addition, the changed functionality of myofibrillar proteins caused by oxidation may affect water binding capacity and hereby reduce juiciness of the meat (Huff-Lonergan & Lonergan, 2005). The most abundant meat protein, myosin, is highly susceptible to oxidation and is known to polymerize through intermolecular cross-linking in model systems (Bhoite-Solomon *et al.*, 1992; Decker *et al.*, 1993; Hanan & Shaklai, 1995b; Hanan & Shaklai, 1995a; Liu & Xiong, 2000a; Ooizumi & Xiong, 2004). Additionally, myosin is known to cross-link during aging of meat (Martinaud *et al.*, 1997), a reaction which may be increased by oxidation and influence meat quality negatively. The reaction mechanism of myosin oxidation has not previously been described in details.

The aim of the present PhD study has been to investigate the importance of protein oxidation in meat during storage in modified atmosphere containing high concentrations of oxygen. Different methods used to detect protein oxidation products in meat samples have been evaluated in order to select the best marker for protein oxidation in meat. The effect of protein oxidation on meat texture has been investigated from two hypotheses: i) that oxidation causes inactivation of proteolytic enzymes responsible for meat tenderization and hereby reduced meat tenderness (Rowe *et al.*, 2004a), and ii) that oxidation causes protein cross-linking of myofibrillar proteins and hereby tighten or strengthen the myofibrillar structure resulting in less tender meat. Furthermore, the oxidation of myosin by heme proteins has been investigated from a mechanistic point of view. Finally, to which extent proteins oxidize relative to lipids in meat has been studied including the protective effect on protein and lipid oxidation of antioxidants traditionally used in foods in order to improve the control of oxidation in meat during storage.

The pigments myoglobin and hemoglobin are the quantitatively most important heme proteins in mammals. These heme proteins may initiate oxidative damage in meat by various activation mechanisms and reaction pathways and has accordingly attracted considerable attention (Baron & Andersen, 2002; Kroger-Ohlsen, Carlsen, Andersen, & Skibsted, 2002). Myoglobin is probably the most thoroughly investigated meat protein in relation to oxidation due to its close connection with meat colour and the fact that it is highly sensitive to oxidation (Mancini & Hunt, 2005; Renerre, 2003; Skibsted *et al.*, 1998). Nevertheless, as meat colour has not been the focus of attention during the present PhD study only an introductory section is included in this thesis. Hence, the term protein oxidation refers to oxidation of other meat proteins than myoglobin and hemoglobin. Likewise, the impact of protein oxidation on flavour and nutritional value of meat has not been investigated either even though they without a doubt are interesting research subjects as well.

The present thesis constitutes a literature review on protein oxidation in relation to meat (Chapter 2-4). Whenever appropriate, the results from the experimental part of the PhD thesis (paper I-VI) are also included in this literature discussion. Chapter 2 focuses on the oxidizing systems present in meat, and on the protein oxidation products formed, and includes a detailed description of the oxidation of myosin. Chapter 3 deals with the effect of protein oxidation on meat texture. Chapter 4 discusses the coupling between lipid and protein oxidation in meat during storage together with protective effects of antioxidants traditionally

used in meat. Additionally, an important part of the study has been establishment of analytical methods for evaluation of protein oxidation and a fifth chapter describes these methods from a practical point of view. Finally, chapters 6-8 include a final discussion, conclusions and perspectives of the results obtained, which are included in the appendix as papers I-VI.

2 Protein Oxidation in Meat

Protein oxidation in meat is discussed in relation to i) initiation of oxidation with special emphasis on the prooxidative systems relevant for meat, ii) formation of protein oxidation products and markers for evaluation of protein oxidation in fresh meat, and iii) oxidation of specific meat proteins with focus on myosin as the most abundant meat protein.

2.1 Initiation of Oxidation in Meat

The presence of elevated levels of molecular oxygen (or triplet state oxygen) in packaging atmospheres holds the risk for increased oxidation. However, molecular oxygen requires energy (as from exposure to light or other irradiation) or occurrence of reactive species (as reactive radicals, peroxidases, metal ions) to become activated and induce damage. Enzyme-catalyzed redox reactions, metal ion catalyzed reactions, exposure to ionizing irradiation leading to radiolysis, and photochemical processes cause formation of the initial radicals. The nature of protein oxidation products formed is highly dependent on how oxidation is initiated. In general, the more reactive the formed radicals are, the less selective reactions are initiated (Davies, 2003; Garrison, 1987; Irwin *et al.*, 1999; Stadtman & Berlett, 1988).

Myoglobin, the most abundant heme protein in meat, may initiate oxidation by various reaction pathways: i) autoxidation of oxymyoglobin followed by enzymatic reduction of metmyoglobin (Hagler *et al.*, 1979; Satoh & Shikama, 1981), ii) catalytic degradation of lipid hydroperoxides (Tappel, 1955), and iii) pseudoperoxidase activity of myoglobin (Chance *et al.*, 1986; Kroger-Ohlsen *et al.*, 2002; Skibsted *et al.*, 1998). The myoglobin H₂O₂-activation involves formation of hypervalent myoglobin species (King & Winfield, 1963), which include perferrylmyoglobin ($\bullet\text{MbFe(IV)=O}$) and ferrylmyoglobin (MbFe(IV)=O) (Davies, 1991; Davies, 1990; Egawa *et al.*, 2000). The prooxidative activity of myoglobin is directly coupled to the colour cyclus of meat, as illustrated in Figure 2.1.

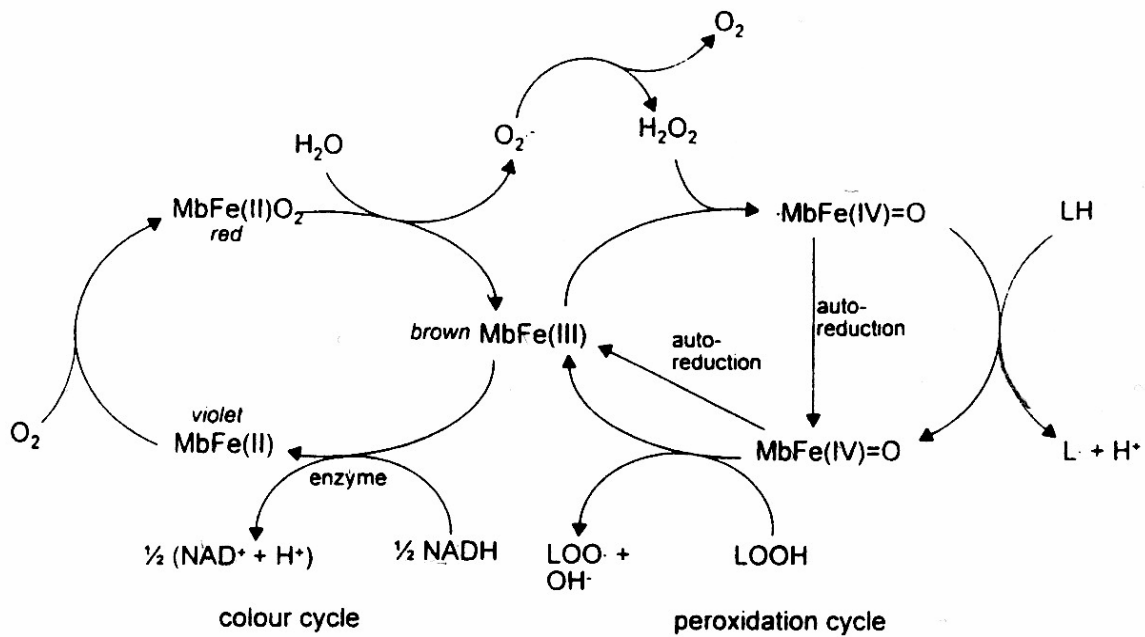


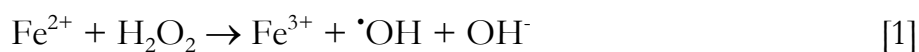
Figure 2.1 Pseudo-peroxidase cycle of myoglobin as linked to the colour cycle of meat (Skibsted *et al.*, 1998).

The oxidation of myoglobin has been studied extensively and reviewed in several papers (Baron & Andersen, 2002; Carlsen *et al.*, 2005; Kroger-Ohlsen *et al.*, 2002). Briefly, the presence of oxygen causes formation of the red oxymyoglobin (MbFe(II)O_2), which oxidizes to the brown metmyoglobin (MbFe(III)) (colour cycle). While there is still enzymatic activity left in the meat, MbFe(III) will be transformed into the violet deoxymyoglobin (MbFe(II)), which again in presence of oxygen will form MbFe(II)O_2 , and is the explanation for preservation of red colour on the surface of meat in high-oxygen atmospheres. When the enzymes in meat is depleted MbFe(III) will accumulate in the meat and by reaction with H_2O_2 hypervalent myoglobin species ($\bullet\text{MbFe(IV)=O}$ and MbFe(IV)=O) are formed (peroxidation cycle).

Hypervalent myoglobin species have been shown to induce both intra- and intermolecular protein cross-linking (Bhoite-Solomon *et al.*, 1992; Hanan & Shaklai, 1995a; Ostdal *et al.*, 1997; Solar *et al.*, 1990)(Paper IV), and radical transfer from $\bullet\text{MbFe(IV)=O}$ to other proteins have been shown to cause formation of long-lived protein radicals (Ostdal *et al.*, 1997)(Paper IV). However, oxidation of proteins is not only caused by $\bullet\text{MbFe(IV)=O}$ but in some cases also by MbFe(IV)=O , as demonstrated by the reduction of MbFe(IV)=O by β -lactoglobulin (Ostdal *et al.*, 1996) and myosin (Paper V). The initiation of lipid oxidation by reaction between hypervalent myoglobin and lipid (LH) and/or lipid hydroperoxides (LOOH), as

demonstrated in Figure 2.1 (Davies, 1991; Kanner & Harel, 1985; Skibsted *et al.*, 1998), as well as oxidation of bovine serum albumin (BSA), is pH dependent (Baron & Andersen, 2002; Kroger-Ohlsen *et al.*, 2003). The presence of hypervalent myoglobin species in meat has been questioned, but it seems that MbFe(IV)=O is present in meat and also is able to initiate lipid oxidation under the conditions expected to be found in meats (Baron *et al.*, 1997; Baron & Andersen, 2002; Kroger-Ohlsen *et al.*, 2002; Xu *et al.*, 1990), whereas the ability of MbFe(IV)=O to induce protein oxidation in meat has yet to be elucidated. The presence and importance of •MbFe(IV)=O as a prooxidant in meat may be more speculative as the concentration of •MbFe(IV)=O at pH relevant to meat systems (pH 5-6) has been shown to be extremely small (Xu *et al.*, 1990), and as •MbFe(IV)=O rearranges intramolecularly at pH 5.5-6.5 rather than interacting with lipids (Baron *et al.*, 1997). •MbFe(IV)=O has been found to react rapidly with myosin to form myosin radicals and cross-linked products in a concentration dependent manner at physiological pH (paper IV), and the formation of cross-linked myosin induced by H₂O₂-activated myoglobin does not seem to be greatly affected by lowering pH to values relevant for meat systems (paper V). The oxidation of myosin is described in detail in section 2.3.

Non-heme iron and other transition metal ions also catalyse both protein oxidation and lipid oxidation in the presence of H₂O₂ in muscle tissue (called the Fenton reaction, metal ion catalyzed oxidation or mixed function oxidation) (Carlsen *et al.*, 2005; Kanner, 1994; Levine, 1984; Skibsted *et al.*, 1998; Stadtman, 1990; Stadtman & Berlett, 1988). A finite mechanism of the reaction between iron and H₂O₂ is still not established, but in general, the Fenton reaction is described by Eq. 1 and is regarded as a common source of the highly reactive hydroxyl radicals (•OH):



The reaction mechanism is far more complicated with several reaction intermediates and reviews by Dunford (2002) or Carlsen *et al.* (2005) should be studied for further information. Formation of carbonyl compounds on amino acid side chains is a well-documented result of metal ion-catalyzed oxidation (Levine, 1984; Oliver *et al.*, 1987; Stadtman & Oliver, 1991), and the reaction mechanism suggested by Stadtman (1990) (Figure 2.2) is generally accepted.

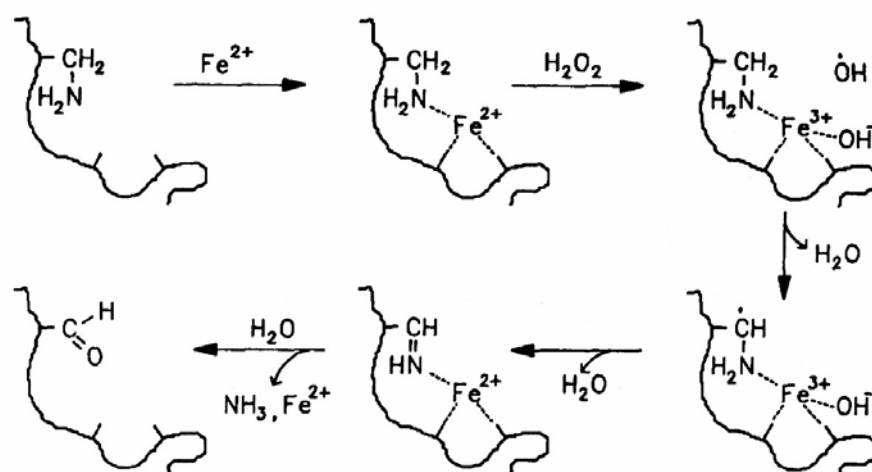


Figure 2.2 Mechanism for the site-specific metal-ion catalyzed oxidation of proteins suggested by Stadtman (1990).

The amount of non-heme iron in meat has been shown to increase significantly during cooking as iron is released from myoglobin due to denaturation. Non-heme iron has therefore been proposed to increase its prooxidative potential in cooked meat (Schrickler & Miller, 1983). However, several studies have shown that the prooxidative activity of myoglobin (in both heated and non-heated systems) is higher than that of non-heme iron at concentrations relevant to meat (Johns *et al.*, 1989; Kristensen & Andersen, 1997; Monahan *et al.*, 1993). To what extent myoglobin and non-heme iron act individually as prooxidants in cooked and uncooked meat is indefinite, but it is generally accepted that both systems induce oxidation in meat (Carlsen *et al.*, 2005; Kanner, 1994; Monahan *et al.*, 1993; Skibsted *et al.*, 1998).

Hydrogen peroxide is formed in cells and accumulates in meat *post-mortem* (Harel & Kanner, 1985; Kanner, 1994). Cysteine residues are highly susceptible to oxidation by H_2O_2 (Neumann, 1972), but the rate of reaction between H_2O_2 and cysteine-containing peptides or proteins is rather slow, e.g. with cysteine and BSA the rate constants are $k = 1.0$ and $1.14 \text{ M}^{-1}\text{s}^{-1}$ (pH 7.4, 25°C), respectively (Radi *et al.*, 1991; Winterbourn & Metodiewa, 1999). It is also noteworthy, that oxidation of myosin ($13 \mu\text{M}$) with H_2O_2 in the concentration range of $25 \mu\text{M} - 10 \text{ mM}$ does not cause loss of free thiol groups in myosin in comparison to oxidation with MbFe(III) activated by H_2O_2 (paper IV). However, oxidation with H_2O_2 of proteolytic enzymes from meat (Carlin *et al.*, 2006; Guttman *et al.*, 1997) and fruits (Mikkelsen & Skibsted, 1998) containing active sites based on cysteine has been shown to decrease enzymatic activity.

Oxidation may also be initiated by γ -irradiation, which is used to eliminate spoilage and pathogenic bacteria in meat (Dempster, 1985), but at the same time induces oxidation as the very reactive hydroxyl radicals are formed (Garrison, 1987). However, meat is not irradiated in Denmark, and this preservation method has therefore not been investigated further as initiator of protein oxidation in the present study. Light-induced oxidation of proteins has been reviewed by Davies (Davies, 2003), and even though it is a highly relevant topic in relation to oxidation of meat it is outside the scope of the present study, and will not be described in any details. In paper II the meat was exposed to light in order to imitate retail storage but as no controls were included without light exposure it is not possible to separate any effects of light-induced oxidation.

Finally, the oxygen penetration depth in muscle *post-mortem* may also influence oxidation in meat during storage, and has recently been positively correlated ($R = 0.42$) to lipid oxidation in 19 bovine muscles (McKenna *et al.*, 2005). The depth of oxygen penetration increases slowly with time, but rarely exceeds one centimetre even after long periods of storage (Bendall & Taylor, 1972; Brooks, 1938; Kebede *et al.*, 1998; McKenna *et al.*, 2005). The oxygen penetration depth increases with increasing oxygen concentrations in the packaging atmosphere (Brooks, 1938), but also depends on temperature, pH, species, muscle type, and competition for oxygen by other respiratory processes (Kebede *et al.*, 1998; Kilic & Cassens, 1998; Mancini & Hunt, 2005; McKenna *et al.*, 2005; Millar *et al.*, 1994). The oxidation of turkey muscles has been shown to take place predominantly in the surface layer of about 2 mm of the muscle, determined in a model system where the lipid soluble compound, β -carotene, was used as a marker of oxidation (Kebede *et al.*, 1998), but the extent of radical formation and of lipid and protein oxidation in various layers from the surface of the muscle to the inside is fundamentally unknown.

In summary, storage of meat in high-oxygen atmosphere holds a potential risk for elevated oxidation but the oxidation is dependent on presence of prooxidants in meat or activation of the molecular oxygen by other means, and perhaps also to which degree oxygen penetrates the muscle.

2.2 Protein Oxidation Products and Markers

Oxidation of amino acids and proteins has been reviewed in several scientific papers (Davies, 1987; Davies, 2003; Dean *et al.*, 1997; Garrison, 1987; Hawkins & Davies, 2001; Stadtman, 1993; Stadtman & Berlett, 1997; Stadtman & Levine, 2003) and a recent monograph (Davies & Dean, 2003). Hence, this section will include a

general overview of the most common products formed during protein oxidation. For more detailed information about the mechanism of formation of protein oxidation products the above literature should be consulted.

Reaction of radicals with proteins and peptides in the presence of oxygen gives rise to alterations of both the backbone and of the amino acid side chains. These oxidative changes includes cleavage of peptide bonds, modification of amino acid side chains, and formation of covalent intermolecular cross-linked protein derivatives (Davies *et al.*, 1987a; Davies & Dean, 2003; Dean *et al.*, 1997; Garrison, 1987; Stadtman & Berlett, 1997), as shown in Figure 2.3. Some of the most general amino acid modifications are the formation of protein carbonyl groups and protein hydroperoxides, while cross-linking has mostly been described as formation of disulfide and dityrosine (Davies *et al.*, 1999).

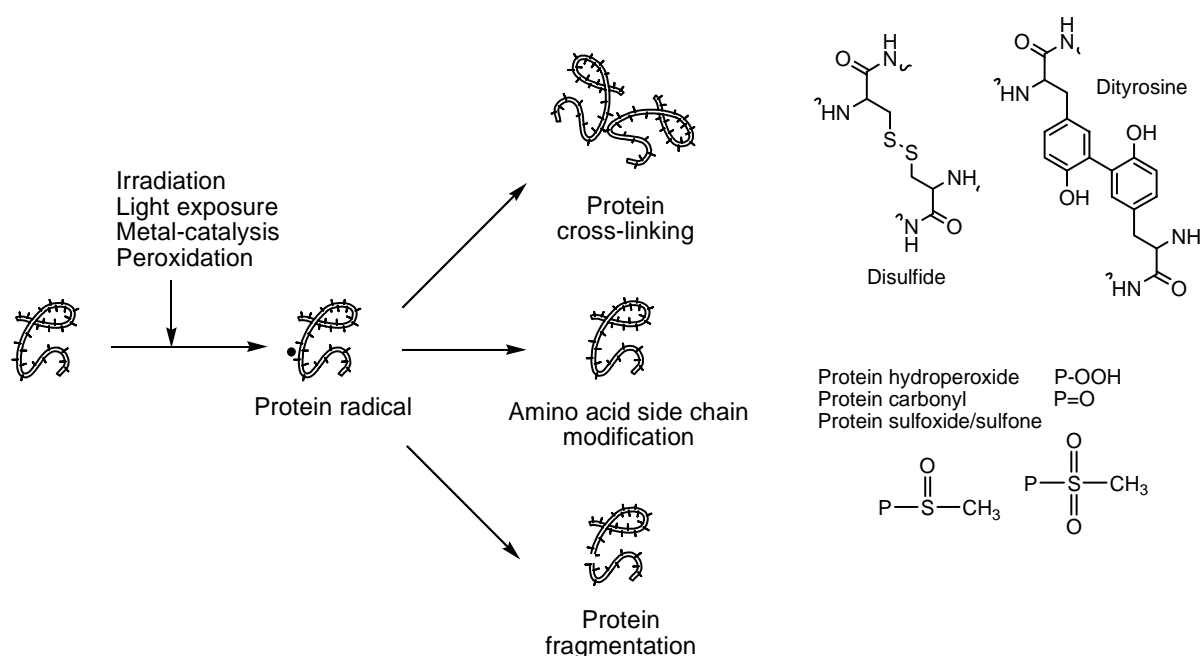


Figure 2.3 Different consequences of oxidation of proteins.

Oxidation of proteins may cause changes in protein hydrophobicity and conformation, inactivation of proteolytic enzymes, and altered susceptibility of protein substrates to proteolytic enzymes (Davies *et al.*, 1987b; Dean *et al.*, 1986b; Wolff & Dean, 1986).

The amino acids, which are generally most susceptible to oxidation are cysteine, tyrosine, phenylalanine, tryptophan, histidine, proline, arginine, lysine, and methionine (Stadtman, 1992), and the most common oxidation products of these amino acids are listed in Table 2.1.

Table 2.1 Amino acid oxidation products (Davies *et al.*, 1999; Stadtman & Berlett, 1997).

Amino acid	Oxidation product
Arginine	Glutamic semialdehyde*, 5-hydroxy-2-amino-valeric acid
Cysteine	Disulfide cross-links, oxy acids
Histidine	Aspartate, asparagine, 2-oxo-histidine*
Lysine	3-, 4-, or 5-Hydroxylysine, 2-amino-adipylsemialdehyde*
Methionine	Methionine sulfoxide/sulfone
Phenylalanine	<i>o</i> -, <i>m</i> -Tyrosine
Proline	3- or 4-Hydroxyproline, 5-hydroxy-2-amino-valeric acid, glutamic semialdehyde*
Tryptophan	2-, 4-, 5-, 6-, or 7-Hydroxytryptophan, kynurenine, N-formylkynurenine, 3-hydroxykynurenine
Tyrosine	Dityrosine, 3,4-dihydroxyphenylalanine (DOPA) 3-chlorotyrosine, 3,5-dichlorotyrosine 3-nitrotyrosine, 3,5-dinitrotyrosine

* carbonyl compounds.

Additionally, many of the amino acid side chains are susceptible to hydroperoxide formation but as these are unstable products and not easily identified they have not been included in Table 2.1 (Davies *et al.*, 1999).

Formation of carbonyl compounds on amino acid side chains is a well-described result of metal-catalyzed oxidation of proteins (marked by * in Table 2.1), and is shown in Figure 2.2 (Levine, 1984; Oliver *et al.*, 1987; Stadtman & Oliver, 1991). The content of protein carbonyl groups is used as a general measure for protein oxidation in biological samples. However, formation of protein carbonyl groups also occurs through non-oxidative pathways such as reactions with proteins and aldehydes or reducing sugars causing overestimation of the oxidation level of the sample (Adams *et al.*, 2001; Xiong, 2000). In contrast, carbonyl groups are not formed during oxidation of all amino acid side chains (Davies *et al.*, 1999), which may cause underestimation of the oxidative status of the sample.

Detection and quantification of carbonyl content in proteins is most often done by derivatization with 2,4-dinitrophenylhydrazine (DNPH) and this analytical method is discussed in section 5.1. In unoxidized biological samples the carbonyl content is 1-2 nmol/mg protein, which has been estimated to represent modification of about 10% of the total cellular protein (Starke-Reed & Oliver, 1989). The carbonyl content in oxidized meat samples varies dependent on nature of the sample and how oxidation is initiated. An overview of the reported results from chill storage experiments available in the literature is given in Table 2.2.

Table 2.2 Maximum carbonyl content obtained in chill storage experiments with meat of different species. HI-OX; high-oxygen atmosphere, LD; *longissimus dorsi*, LTL; *longissimus thoracis et lumborum*, LL; *longissimus lumborum*, DP, *diaphragma pedialis*, P, *pectoralis*; S, *sartorius*.

Meat	Experimental and storage conditions	Storage time	Max. carbonyl content (nmol/mg protein)	Reference
Beef LD	Irradiation	15 days	14	(Rowe <i>et al.</i> , 2004b)
Beef LL	Not given	10 days	5.1	(Martinaud <i>et al.</i> , 1997)
Beef DP	Not given	10 days	6.9	(Martinaud <i>et al.</i> , 1997)
Beef patties	HI-OX	6 days	1.9	Paper I
Pork LD	HI-OX	14 days	1.1	Paper II
Pork patties	HI-OX	7 days	0.95	Paper III
Pork patties	Cooking	9 days	< 2.0	(Vuorela <i>et al.</i> , 2005)
Liver pâté	Not given	90 days	22.5	(Estevez & Cava, 2004)
Lamb LTL	Not given	8 days	1.42	(Petron <i>et al.</i> , 2007)
Turkey P	Atmospheric air	9 days	3.41	(Mercier <i>et al.</i> , 1998)
Turkey S	Atmospheric air	9 days	3.15	(Mercier <i>et al.</i> , 1998)
Chicken	Irradiation	12 days	2.70 μ mol/10g meat	(Rababah <i>et al.</i> , 2004)

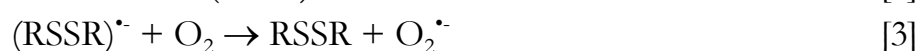
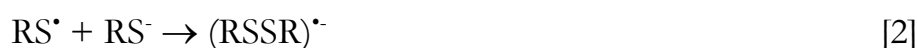
Irradiation of beef *longissimus dorsi* (LD) and storage for 15 days has shown to cause formation of up to 14 nmol carbonyls/mg protein (Rowe *et al.*, 2004b), which is the highest reported value obtained in fresh meat. In contrast, high-oxygen atmosphere storage of pork LD for 14 days only resulted in a maximum carbonyl content of 1.1 nmol/mg protein (paper II). Chill storage of beef *longissimus lumborum* (LL) and *diaphragma pedialis* (DP) increased the carbonyl content from 3.1 to 5.1 nmol/mg protein and from 4.8 to 6.9 nmol/mg protein, respectively, during storage for 10 days (Martinaud *et al.*, 1997) (no information was given about the storage atmosphere). The relatively high carbonyl content in this storage experiment indicates that the beef was already oxidized to some extent before chill storage as also stated by the authors. Minced meat might be more vulnerable to high-oxygen atmospheres either due to disruption of the normal structure of the cells and hereby release of prooxidants present in meat, or to the incorporation of more oxygen in the tissues. However, the carbonyl content of minced beef was found to be < 2 nmol/mg protein after 6 days of storage in a high-oxygen atmosphere (paper I). The content of protein carbonyls seems to depend on the level of antioxidants and heme pigment in the meat to a greater extent than mincing of the meat (Petron *et al.*, 2007)(paper III), which will be discussed in section 4.2. In porcine liver pâté the carbonyl content is high (22.5 nmol/mg protein) after 90 days of storage, but a dramatic increase in the carbonyl content was observed within the last 30 days of storage as the carbonyl content was only about 6.5 nmol/mg after 60 days of storage (Estevez & Cava, 2004). Oxidation of muscle proteins in model systems by metal-catalyzed initiation has been found to increase the protein carbonyl content to values above 30 nmol/mg protein dependent on

reaction conditions (Decker *et al.*, 1993; Liu *et al.*, 2000; Liu & Xiong, 2000b; Martinaud *et al.*, 1997; Morzel *et al.*, 2006; Park *et al.*, 2006b; Park *et al.*, 2006a; Srinivasan & Hultin, 1997).

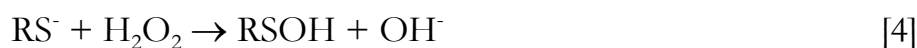
The thiol group of cysteine (RSH) is highly susceptible to oxidation. The oxidation of thiol groups leads to a series of complex reactions resulting in formation of various oxidized products such as sulfenic acid (RSOH), sulfinic acid (RSOOH), and disulfide cross-links (RSSR). The total content of thiol groups in meat does not seem to be affected by *post-mortem* aging (Hay *et al.*, 1972; Hay *et al.*, 1973; Hofmann & Reiner, 1978) suggesting that the content of thiol groups is a good marker of oxidation. However, underestimation of the thiol content is possible as availability of the thiol groups to the thiol-selective reagents is dependent on denaturation of the protein (can be overcome by addition of SDS or urea to the sample), while overestimation may be caused by the presence of other thiol-containing molecules in meat like aroma compounds (although the aroma compounds are not normally present in raw meat but mostly formed during heating) (Hofmann & Reiner, 1978; Kato *et al.*, 1973; Mottram, 1994).

Determination of loss of thiol groups in proteins from biological samples is often based on Ellman's reagent (Ellman, 1959) (discussed in section 5.2). However, a certain amount of animals should be included in the experiment in order to obtain statistical significance of the measured effects. Only a 6% loss of thiol groups were found in LD slices stored in high-oxygen atmospheres from day 1 to day 14 *post-mortem* (paper II), while worst-case scenario a 37% loss of thiol groups in minced pork stored in high-oxygen atmospheres for 7 days was observed (paper III). In the study by Petron *et al.* (2007), a maximum of 23% loss of thiol groups from day 4 to day 8 *post-mortem* in lamb meat was found, while the study by Martinaud *et al.* (1997) showed a 10-17% loss in beef from day 1 to day 10 *post-mortem* dependent on muscle type. In model systems with oxidation of myosin by 100 μM MbFe(III)/H₂O₂ for 2 min a 92% loss of thiol groups in myosin was found (paper IV). Oxidation of muscle proteins by metal-catalyzed initiation for 2 hours caused a 29% loss of thiol groups (Srinivasan & Hultin, 1997), and greater losses in thiol content has been found in other studies under different conditions (Liu *et al.*, 2000; Liu & Xiong, 2000b). However, as model systems are designed to study the effect of oxidation and the concentration of oxidants varied to obtain maximum effect, the extent of oxidation should not be directly compared to real meat products.

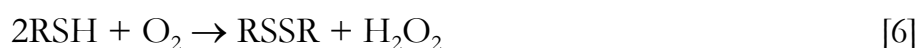
Intra- and intermolecular cross-linking of proteins may also occur during oxidation, which causes formation of a variety of cross-linked oxidation products and subsequently polymerization of the proteins. Formation of disulfide cross-links has been observed in meat model systems, e.g. (Decker *et al.*, 1993)(paper IV, V) and in fresh meat (paper II), and can be formed by oxidation of cysteine residues through various reactions, some of which are mentioned below. Reaction between a thiyl radical (RS[•]) and a thiolate ion (RS⁻) causes formation of the intermediate disulfide radical ((RSSR)^{•-}), which results in the formation of a disulfide (RSSR) in the presence of oxygen (reaction 2-3) (Davies & Dean, 2003; Garrison, 1987).



Formation of disulfide cross-links also occurs as a result of direct reaction between a thiolate ion and hydrogen peroxide via formation of sulfenic acid (reaction 4-5) (Garrison, 1987).



Cysteines can also react directly with oxygen to form disulfide cross-links (reaction 6) (Garrison, 1987):



Another cross-linked compound, which has been observed in meat model systems, e.g. (Bhoite-Solomon *et al.*, 1992)(paper IV), is the formation of dityrosine by oxidation of tyrosine through the formation of a tyrosyl phenoxyl radical (Figure 2.4) (Davies & Dean, 2003). Transfer of oxidative damage from cysteine, tryptophan, and methionine to tyrosine is well characterized (Prutz *et al.*, 1986), and it has therefore been suggested that tyrosine residues act as the ultimate “sink” for oxidizing equivalents in proteins (Davies & Dean, 2003).

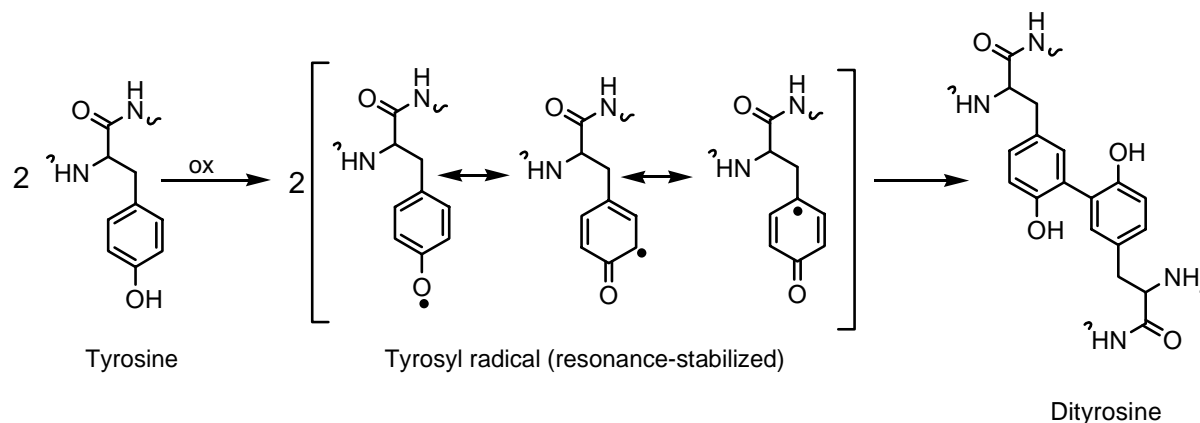


Figure 2.4 Dityrosine formation through the mediation of resonance-stabilized tyrosyl phenoxyl radicals.

The tyrosyl radical is resonance-stabilized due to its aromatic nature, which may cause the formation of long-lived tyrosyl radicals in some proteins, e.g. bovine serum albumin (BSA) (Ostdal *et al.*, 1997) and myosin (paper IV).

Cross-linking can also occur by the reaction between a carbonyl group and an ϵ -amino group of lysine (Buttkus, 1967), by the cross-linking of two ϵ -amino groups through a dialdehyde (eg. malondialdehyde or dehydroascorbate), and by the condensation reaction of protein radicals (Xiong, 2000). The formation of mainly disulfide and dityrosine cross-linking has been investigated. The disulfide bonds are reducible, while the other cross-linked compounds are non-reducible. Thiol determination in combination with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-page) under reducing and non-reducing conditions (discussed in section 5.4) makes it possible to determine oxidation of thiol groups and formation of disulfide cross-linking in meat (Paper II). Dityrosine formation can be determined by hydrolysis followed by HPLC analysis using fluorescent detection (Daneshvar *et al.*, 1997).

Fragmentation of proteins due to oxidation is complicated to measure, and has in most cases only been done qualitatively (Dean *et al.*, 1997). Fragmentation will often occur through the formation of a radical on the α -carbon of the peptide backbone, but the yield of such backbone-derived radicals decreases markedly when there are side chains present which can form stabilised radicals (as tyrosine, tryptophan and histidine) or when steric factors play a role (Hawkins & Davies, 2001). Quantification of protein fragmentation by SDS-page has also been discussed in section 5.4.

2.3 Oxidation of Myosin

This section will include a brief introduction to muscle composition including an overview of the proteins present in meat, and subsequently a detailed description of the oxidation of myosin.

2.3.1 Introduction to Muscle Composition

Meat consists of mainly water (75%) and proteins (19%) and to a lesser extent of lipids (2.5%), carbohydrates (1.2%), and other non-protein substances as inorganic compounds, vitamins and amino acids (2.3%). The muscle structure is extremely complex and for detailed information about this issue several excellent books can be studied as Pearson & Young (1989) or Lawrie & Ledward (2006). Briefly, skeletal muscle consists of bundles of muscle fibres (muscle cells) and associated connective tissue (epimysium, perimysium and endomysium). The muscle is surrounded by the epimysium and the muscle fibre bundles by the perimysium. The muscle fibres consist of bundles of myofibrils surrounded by a plasma membrane (the sarcolemma) and the endomysium. A schematic structure of a muscle fibre is shown in Figure 2.5A.

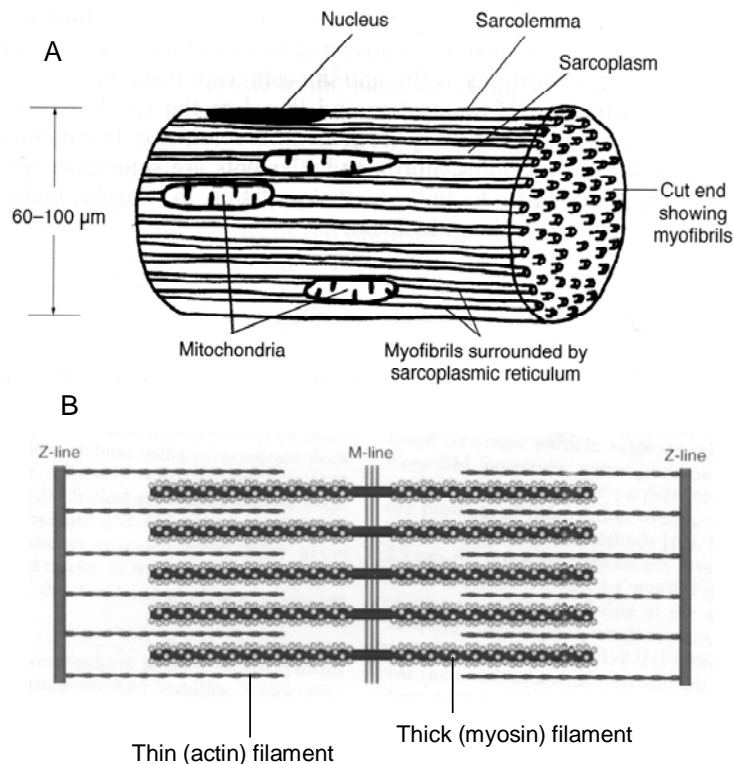


Figure 2.5 Schematic structure of A) a skeletal muscle fibre (muscle cell) (Warriss, 2000), and B) a sarcomere (Craig & Woodhead, 2006).

The myofibrils can be divided into thin and thick filaments consisting of actin and myosin, respectively. The functional contractile unit of a skeletal muscle myofibril, called a sarcomere, repeats every 2.3 μm (at rest) along the fibril axis and is bound between two Z discs (Lawrie & Ledward, 2006) (Figure 2.5B).

Meat proteins are divided into three groups: sarcoplasmic proteins, myofibrillar proteins, and connective tissue. The most abundant meat proteins are listed in Table 2.3.

Table 2.3 Protein composition of typical adult mammalian muscle after rigor mortis but before degradative changes *post-mortem*, adapted from Lawrie & Ledward (2006).

Protein	% wet weight
Myofibrillar (salt-soluble proteins)	11.5
Myosin	5.5
Actin	2.5
Titin	0.9
Nebulin	0.3
Tropomyosins	0.6
Troponins	0.6
Actinins	0.5
Myomesin and C-proteins	0.2
Desmin, filamin, F- and I-proteins, vinculin, talin	0.4
Sarcoplasmic (water-soluble proteins)	5.5
Soluble enzymes	4.5
Myoglobin	0.2
Hemoglobin and other unspecified extracellular proteins	0.6
Connective tissue (insoluble proteins)	2.0
Collagen	1.0
Elastin	0.05
Other insoluble proteins	0.95

In the present study myofibrillar and sarcoplasmic proteins have been in focus, and oxidation of connective tissue will thus not be commented.

Myosin, which is the most abundant protein in meat, is highly susceptible to oxidation as shown in several studies, e.g (Decker *et al.*, 1993; Liu *et al.*, 2000; Ooizumi & Xiong, 2004), and from preliminary experiments of metal-catalyzed oxidation of muscle homogenates performed in the present PhD study. Furthermore, myosin heavy chain was found to form intermolecular disulfide cross-links in pork LD stored in high-oxygen atmospheres (paper II). Hence, the oxidation of myosin was studied in more detail.

2.3.2 The Myosin Molecule

Myosin is a contractile protein located in the thick filaments in the myofibrillar part of the muscle (see Figure 2.5). The myosin molecule (~520 kDa) consists of two

myosin heavy chains (MHC) (each ~220 kDa) and two pairs of myosin light chains (MLC) (ranging from 17 to 22 kDa), and is schematically depicted in Figure 2.6.

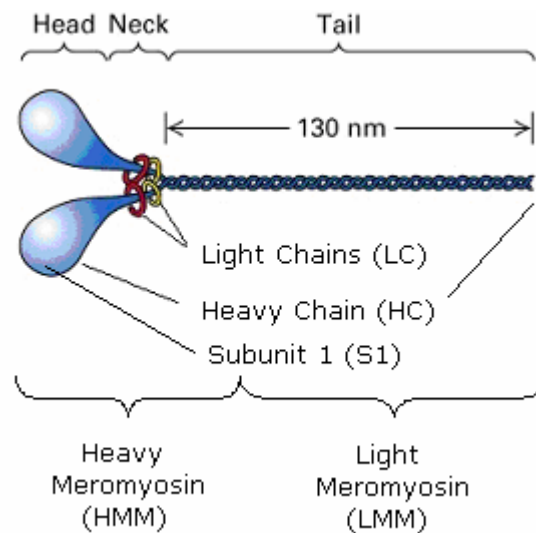


Figure 2.6 Schematic figure of the rod-shaped myosin molecule consisting of two myosin heavy chains and four associated myosin light chains.

The tail (or rod) region forms the backbone of the thick filament and the globular head region extends from the thick filament and interacts with actin in the thin filament. The globular head region also exhibits adenosine triphosphatase (ATPase) activity; it can hydrolyze ATP and liberate energy for muscle contraction (Engelhardt & Ljubimowa, 1939; Gergely, 1953; Moss *et al.*, 1995). Numerous studies show that myosin is highly susceptible to oxidation by metal-catalyzed oxidation and H₂O₂-activated myoglobin, e.g. (Bhoite-Solomon *et al.*, 1992; Decker *et al.*, 1993). The light meromyosin (LMM) part of myosin has been found to be most susceptible to oxidation and subsequent formation of disulfide cross-links (Ooizumi & Xiong, 2006). However, as the ATPase activity of myosin has proven to be strongly affected by oxidation (Ooizumi & Xiong, 2004; Park *et al.*, 2006b; Park *et al.*, 2006a), the head region is most likely also susceptible to oxidation.

Mammalian muscle contains nine MHC isoforms of which four of the isoforms (1, 2A, 2X, 2B) are predominant in adult skeletal muscle (Talmadge, 2000). The distribution of the isoforms in muscle tissue is highly dependent on species, breed, and muscle type (Chang *et al.*, 2003; Talmadge, 2000). Generally, the same isoform type is found singularly in the same fiber type, but hybrid fibres containing multiple isoforms also exist (Pette & Staron, 2000; Talmadge, 2000). Generally, porcine muscle contains more than 50% of the MHC 2B isoform

(Chang *et al.*, 2003), while the presence of MHC 2B in bovine muscle has been questioned (Chikuni *et al.*, 2004). Myosin was purified from pork LD in the present PhD study, and therefore the amino acid distribution of MHC 2B is given in Table 2.4. The variation in amino acid distribution between MHC isoforms is relatively low.

Table 2.4 The amino acid distribution of porcine myosin heavy chain (MHC) 2B from skeletal muscle (Expasy Proteomics Server, 2007).

Amino acid	Amount in total MHC	Percentage (%) of total	Amount in head-like MHC	Amount in tail-like MHC
Ala	166	8.6	56	110
Arg*	104	5.4	28	76
Asn	79	4.1	35	44
Asp	95	4.9	33	62
Cys	16	0.8	8	8
Gln	131	6.8	38	93
Glu	266	13.7	67	199
Gly	71	3.7	50	21
His*	39	2.0	17	22
Ile	93	4.8	46	47
Leu	191	9.9	62	129
Lys*	209	10.8	72	137
Met*	55	2.8	22	33
Phe*	60	3.1	47	13
Pro*	32	1.7	30	2
Ser	96	5.0	42	54
Thr	99	5.1	50	49
Trp	9	0.5	5	4
Tyr	38	2.0	30	8
Val	88	4.5	44	44
Total	1937	100	782	1155

* The amino acids most susceptible to carbonyl formation.

The carbonyl content of myosin increases dramatically when oxidation is induced (Liu *et al.*, 2000; Liu & Xiong, 2000b), which is consistent with the presence of a high amount of amino acids susceptible to carbonyl formation (marked by * in Table 2.4). Additionally, myosin heavy chain contains 16 free cysteines evenly distributed between the head and tail part of the molecule, and a relatively high amount of tyrosine residues which is predominantly located in the MHC head (high-lighted in Table 2.4). The Cys-707 and Cys-697 are located in the catalytic domain of the myosin head, and modification of both of these cysteines with thiol-selective reagents or by oxidation has been shown to inhibit ATPase activity (Crowder & Cooke, 1984; Kielley & Bradley, 1956; Lowe *et al.*, 2001; Ooizumi & Xiong, 2004; Park *et al.*, 2006b; Park *et al.*, 2006a). The amount of cysteine and tyrosine residues in the MLCs is relatively small (1-2 cysteines and 1-2 tyrosines for

each MLC). Since the size of MLC compared to MHC is very small, the amino acid content of MLC will not contribute significantly to the overall amino acid distribution of myosin. A total amount of 42 cysteines in the complete myosin molecule has been reported (Crowder & Cooke, 1984; Kominz *et al.*, 1954; Lowey & Cohen, 1962). The cysteine and tyrosine residues are responsible for the formation of intermolecular disulfide and dityrosine cross-links upon oxidation with various oxidation systems (reviewed in Table 2.5).

Table 2.5 Type of cross-links formed in myosin during oxidation with various oxidizing systems.

Oxidizing system	Protein fraction	Type of cross-linking	Reference
Fe ³⁺ /ascorbate/H ₂ O ₂	Myofibrillar proteins	Disulfide ^a	(Ooizumi & Xiong, 2004), (Srinivasan & Hultin, 1997), (Liu <i>et al.</i> , 2000), (Park <i>et al.</i> , 2006b)
Fe ³⁺ /ascorbate/H ₂ O ₂	Purified myosin	Disulfide ^a	(Liu & Xiong, 2000b), (Liu & Xiong, 2000a)
Fe ³⁺ /H ₂ O ₂	Myofibrillar proteins	Disulfide/Non-disulfide ^a	(Park <i>et al.</i> , 2006a)
Fe ³⁺ /ascorbate Cu ²⁺ /ascorbate	Myofibrillar proteins	Disulfide/Non-disulfide ^a	(Decker <i>et al.</i> , 1993)
Fe ²⁺ /DETAPAC/H ₂ O ₂	Myofibrillar proteins	Dityrosine ^{a,b}	(Morzel <i>et al.</i> , 2006)*
MbFe(III) Linoleic acid/lipoxidase	Myofibrillar proteins	Disulfide ^a	(Park <i>et al.</i> , 2006b)
MbFe(III) H ₂ O ₂	Purified myosin	Disulfide ^a	(Bhoite-Solomon <i>et al.</i> , 1992)
Hb/H ₂ O ₂	Myofibrillar proteins	Dityrosine ^c	(Bertram <i>et al.</i> , 2007)
MbFe(III)/H ₂ O ₂	Myofibrillar proteins	Non-disulfide ^a	(KaminBelsky <i>et al.</i> , 1998)
MbFe(III)/H ₂ O ₂	Purified myosin	Non-disulfide ^a	(Bhoite-Solomon <i>et al.</i> , 1992)
HRP/H ₂ O ₂	Purified myosin	Dityrosine ^{a,b}	(Hanan & Shaklai, 1995a)*
MbFe(III)/H ₂ O ₂ MbFe(II)O ₂ / H ₂ O ₂ MbFe(II)/ H ₂ O ₂	Purified myosin	Dityrosine ^{a,b}	(Hanan & Shaklai, 1995b)*
MbFe(III)/H ₂ O ₂ HRP/H ₂ O ₂	Purified myosin	Disulfide/Non-disulfide ^a Dityrosine ^c	Paper IV

Investigated by a) SDS-page, b) fluorescence spectroscopy, c) HPLC. * Disulfide formation not investigated.

From the results listed in Table 2.5 it seems that oxidation of myosin irrespective of oxidizing system results in formation of both disulfide and non-disulfide cross-links in myosin, even though some studies show only formation of disulfide cross-links upon metal-catalyzed oxidation (Liu & Xiong, 2000a; Liu & Xiong, 2000b; Srinivasan & Hultin, 1997), while in other studies the potential formation of both types of cross-links has not been investigated. It should be noted that detection of dityrosine based on direct fluorescence measurements (marked by a 'b' in Table 2.5) is questionable as other oxidation products also exhibits fluorescence emission in the same wavelength region (Guptasarma *et al.*, 1992). Proof of dityrosine formation can therefore only be done by e.g. hydrolysis of the protein and subsequent HPLC separation using fluorescence detection (marked by a 'c' in Table 2.5) (Daneshvar *et al.*, 1997).

2.3.3 Reaction Mechanism of the Oxidation of Myosin

The mechanisms by which the cross-linking reactions occur, and the nature of the reactive intermediates involved, have been examined in detail by using EPR spectroscopy (described in section 5.3), amino acid consumption and product analysis by high-performance liquid chromatography (HPLC) and SDS-page (paper IV). Oxidation of myosin with H₂O₂-activated myoglobin resulted in the direct detection of myosin-derived radicals with extremely long lifetimes (detectable for up to five hours after initiation of oxidation) (Figure 2.7A). This is a remarkable observation as radicals are generally very reactive and therefore not easily detected directly at room temperature. However, BSA and β -lactoglobulin have also been reported to form long-lived radicals by oxidation with H₂O₂-activated myoglobin (Ostidal *et al.*, 1997). The most likely nature of a long-lived protein radical is tyrosine, tryptophan or histidine-derived due to the possible formation of resonance-stabilized radicals in the aromatic side chains of these amino acids (Agon *et al.*, 2006; Davies, 1990; Gunther *et al.*, 1995; Ostidal *et al.*, 1999). Even so, blocking of thiol groups in myosin using N-ethylmaleimide (NEM) resulted in a loss of radical intensity suggesting that thiol groups in myosin is involved in the reaction mechanism of myosin oxidation (Figure 2.7B).

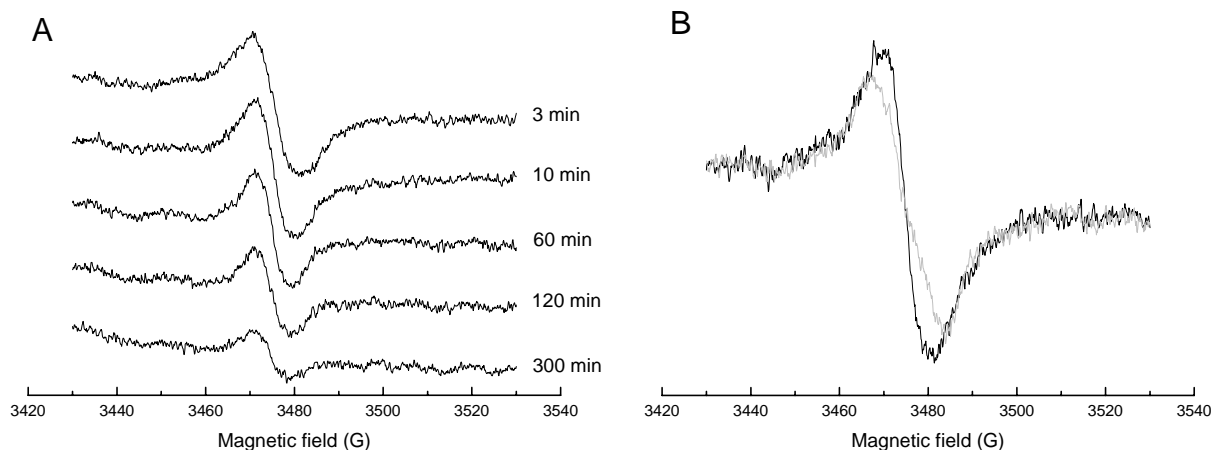


Figure 2.7 A) Myosin radical directly detected at room temperature by EPR spectroscopy formed by oxidation of $13 \mu\text{M}$ myosin with $300 \mu\text{M}$ MbFe(III)/ H_2O_2 . B) Myosin radical (native, black spectrum; thiol-groups in myosin blocked by NEM, grey spectrum) detected 2 min after initiation of oxidation (paper IV).

Amino acid consumption and product analysis of oxidized myosin showed loss of cysteine and tyrosine in myosin and formation of intermolecular disulfide and dityrosine cross-links in myosin. These observations resulted in a proposed reaction mechanism for the oxidation of myosin by activated heme proteins (Figure 2.8).

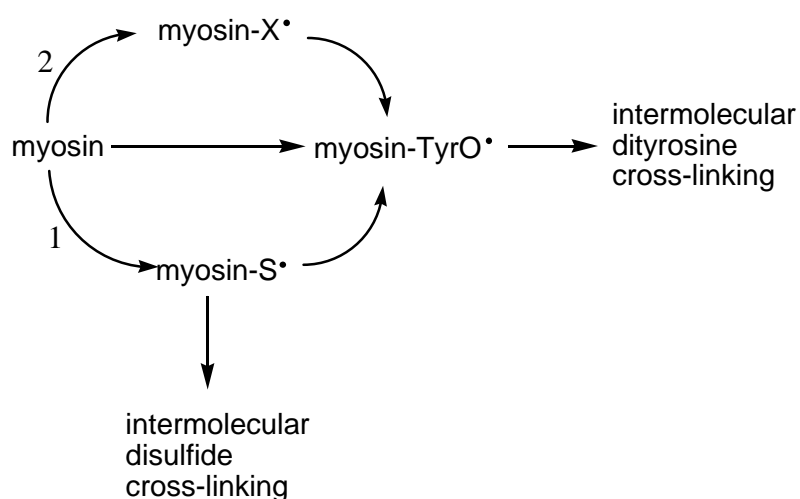
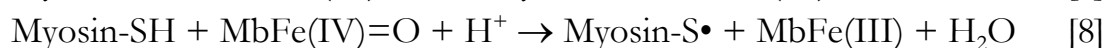
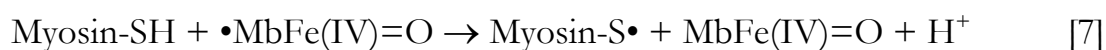


Figure 2.8 A proposed reaction mechanism for the oxidation of myosin by H_2O_2 -activated heme proteins at physiological pH (paper IV).

The reaction mechanism includes a direct formation of a long-lived tyrosyl phenoxyl radical on myosin (myosin-TyrO•) including two other reaction pathways

(1 and 2) for the formation of this long-lived myosin radical. One pathway (1) involves the direct oxidation of thiol groups to give thiyl radicals (myosin-S[•]), which results in formation of intermolecular disulfide cross-linking, but also includes formation of the long-lived tyrosyl radical on myosin and dityrosine cross-linking. A second pathway (2) was suggested to involve the mediation of other short-lived radicals (myosin-X[•]). These radical species were not identified but could be another population of transient tyrosine-derived phenoxyl radicals or possibly tryptophan-derived species. The first pathway (1) seems to be the most likely to occur in meat during storage in high-oxygen atmospheres as only reducible (disulfide) cross-links and not non-reducible (as dityrosine) cross-links were formed after 14 days of storage (paper II).

Oxidation of thiol groups in myosin by MbFe(III)/H₂O₂ to form disulfide cross-linking was found to be an extremely fast reaction, which is consistent with the formed myosin-derived thiyl radicals being highly reactive and short-lived. The rate constant for reaction between myosin thiol groups and H₂O₂-activated myoglobin was not possible to determine in the present study as oxidation caused alterations in the protein composition (tertiary structure) and hereby changed the susceptibility of thiol groups in myosin towards the thiol-selective reagent (DTNP) (paper IV). Reactivity of the hypervalent myoglobin species (•MbFe(IV)=O and MbFe(IV)=O) towards myosin was investigated in more detail (paper V), and may be summarized in two separate reactions:



Reaction 7 describes the fast reaction between perferrylmyoglobin and myosin, which was found to be concentration dependent in studies using low temperature EPR spectroscopy (paper IV). Ferrylmyoglobin may be reduced by myosin if myosin is present in excess concentrations relative to perferrylmyoglobin (reaction 8), showing that both hypervalent myoglobin species were able to oxidize myosin (paper V).

2.3.4 pH-dependency of the Oxidation of Myosin

The study on oxidation of myosin was initially performed at physiological pH and at high ionic strength (I = 0.5-1.0) to keep myosin into solution. In meat, ionic strength is I = 0.16 and *post-mortem* pH decreases to ~5.6 (Koohmaraie *et al.*, 1987).

Decreasing pH from 7.0 to 5.5 of beef myofibrillar proteins and subsequent storage at 0°C has been shown to increase protein oxidation measured by protein carbonyl content (Srinivasan *et al.*, 1996). Therefore, studies on the oxidation of myosin were attempted at lower pH values and ionic strengths (paper V).

Myosin solubility is dependent on ionic strength and pH, which is shown in Figure 2.9A. The solubility phase diagram is given for light meromyosin (LMM), which confers the solubility and aggregation properties to the myosin heavy chain (Tsunashima & Akutagawa, 2006; Wick, 1999). Myosin is mostly soluble in high concentrations of salt. Reducing the ionic strength of a myosin solution causes myosin to assemble first into dimers, then minifilaments and finally to synthetic filaments, which are insoluble (Figure 2.9B) (Wick, 1999).

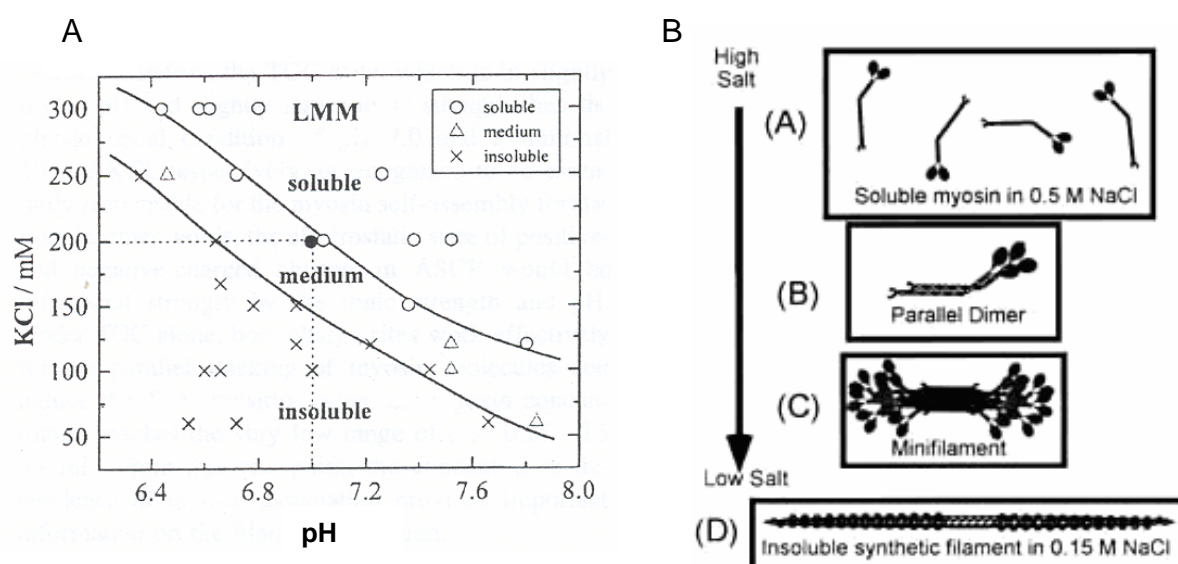


Figure 2.9 A) Solubility phase diagram of light meromyosin (LMM) in solution (Tsunashima & Akutagawa, 2006). B) A putative mechanism of myosin assembly. Myosin monomers in high salt concentrations (A), spontaneously assemble into parallel dimers (B), minifilaments (C), and synthetic filaments (D) as the ionic strength of the solution is reduced (Wick, 1999)

Reduction of pH during oxidation of myosin to values relevant for meat systems caused the protein to precipitate even though the ionic strength was kept high ($I > 0.50$) (pI of myosin heavy chain is 5.4), which is consistent with other reported studies (Stefansson & Hultin, 1994). SDS was added to all reaction solutions to prevent precipitation during spectrophotometric measurements, although this treatment also resulted in unfolding of the protein. Presence of SDS in a model

system does not prevent oxidation of proteins but might decrease the rate of oxidation slightly (Dean *et al.*, 1991).

No obvious pH dependency of oxidative cross-linking (both reducible and non-reducible) was observed in the pH interval from 5.0 to 7.8 evaluated by SDS-page analysis (paper V). Studying the loss of thiol groups in myosin due to oxidation with H₂O₂-activated myoglobin (in the presence of SDS) showed a slight pH dependency. The loss of thiol groups in myosin was found to be faster at the higher pH values (pH 6.4-7.8) compared to the lower pH values (pH 5.0-5.6). Correspondingly, formation of myosin spin adducts (suggested to be thiyl-derived myosin radicals) increased slightly with increasing pH (in the absence of SDS) (paper V). The increase in oxidation of myosin at higher pH values is consistent with the thiolate ion (RS⁻) being more reactive than the protonated thiol group (RSH) (Barton *et al.*, 1973). Conversion of myosin thiols into the deprotonated form (RS⁻) is more pronounced at physiological pH in comparison to pH representative for meat systems, as pK_a of the thiol group of cysteine is 8.37, even though this value may be higher or lower dependent on the environment of the cysteine group in the protein (Stryer, 1997). However, the pH dependency of oxidation of myosin by H₂O₂-activated myoglobin was found to be relatively small compared to the fast reaction between perferrylmyoglobin and myosin. This is consistent with the observation reported by Kröger-Ohlsen *et al.* (2003) showing that the reactivity of perferrylmyoglobin towards other proteins has been found to be less pH dependent compared to the reactivity of ferrylmyoglobin.

Intermolecular disulfide cross-linking of myosin heavy chain was found in pork LD (pH in the meat was 5.5 - 5.6) after storage for 8 days in high-oxygen atmospheres (paper II). Interestingly, dityrosine formation in isolated muscle myofibrils has been shown to increase considerably when pH is lowered from 7.0 to 5.4 (Bertram *et al.*, 2007), but no non-reducible cross-links (as dityrosine) was found in the pork LD stored in high-oxygen atmosphere for up to 14 days (paper II) indicating that comparison of studies between fresh meat and isolated muscle myofibrils should be done with care.

In summary, myosin is found to be highly susceptible to oxidation by various oxidation systems, which causes formation of several radical species in myosin and formation of both disulfide and dityrosine cross-links. The reaction between myosin and perferrylmyoglobin is fast and exhibits relatively small pH dependency. Hence, choice of pH of the model system to simulate conditions of meat was suggested to be of minor importance for the oxidative sensitivity of myosin. The

formation of disulfides in myosin through intermolecular cross-linking reactions has been observed in pork LD, and the significance of this reaction and protein oxidation in general in relation to meat texture is described in the following chapter.

3 Effects of Protein Oxidation on Meat Texture

Oxidation has a major impact on quality deterioration during processing and storage of muscle foods (Kanner, 1994; Skibsted *et al.*, 1998). The implications for muscle food quality by protein oxidation has been described in a review by Xiong (2000). Focus has increased on this research area since 2000 and the most recent findings are discussed in this chapter in relation to meat texture.

3.1 Meat Tenderness

Improvement of tenderness in meat occurs mainly during *post-mortem* storage with the rate of tenderization depending on species, gene, age, etc. (Dransfield *et al.*, 1981). A time course of meat tenderization is shown in Figure 3.1, evaluated by Warner-Bratzler shear force (WBSF), which is a mechanical and objective determination often used to evaluate meat tenderness (Bratzler, 1932). WBSF has been shown to give reasonable correlations (up to $R = -0.83$) with sensory assessed tenderness (Safari *et al.*, 2001; Sivertsen *et al.*, 2002).

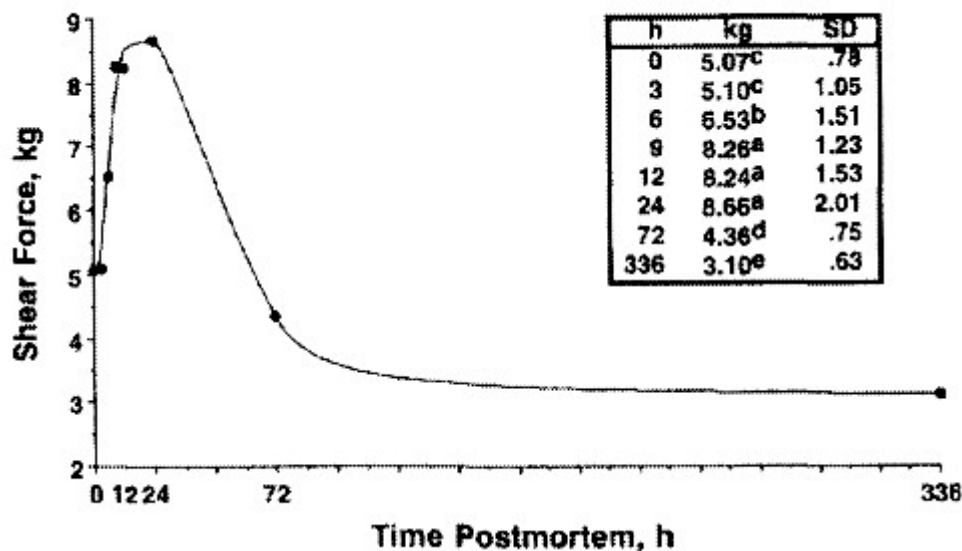


Figure 3.1 Tenderness of lamb *longissimus* measured by Warner-Bratzler shear force of at various *post-mortem* times (Wheeler & Koohmaraie, 1994).

Shear force increases (the meat becomes more tough) within the first ~24 hours *post-mortem* due to muscle contraction (development of rigor). The time of rigor is very dependent on species, muscle energy level at slaughter, muscle type, and temperature during the initial chilling period of the carcass (Dransfield *et al.*, 1981;

Hopkins & Thompson, 2002a; Ouali, 1990; Ouali, 1992). After rigor (when the curve drops again) tenderness increases, which is mainly believed to be caused by *post-mortem* proteolysis of key myofibrillar proteins (Koohmaraie, 1992; Ouali, 1992). A number of studies have shown that titin, nebulin, filamin, desmin, vinculin, troponin-T, myosin heavy chain, and actin are degraded during *post-mortem* storage. The degradation of most of these proteins has been associated with the *post-mortem* tenderization process, but their direct significance in relation to tenderization is still unclear (Hopkins & Thompson, 2002a; Hopkins & Thompson, 2002b; Huff-Lonergan *et al.*, 1995; Huff-Lonergan *et al.*, 1996; Koohmaraie & Geesink, 2006; Lametsch *et al.*, 2002; Lametsch *et al.*, 2003; Taylor *et al.*, 1995).

Mainly two proteolytic systems have been implicated in *post-mortem* tenderization; calpains (μ - and m-calpain) and cathepsins (cathepsins B, D, H, and L) (Koohmaraie, 1992; Ouali, 1992). However, it has recently been suggested that other proteases such as caspases and the proteasome 20S might also influence tenderization in meat (Dutaud *et al.*, 2006; Kemp *et al.*, 2006), and that tenderization may be a multienzymatic process implying some or all of the enzymatic systems present in meat (Ouali *et al.*, 2006). Hence, the detailed mechanisms responsible for meat tenderization are not fully understood.

The calpain system is by many considered to be responsible for most of the *post-mortem* tenderization of meat (Hopkins & Thompson, 2002a; Huff-Lonergan *et al.*, 1996; Koohmaraie, 1992; Koohmaraie & Geesink, 2006). In skeletal muscle, the calpain system includes at least three proteases, μ -calpain, m-calpain and skeletal muscle-specific calpain, p94, and an inhibitor of μ - and m-calpain, calpastatin. The role of μ -calpain in meat tenderization is well-established, while the importance of m-calpain is highly debated and the role of the p94 calpain has been ascribed a negligible role in *post-mortem* proteolysis of muscle proteins (Geesink *et al.*, 2005; Hopkins & Thompson, 2002a; Koohmaraie, 1992; Koohmaraie & Geesink, 2006). μ -Calpain and m-calpain are calcium dependent cysteine proteases. Because of the presence of a cysteine residue at the active site, reducing conditions are required for the activity of the calpain. Due to the importance of μ -calpain in meat tenderization focus will be on this protease in the present PhD thesis although the other proteolytic enzymes may also be susceptible to oxidation and thus play a role during *post-mortem* tenderization in oxidative environments.

μ -Calpain is a heterodimer composed of two distinct subunits, a 80 kDa catalytic subunit and a 28 kDa regulatory subunit divided into four (I-IV) and two

(V-VI) domains, respectively (Goll *et al.*, 2003). The schematic structure of μ -calpain is shown in Figure 3.2.

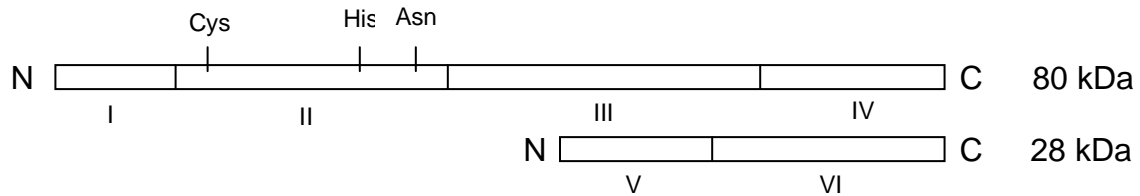


Figure 3.2 Schematic diagram showing the domain structure of μ -calpain, adapted from Goll *et al.* (2003).

The 80 kDa subunit is responsible for protease activity, and domain II contains the active site consisting of a cysteine, a histidine, and an asparagine residue. The activity of μ -calpain is influenced by several factors including calcium concentration, temperature, pH, ionic strength, oxidative environment, and the inhibitor, calpastatin (Carlin *et al.*, 2006; Goll *et al.*, 2003; Guttman *et al.*, 1997; Guttman & Johnson, 1998; Koohmaraie, 1992; Maddock *et al.*, 2005).

An important characteristic of μ -calpain is that it undergoes autolysis in the presence of calcium resulting in the initial conversion of i) the 80 kDa subunit to a 76 kDa form through a 78 kDa intermediate, and ii) the 28 kDa subunit to a 18 kDa form through several intermediates (Cong *et al.*, 1989). The autolytic fragmentation occurs from the N-terminal in domain I and V. This initial autolysis of the calpains reduces the calcium concentration required for half-maximal proteolytic activity without affecting the specific activity of the enzyme (Edmunds *et al.*, 1991). Further autolysis of μ -calpain leads to further fragmentation of the 76 kDa subunit, and hereby loss of activity (Hopkins & Thompson, 2002a; Koohmaraie, 1992; Koohmaraie & Geesink, 2006). In meat, the activity of μ -calpain decreases within 1-3 days *post-mortem* due to autolysis (Boehm *et al.*, 1998; Koohmaraie *et al.*, 1995; Koohmaraie *et al.*, 1987; Koohmaraie *et al.*, 1991; Veiseth *et al.*, 2001), which is shown in Figure 3.3.

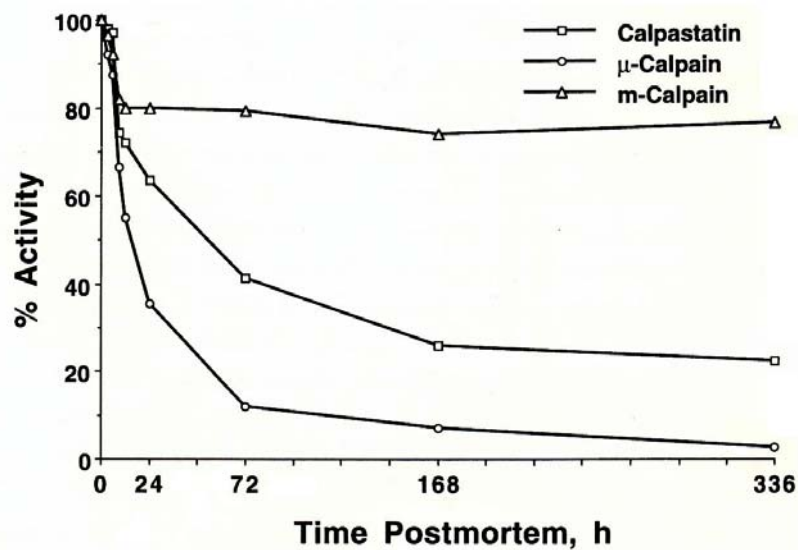


Figure 3.3 Effect of *post-mortem* storage on μ -calpain, m-calpain and calpastatin activities in bovine longissimus muscle (Koochmaraie *et al.*, 1995).

The loss of μ -calpain activity due to autolysis indicates proteolytic activity of the enzyme. The amount of μ -calpain remaining at any time *post-mortem* reflects the extent to which the enzyme has been activated, and is therefore inversely proportional to the amount of proteolysis that has occurred during *post-mortem* storage (Koochmaraie *et al.*, 1987).

μ -Calpain is located within skeletal muscle cells and has direct access to myofibrillar proteins. Specifically, μ -calpain has been shown to degrade the myofibrillar proteins titin, nebulin, filamin, desmin, and troponin-T in model systems similarly to degradation in muscle *post-mortem*. Additionally, the rate and degree of degradation of the five myofibrillar proteins have been shown to relate to tenderness determined by WBSF (Huff-Lonergan *et al.*, 1996).

The effect of protein oxidation on meat tenderness has already been outlined by two hypotheses in the introduction of this thesis: i) that oxidation causes inactivation of proteolytic enzymes responsible for meat tenderization and hereby reduced meat tenderness (Rowe *et al.*, 2004a) and ii) that oxidation causes protein cross-linking of myofibrillar proteins and hereby strengthening of the myofibrillar structure to cause less tender meat (paper II). Decrease of meat tenderness by oxidation was reported as early as in 1962 by Chajuss & Spencer in two separate research notes concerning the effect of aging in oxidative environments on the tenderness of chicken muscle. Additionally, the authors observed a loss of free thiol

groups in proteins extracted from aged muscle tissue subjected to air oxidation (Chajuss & Spencer, 1962a; Chajuss & Spencer, 1962b) indicating a negative effect of protein oxidation on meat tenderness.

3.1.1 Inactivation of μ -Calpain by Oxidation

μ -Calpain requires reducing conditions to be active due to its nature of being a cysteine protease. Oxidation by H_2O_2 (Carlin *et al.*, 2006; Guttman *et al.*, 1997), sodium hypochlorite (Guttman *et al.*, 1997), and a superoxide radical generating system (Guttman & Johnson, 1998) have been shown to inactivate μ -calpain *in vitro*. However, in the study by Carlin *et al.* (2006) oxidation was also found to decrease the ability of calpastatin to inhibit μ -calpain. It was suggested that calpastatin binds to μ -calpain to block H_2O_2 from access to the active cysteine residue site and hereby preventing oxidative inactivation of μ -calpain. This indicates a necessity to evaluate the oxidative susceptibility of μ -calpain in the presence of calpastatin.

In meat, irradiation early *post-mortem* and subsequent chill storage has been shown to decrease tenderness (measured by WBSF) by inactivation of μ -calpain in beef (Rowe *et al.*, 2004a). Usually, μ -calpain autolyzes within 1-3 days *post-mortem* in tissue with the eventual loss of activity (Veiseth *et al.*, 2001). However, in the irradiated beef steaks μ -calpain activity was detected on day 15 *post-mortem* (14 days after irradiation), which is a remarkable effect of irradiation. Additionally, irradiation also influenced calpastatin activity in the beef steaks. During non-oxidative conditions calpastatin is degraded and hereby inactivated *post-mortem* as it is a substrate for μ -calpain, as shown in Figure 3.3 (Doumit & Koochmariaie, 1999), but irradiation diminished the rate of calpastatin inactivation compared to non-irradiated beef, which might also have influenced the tenderization of the beef steaks (Rowe *et al.*, 2004a). Increased protein oxidation (measured by carbonyl content) in both sarcoplasmic and myofibrillar protein extracts was found in the irradiated steaks compared to the non-irradiated steaks, which was positively correlated to WBSF. In fact, carbonyl content measured 1 day after irradiation was positively correlated with WBSF measured 14 days after irradiation, which means that increased early *post-mortem* protein oxidation is associated with decreased tenderness at later times *post-mortem* due to compromised tenderization through μ -calpain inactivation (Rowe *et al.*, 2004b). Cross-linking of myofibrillar proteins has not been investigated in the two studies by Rowe and colleagues, and thus it is not

possible to determine to what extent μ -calpain inactivation and protein cross-linking individually influence the observed decrease in tenderness.

During the present PhD study it was not possible to detect any activity of μ -calpain in both beef and pork on day 3 (paper VI) and in pork on day 4 (paper II) *post-mortem*. Therefore, the effect of high-oxygen atmosphere packaging compared to storage without oxygen on μ -calpain activity could not be determined in the present study. In paper II, a novel method was used for determination of myofibrillar fragmentation using light scattering to determine the surface mean diameter (D(3,2)) of the myofibrillar particles in the sample. D(3,2) has been found to correlate with WBSF ($R = 0.40$) (Lametsch *et al.*, 2007). Myofibrillar fragmentation was found to increase (D(3,2) decreased) especially from day 1 to day 4 *post-mortem*. This proves persistent proteolytic activity in the meat from day 1 to day 4 *post-mortem*, but no significant difference between the packaging atmospheres with and without oxygen was found on day 4. These results are discussed in more details in Chapter 6.

3.1.2 Protein Cross-linking by Oxidation

The fact that oxidation of myosin in model systems causes intermolecular cross-linking has been investigated in a number of studies in relation to processed meat products (results are reviewed in Table 2.5). The functional properties of myosin are influenced by oxidative conditions by reducing the gel-forming ability, emulsifying ability, water-binding capacity, and solubility (Decker *et al.*, 1993; Liu & Xiong, 1996a; Ooizumi & Xiong, 2004; Smith, 1987; Srinivasan & Hultin, 1997; Wan *et al.*, 1993; Xiong *et al.*, 1993; Xiong & Decker, 1995). However, very few studies have investigated oxidation of myosin in fresh meat. Storage of beef from day 1 to day 10 *post-mortem* has been found to cause myosin cross-linking (Martinaud *et al.*, 1997), but no information was given about the packaging atmosphere of the meat during the storage period. Hence, it is noteworthy that myosin heavy chain was found to form intermolecular cross-linkings in pork LD stored in modified atmospheres containing high concentrations of oxygen whereas no myosin cross-linking was observed without presence of oxygen (paper II). In the same study, storage in high-oxygen atmospheres was also found to cause significantly less tender meat compared to storage without oxygen already on day 4 *post-mortem* evaluated by a subjective sensory assessment, as demonstrated in Figure 3.4.

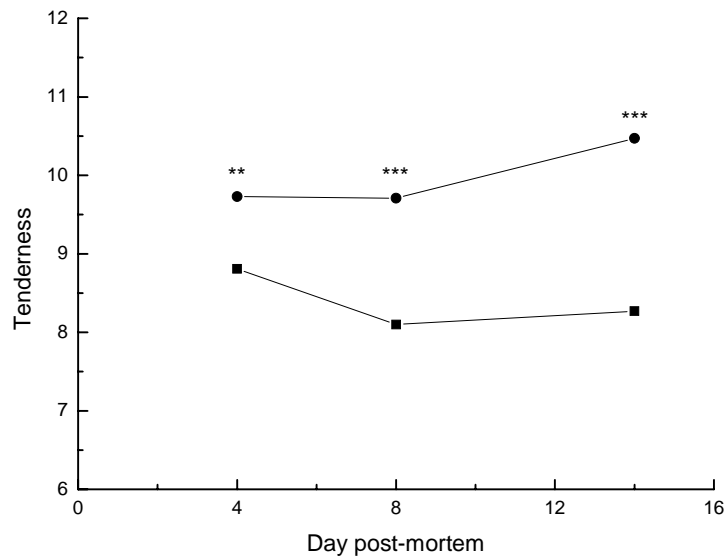


Figure 3.4 Sensory assessed tenderness of LD slices stored in modified atmosphere (70% O₂/30% CO₂) (■) and vacuum skin packaging (no oxygen) (●) for up to 14 days at 4°C. Tenderness was evaluated on a scale from 1 to 15, with 15 being most tender and 1 being least tender. Significance levels between packaging atmosphere on each storage day are given by ***($p < 0.001$); ** ($p < 0.01$); * ($p < 0.05$), and NS (non-significant) (paper II).

The tenderness of LD slices stored without oxygen increased during the storage period from day 4 to day 14 *post-mortem* ($p = 0.0374$) indicating that development of tenderness still occurs after day 4. In contrast, tenderness of LD slices stored in high-oxygen atmosphere decreased further over time ($p = 0.0436$), which indicates that presence of oxygen not only inhibits development of tenderness but strengthens the myofibrillar structure as well, for example by myosin cross-linking. However, the part of myosin heavy chain cross-linking after 14 days *post-mortem* was quite small and whether cross-linking of myosin was the only cause for the observed tenderness decrease remains to be investigated.

Storage in high-oxygen atmospheres has previously been found to decrease sensory assessed tenderness of beef compared to storage without oxygen (Seyfert *et al.*, 2005). However, the beef steaks packed in high-oxygen atmosphere were stored for 9 days compared to 16 days for the beef steaks stored without oxygen. The decreased tenderness in beef steaks stored in high-oxygen atmospheres may thus also be caused by a limited aging period and not only oxidation. The effect of high-oxygen atmospheres on structural changes of buffalo meat has recently been investigated by determination of the myofibrillar fragmentation index (determined as weight of fragments larger than 250 μm left on filter paper after filtration of

muscle homogenate), fibre diameter and sarcomere length. Myofibrillar fragmentation index and fibre diameter was found to increase, while sarcomere length decreased significantly during storage in high-oxygen atmospheres compared to vacuum (Sekar *et al.*, 2006). This indicates that aging, and hereby tenderization, of the buffalo meat was faster in vacuum compared to high-oxygen atmosphere packaging.

The effect of oxygen in the packaging atmosphere specifically on the myofibrillar component of meat was investigated using a unique method (Christensen *et al.*, 2006) to determine the breaking strength of single muscle fibres isolated from meat (Paper VI). Increasing oxygen concentrations in the packaging atmosphere caused an increase in the breaking strength of bovine single muscle fibres after 2 days of storage indicating a strengthening of the myofibrillar component due to presence of oxygen. Unfortunately, no effect of packaging atmosphere was found on protein oxidation and myosin cross-linking indicating a need for longer storage periods and the use of a higher number of animals (only one animal was used in the current study) to detect any variation in protein oxidation in meat samples. No effect of packaging atmosphere was found on porcine single muscle fibres, which may be due to the fact that the porcine fibres were quite weak already on day 1 *post-mortem* as pork tenderizes faster than beef (Dransfield *et al.*, 1981). Likewise, irradiation of pork LD did not show the same effect on WBSF (Davis *et al.*, 2004) as irradiation of beef (Rowe *et al.*, 2004a), but the irradiation dose was only max. 4.4 kGy compared to 6.4 kGy, respectively. Bovine muscles have a higher content of myoglobin than porcine muscles (Hazell, 1982; Lawrie, 1952), and may therefore be more susceptible to oxidation. The content of myoglobin in bovine and porcine muscle fibres could explain the effect of high-oxygen atmosphere on the breaking strength of muscle fibres isolated from beef and not pork.

In summary, oxidative environment may cause both inactivation of μ -calpain and protein cross-linking of myofibrillar proteins resulting in decreased tenderness depending on species, type of oxidation, and storage time. To what extent protein cross-linking and inactivation of μ -calpain individually affect meat tenderization is still an unanswered question.

3.2 Meat Juiciness

Juiciness is related to the water-holding capacity of meat, which is defined as the ability of meat to hold its own or added water during application of any force

(Offer & Knight, 1988a). Water-holding capacity is often evaluated by determination of percentage drip loss of fresh meat during storage. Sensory assessed juiciness is evaluated after cooking, which induces further loss of water, and a sensory assessment of juiciness will therefore include both drip loss and cooking loss (Lawrie & Ledward, 2006). Reduced water-holding capacity of meat also has economic consequences by reducing processing yields as loss of water means a reduction of product weight.

Water is lost from fresh meat from the beginning of the slaughter process and onwards. The capability of fresh meat to retain water is affected by various factors including rate and extent of pH decline, the cooling process *post-mortem*, proteolysis, cutting and subsequent formation of exudate leaks, packaging, chill storage temperature and storage time (Lawrie & Ledward, 2006; Melody *et al.*, 2004; Offer & Knight, 1988a; Offer & Knight, 1988b; Offer & Trinick, 1983; Smulders *et al.*, 2006), but possibly also protein oxidation (Huff-Lonergan & Lonergan, 2005). The majority of water in muscle is entrapped in the structure of the muscle and muscle fibres (Huff-Lonergan & Lonergan, 2005; Offer & Cousins, 1992; Offer & Trinick, 1983). Water loss has been described as a process of i) loss of water from within the myofibrils due to *post-mortem* shrinkage, ii) relocation of water from intra- to extracellular compartments, and iii) subsequent flow of this liquid to the surface (Offer & Knight, 1988a; Offer & Knight, 1988b).

It has been estimated that as much as 85% of the water inside living muscle cells is located within the myofibrils (Offer & Knight, 1988a). Therefore, key changes in the intracellular structure of the fibre influence the ability of muscle fibres to retain water. As pH decreases in meat *post-mortem* and approaches the isoelectric point of the major proteins (pI of myosin is 5.4), the electrostatic repulsion of the filaments within the myofibril is reduced, and the filaments become more densely packed. Hereby the space for water to be held in the myofibrils is reduced, and fluid can be forced into the extramyofibrillar spaces (Bendall & Swatland, 1988; Diesbourg *et al.*, 1988; Offer & Cousins, 1992; Offer & Knight, 1988a). The subsequent movement of fluid from the extramyofibrillar spaces to the extracellular space and ultimately out of the fibre may be increased by limited proteolysis (Bee *et al.*, 2007; Huff-Lonergan & Lonergan, 2005; Melody *et al.*, 2004; Zhang *et al.*, 2006). The linkages responsible for attaching the myofibrils to each other and to the sarcolemma (Wang & Ramirez-Mitchell, 1983) (include a.o. desmin, filamin, vinculin) are degraded during proteolysis (Bee *et al.*, 2007; Morrison *et al.*, 1998; Zhang *et al.*, 2006). If these linkages remain intact *post-mortem*

due to limited or no proteolysis, shrinkage of the myofibrils during rigor would be transferred to the entire fibre resulting in increased shrinking of the overall muscle fibre (depicted in Figure 3.5). This would ultimately result in increased drip loss (Kristensen & Purslow, 2001; Morrison *et al.*, 1998; Rowe, Huff-Lonergan, & Lonergan, 2001).

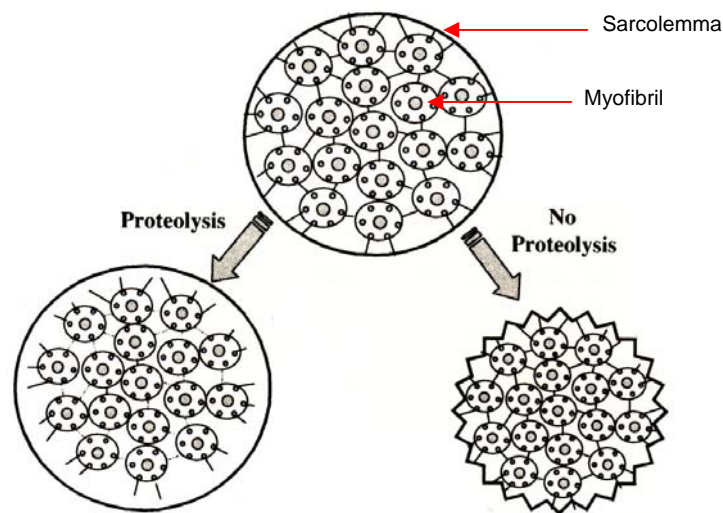


Figure 3.5 Schematic over viewing the potential changes in muscle fibre (muscle cell) diameter during *post-mortem* aging as influenced by proteolysis (Huff-Lonergan & Lonergan, 2005).

Taking the effect of proteolysis on water-holding capacity into account, it has been suggested that degradation of key myofibrillar proteins by calpains play a role in determining water-holding capacity of meat (Bee *et al.*, 2007; Huff-Lonergan & Lonergan, 2005; Melody *et al.*, 2004; Zhang *et al.*, 2006). Because desmin is a known μ -calpain substrate (Baron *et al.*, 2004; Huff-Lonergan *et al.*, 1996), it has been hypothesized that calpain activation and autolysis may explain a portion of the variation of desmin degradation and could subsequently influence drip loss (Bee *et al.*, 2007; Huff-Lonergan & Lonergan, 2005; Melody *et al.*, 2004; Zhang *et al.*, 2006).

Modified atmosphere packaging with high concentrations of oxygen was found to increase drip loss dramatically and decrease sensory assessed juiciness compared to storage without oxygen, as demonstrated in Figure 3.6 (paper II).

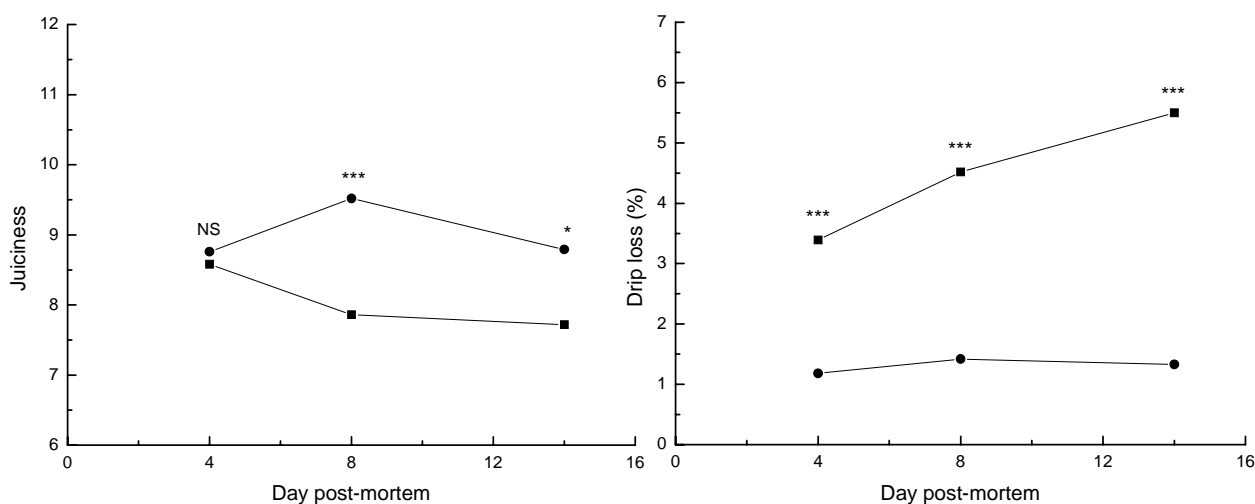


Figure 3.6 Sensory assessed juiciness and drip loss of LD slices stored in modified atmosphere (70% O₂/30% CO₂) (■) and vacuum skin packaging (no oxygen) (●) for up to 14 days at 4°C. Significance levels between packaging atmosphere on each storage day are given by ***($p < 0.001$); ** ($p < 0.01$); * ($p < 0.05$), and NS (non-significant) (paper II).

An effect on drip loss of high-oxygen atmospheres was detected already on day 4 *post-mortem* while juiciness was only significantly affected after 8 days of storage. This observation indicates that the drip loss has to reach a certain level before juiciness is found to be affected. The fact that drip loss and juiciness is affected by presence of oxygen in the packaging atmosphere indicates that loss of water from meat is associated with oxidation. Hence, if oxidation inhibits proteolysis and hereby degradation of key myofibrillar proteins, as shown by Rowe *et al.* (2004a), the results shown in Figure 3.6 is in agreement with the hypothesis outlined above about water-holding capacity being associated with degradation of myofibrillar proteins. Additionally, as the myofibrils entrap most of the water in meat, other changes in the structure of the myofibrillar proteins as cross-linking of myosin heavy chain, as shown to occur in paper II, may also influence the water-holding capacity and juiciness similarly to inactivation of μ -calpain. The effect of oxidation on water-holding capacity of purified myofibrils in a model system has recently been investigated (Bertram *et al.*, 2007). Reduced water-holding capacity of the myofibrils upon oxidation with haemoglobin and H₂O₂ was found together with an increase in formation of the cross-linked oxidation product, dityrosine, illustrating that the effect of oxidation on water-holding capacity is not solely related to proteolysis. However, a direct relationship between water-holding capacity and degree of oxidation was not found with various pH and ionic strengths indicating a need for further studies.

Conversely, it should be noted that the effect of packaging atmosphere on drip loss and juiciness described in Figure 3.6 could be a result of packaging type (container versus vacuum packaging) and not only oxygen existence. In a recent study with beef stored in containers with 70% N₂/30% CO₂ and vacuum, a higher drip loss was found when beef was stored in the container (Smulders *et al.*, 2006). However, contradictory observations have been reported as some studies show increased drip loss in meat during storage in vacuum compared to high-oxygen atmosphere packaging (Cayuela *et al.*, 2004; Sekar *et al.*, 2006), which is probably due to varying pressure in vacuum packages. Furthermore, presence of carbon dioxide in a packaging atmosphere might affect water-holding capacity of meat. As pH *post-mortem* reduces, it is assumed that the myofibrillar proteins will release more bound water due to movement of intracellular water towards the extracellular space (Ouali *et al.*, 2006). Presence of carbon dioxide in the packaging atmosphere of pork and beef has been shown to decrease pH of the meat after storage, and water-holding capacity might therefore be negatively affected by this additional decrease in pH (Sorheim *et al.*, 1996; Sorheim *et al.*, 2004).

The implications of oxidation on juiciness of meat is not understood, but a few recent studies describe a connection between protein cross-linking and decreased water-holding capacity (Bertram *et al.*, 2007)(paper II), an effect that should be investigated further in the future. Furthermore, the relationship between μ -calpain activity and water-holding capacity of meat should also be further elucidated.

4 Control of Oxidation in Meat during Storage

In this chapter control of oxidation in meat during storage is discussed in relation to progression of both lipid oxidation and of protein oxidation, and in relation to the effect of antioxidants traditionally used for protection of meat during storage on protein oxidation. The mechanism of lipid oxidation has been described in detail in many literature reviews and text books, e.g. (Frankel, 1980; Skibsted, 2000; Skibsted *et al.*, 1998), and will not be further touched upon in this thesis. Only a limited amount of studies is available regarding the coupling between lipid and protein oxidation in meat, and therefore examples from biochemical studies are included in this chapter to explain the complexity of the reactions.

4.1 Coupling between Lipid Oxidation and Protein Oxidation

In order to control oxidative reactions during storage it is important to consider the initiating reactions behind oxidation. Progression of oxidation in meat during storage has been studied over decades and is well-described in relation to oxidation of lipids but the literature is rather scarce regarding the mechanism behind oxidation of proteins during storage of foods in general. The mechanism for lipid oxidation includes the initial formation of reactive radicals followed by formation of primary and subsequently secondary lipid oxidation products, which can react with proteins to cause formation of Schiff bases which is marked as protein damage in Figure 4.1.

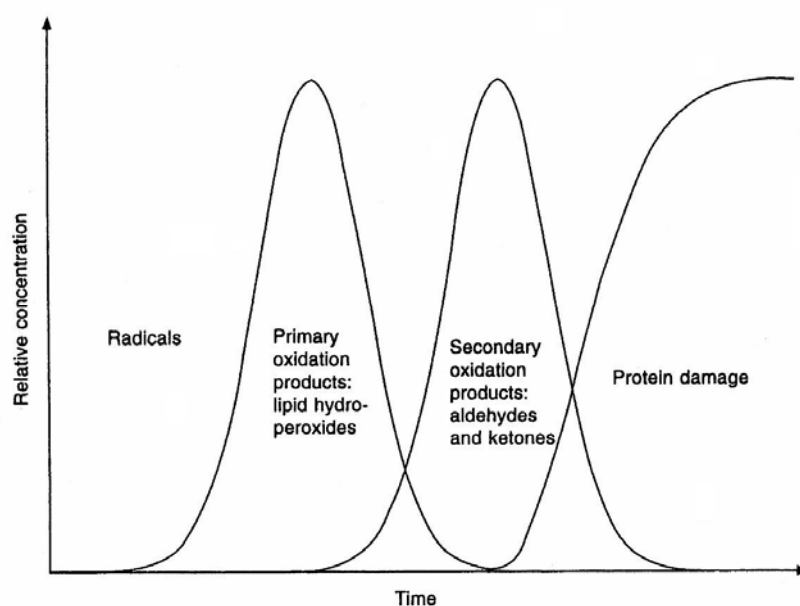


Figure 4.1 Progression of lipid oxidation in a food system from formation of radicals through primary and secondary lipid oxidation products to protein damage (Andersen *et al.*, 2003).

Damage of meat proteins by reaction with single lipid oxidation products (e.g. malonaldehyde) or oxidized lipids to form Schiff bases and/or cause protein cross-linking and aggregation has been shown to occur for myofibrillar proteins (Chelh *et al.*, 2007; Jarenbäck & Liljemark, 1975; Tironi *et al.*, 2002), purified myosin (Buttkus, 1967; Saeed *et al.*, 1999), and myosin subfragment 1 (Li & King, 1999). However, protein damage can also occur via direct reaction of the initial radicals with the protein independently of lipid oxidation products (Davies, 2003; Davies & Dean, 2003; Garrison, 1987; Stadtman & Berlett, 1988). These reactions are not included in Figure 4.1.

The site for initial oxidation in lipids is the double bond(s) of unsaturated fatty acids in a lipid molecule. In contrast, a protein molecule contains numerous and different functional groups which may be affected by oxidation depending on the amino acid composition and conformation of the protein. Oxidation of proteins can occur both at the peptide backbone and amino acid side chains and can result in modification of side chains, fragmentation or polymerization of proteins, as outlined previously in Figure 2.3. The oxidation of proteins will often be initiated through formation of protein radicals which can be located both at the peptide backbone and on individual amino acid side chains. Subsequently, protein hydroperoxides may be generated either from the reaction of a protein radical with oxygen or directly by irradiation or metal-catalyzed oxidation of a protein (Gebicki & Gebicki, 1993; Hawkins & Davies, 2001; Simpson *et al.*, 1992). Protein hydroperoxides are reactive compounds that may react further (Davies *et al.*, 1995). Such a mechanism bears resemblance to the chain reaction for lipid autoxidation. However, protein oxidation products are normally not grouped into primary and secondary oxidation products which are costume for the lipid oxidation products. A general time-dependent formation of protein oxidation products has likewise not been described, as these reactions are highly dependent on the oxidation initiator and the actual oxidation matrix in which the proteins are embedded. Notably, the balance between lipid oxidation and protein oxidation in meat has not been clarified and whether lipid or protein oxidation occurs most rapidly in meat needs to be studied specifically in various meat products under conditions relevant to meat.

Sarcoplasmic proteins are located in the aqueous phase of meat where many of the radicals are formed, so it seems reasonable to expect that proteins are oxidized prior to lipids. This may also apply to myofibrillar proteins which are in

contact with water. Also, catalysts such as ferrous iron bind to proteins, allowing radicals to be formed near protein side chains some of which are sensitive to oxidation resulting in formation of protein carbonyls as seen in Figure 2.2. The initiation of protein oxidation and subsequent interaction with lipids is also poorly described. It seems unknown whether protein oxidation initiates lipid oxidation or whether lipid oxidation initiates protein oxidation in meat or if the two types of oxidation are coupled. However, protein radicals (other than the pseudo-peroxidase myoglobin) have been shown to be able to initiate oxidation of lipids as demonstrated in model systems (Ostdal *et al.*, 2002). In some biological systems (e.g. oxygen-radical oxidation of blood erythrocytes and oxidation of low density lipoprotein by hypochlorous acid) protein oxidation has been found to precede and initiate lipid oxidation (Davies & Goldberg, 1987; Malle *et al.*, 2006; Savenkova *et al.*, 1994), while other systems show the inverse relationship between lipid and protein oxidation (e.g. some membrane proteins and copper-ion-mediated oxidation of low density lipoproteins) (Dean *et al.*, 1986a; Giese *et al.*, 2003; Knott *et al.*, 2002; Tappel, 1973). Hence, initiation of one type of oxidation by the other is dependent on the relative localization of the initially formed radical (lipid or aqueous phase), which again will be dependent on how oxidation is initiated.

The coupling between lipid oxidation and protein oxidation has been studied to some extent in meat model systems and in meat. The reported studies investigating both lipid oxidation and protein oxidation in meat during storage and meat model systems during incubation with various oxidation initiators are summarized in Table 4.1, where the fastest initial rate of oxidation is marked by a '+'. Due to the differences in how these studies have been performed and reported it was necessary to evaluate the results differently, which is marked by footnotes in the table. In some studies the data material is too limited to determine to what extent the two types of oxidation play a role, and these studies are not included in Table 4.1. Surimi is a minced and washed fish or meat product, where blood, membranes, connective tissue and sarcoplasmic proteins are removed, and may therefore be described as a myofibrillar protein concentrate even though removal of lipids may be incomplete and variable (Park & Lin, 2005; Srinivasan *et al.*, 1996).

Table 4.1 Summary of studies where the formation of both protein oxidation and lipid oxidation in meat and meat model systems has been reported. The results are divided into chill storage experiments with meat (upper part) and incubation experiments with meat model systems (lower part). Fat content of the matrix is shown in brackets when it has been reported in the given reference. Protein oxidation is determined as carbonyl content and lipid oxidation as TBARS if nothing else is stated. Thiol groups in proteins (RSH) are determined by UV spectroscopy, hexanal by gas chromatography, and conjugated dienes (CD) by UV spectroscopy. HI-OX: high-oxygen atmosphere, X: xanthine, XO: xanthine oxidase, EDTA: ethyldiaminetetraacetic acid, NADH: nicotinamide adenine dinucleotide, AA: ascorbic acid, MbFe(III): metmyoglobin.

Matrix	Initiator	Oxidation products detected	Fastest initial rate of oxidation ^{a,b}		Reference
			Protein oxidation	Lipid oxidation	
Beef patties (fat 14%)	HI-OX			+ ^a	Paper I
Pork patties (fat 2-15%)	HI-OX	+RSH		+ ^a	Paper III
Pork patties	Cooking	hexanal	Simultaneous		(Vuorela <i>et al.</i> , 2005)
Porcine liver paté (fat > 30%)	-			+ ^a	(Estevez & Cava, 2004)
Turkey	Atmospheric air	+RSH		+ ^a	(Mercier <i>et al.</i> , 1998)
Chicken	Irradiation			+ ^b	(Rababah <i>et al.</i> , 2004)
Chicken myofibrils	-			+ ^a	(Liu & Xiong, 1996b)
Beef heart surimi (fat 1.3-1.9%)	-	+CD	Simultaneous		(Srinivasan <i>et al.</i> , 1996)
Beef heart surimi	-			+ ^b	(Srinivasan & Xiong, 1996)
Beef heart surimi	-			+ ^a	(Parkington <i>et al.</i> , 2000)
Minced cod muscle (fat 0.6%)	X/XO/Fe/EDTA		+ ^a		(Srinivasan & Hultin, 1995)
Dark muscle fish homogenates (four species)	Fe/H ₂ O ₂		+ ^b sardine	+ ^b anchovy, bluefish	(Tokur & Korkmaz, 2007)
			Simultaneous (bonito)		
Cod sarcoplasmic reticulum	NADH/Fe Fe/AA	RSH +LOOH	Simultaneous		(Soyer & Hultin, 2000)
Beef homogenates	Fe/H ₂ O ₂		+ ^b		(Mercier <i>et al.</i> , 2004)
Turkey microsomal membranes	Fe/AA	+RSH		+ ^b	(Mercier <i>et al.</i> , 2001)
Turkey microsomal membranes	MbFe(III)/H ₂ O ₂	+RSH		+ ^b	(Batifoulier <i>et al.</i> , 2002)

^aOne type of oxidation showed significant increases during storage/incubation time prior to the other type of oxidation.

^bBoth types of oxidation increased significantly during storage/incubation time so the percentage of oxidation products were calculated (during initial oxidation when several data points were available).

'Simultaneous' denotes an experiment where both types of oxidation occur simultaneously and no type of oxidation or percentage of oxidation product increases faster than the other.

During chill storage in high-oxygen atmosphere of beef (paper I) and pork (paper III) formation of secondary lipid oxidation products was observed at earlier storage times than formation of protein oxidation products. In a study with turkey meat stored in atmospheric air with an oxygen-permeable film for 9 days, similar results were observed (Mercier *et al.*, 1998). No significant increase in carbonyl content was found during storage for 9 days, while a significant increase in TBARS was observed. A small decrease in free thiol groups in proteins was, however, observed over storage time indicating some oxidation of proteins during storage (Mercier *et al.*, 1998). The other storage experiments reported in Table 4.1 show a similar tendency even though different species and meat products have been investigated (pork, beef, turkey, chicken, liver paté and surimi) and oxidation has been induced differently (oxygen-containing atmosphere, cooking, irradiation). These observations indicate that lipid oxidation precedes protein oxidation in meat when lipid oxidation is evaluated by TBARS and protein oxidation by carbonyl content.

When the coupling between lipid oxidation and protein oxidation in meat model systems are evaluated the results are ambiguous. The coupling between the two types of oxidation seems to be highly dependent on the model system used for investigation, the actual oxidant, and the methods used to monitor formation of lipid and protein oxidation products. For example, in a study of the oxidation of cod sarcoplasmic reticulum, the enzymatic NADH-iron oxidizing system induced greater lipid oxidation (measured by production of lipid hydroperoxides) relative to protein oxidation (measured by loss of thiol groups in proteins), while for non-enzymatic oxidation by ascorbate-iron the situation was clearly reversed (Soyer & Hultin, 2000). In a study with four fish species, the development of lipid and protein oxidation products was highly dependent on species. Hence, the system initiating oxidation and even the concentrations of the reactants (Park *et al.*, 2006b), and the actual methods used for quantification of oxidation products should be carefully considered when interaction between lipid and protein oxidation are investigated in model systems.

The fatty acid composition of the dietary fat has been shown to be of some importance in relation to the interaction between lipid oxidation and protein oxidation in meat. Lipid oxidation generally increases when increased amounts of unsaturated fat are included in the diet (Bremner *et al.*, 1976; Daza *et al.*, 2007; Leszczynski *et al.*, 1992; Rhee *et al.*, 1988; Warnants *et al.*, 1996)(paper III). The extent of cross-linking of oxidized chicken myofibrillar proteins and resultant precipitation has been found to be directly related to the degree of unsaturation of

free fatty acids (Xiong & Decker, 1995). In a recent study with lamb stored for 8 days, lipid and protein oxidation increased from day 4 to day 8, but the individual extent of formed oxidation products was found to depend on the composition of the diet and the fatty acid profile of the diets was not reported (Petron *et al.*, 2007). Contrarily, increased levels of unsaturated fatty acids in meat has been found not to influence protein oxidation (paper III), indicating that lipid and protein oxidation is not coupled. The lipid oxidation and protein oxidation was also found not to be linked together in the microsomal fraction where oxidation was induced by Fe^{3+} /ascorbate in a feeding study where turkeys were fed three different vegetable fats (Mercier *et al.*, 2001). A general conclusion regarding the connection between the level of unsaturated fatty acids in dietary fat and development of protein oxidation cannot be made based on the studies available in the literature.

The content of prooxidants (such as myoglobin) and antioxidants in the meat sample might also influence the development of lipid and protein oxidation products. Generally, beef has been shown to be more susceptible to both lipid oxidation and protein oxidation by storage in high-oxygen atmospheres, which may be due to higher concentration of myoglobin in combination with decreased concentration of α -tocopherol in beef compared to pork (paper I, III). In the study by Mercier *et al.* (1998), the dietary fat caused significant changes in myoglobin content, which may have influenced the formation of protein and lipid oxidation products. A significant effect of dietary fat in combination with α -tocopherol content on protein oxidation measured by carbonyl content in muscles was observed, although contradictory results were obtained with measurement of carbonyl content and free thiol groups in proteins (Mercier *et al.*, 1998). Additionally, a diet containing higher levels of polyunsaturated fatty acids and lower levels of α -tocopherol increased both the protein carbonyl and hexanal content as a lipid oxidation product but not TBARS levels compared to a diet with lower levels of polyunsaturated fatty acids and higher levels of α -tocopherol (Ventanas *et al.*, 2007). The inhibitory effect of antioxidants on protein oxidation will be discussed in more details in section 4.2.

Obviously, the formation of lipid and protein oxidation products is also dependent on the content of lipids and proteins in the meat sample. In washed, minced cod muscle with a lipid content of 0.6%, protein oxidation products were formed faster than lipid oxidation products (Srinivasan & Hultin, 1995). In liver pâtés with >30% fat and 10% protein, formation of lipid oxidation was faster than protein oxidation during 90 days of chill storage (Estevez & Cava, 2004). However,

the results described in paper III was contradictory to these observations as proteins in the high fat (>13% fat) pork patties were oxidized to a greater extent than proteins in the low fat (~2% fat) pork patties, but as the observed differences in thiol oxidation are rather small this result should be considered with care.

In summary, the results reported on a potential coupling between lipid and protein oxidation in meat are ambiguous even though it seems that formation of secondary lipid oxidation products is faster than formation of protein carbonyl groups in various meat types and products during storage. Increased levels of unsaturated fat obtained through feeding increase TBARS but not always carbonyl content in meat during storage. As the feeding strategies most often include varied levels of antioxidants it is at present not possible on the basis of the results reported to conclude which importance unsaturated fat exhibits towards protein oxidation in meat as the presence of antioxidants would be expected to influence the oxidation of both lipids and proteins. Characterization of the oxidative status of the meat (antioxidant level, content of prooxidants, fatty acid composition) is necessary in order to understand the interactions between lipid and protein oxidation. Furthermore, determination of a broader range of oxidation products (and not just TBARS and carbonyl content) most likely will be required in order to make valid conclusions.

4.2 Effect of Antioxidants on Protein Oxidation

Antioxidants are classified as compounds capable of delaying, retarding, or preventing oxidation processes (Shahidi & Wanasundara, 1992), and may be divided into endogenous compounds produced within the organism, or exogenous compounds with origin outside the organism. Exogenous antioxidants can be incorporated into muscle tissue through the diet and hereby increase the oxidative stability of muscle food (Jensen *et al.*, 1998; Monahan *et al.*, 1992). This section will focus on the effect of traditionally used antioxidants only for protection of proteins from oxidation, as the protective effect of the same compounds against lipid oxidation is rather well explained and the mechanism behind antioxidant protection of lipids has been described in many reviews (Andersen *et al.*, 2003; Kamal-Eldin & Appelqvist, 1996; Madsen *et al.*, 1997; Rice-Evans *et al.*, 1996; Shahidi & Wanasundara, 1992).

Establishment of the ability of a compound to function as an antioxidant in foods has been suggested at several levels: i) quantification of radical scavenging activity and reduction potential, ii) evaluation of the ability to inhibit oxidation in

model biological systems, and iii) determination of the ability to prevent or slow down the formation of oxidation products in foods during storage (Becker *et al.*, 2004). Some plant phenolics including vitamin E are examples of established antioxidants which have been extensively studied at all three levels in relation to lipid oxidation. Examples of phenolic compounds from three groups of compounds with antioxidative activity are shown in Figure 4.2.

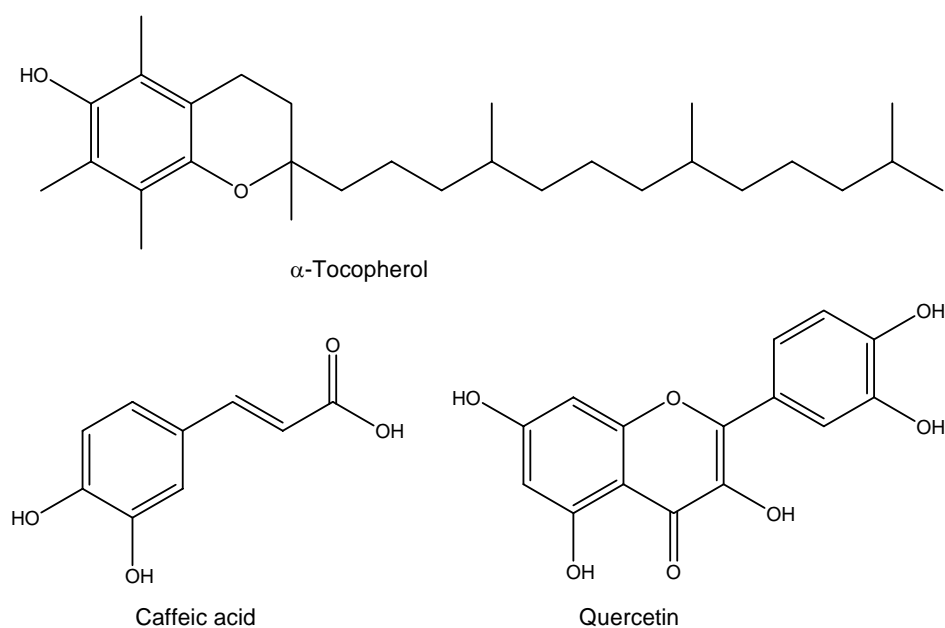


Figure 4.2 Examples of phenolic compounds with antioxidative activity. α -Tocopherol (or vitamin E) is a lipid-soluble chromanol derivate, caffeic acid is a hydroxycinnamic acid derivate soluble in ethanol, and quercetin is a water-soluble flavonoid.

Aqueous radicals have been shown to attack proteins in both the aqueous and lipid phase (Dean *et al.*, 1991). It might thus be predicted that antioxidants would differ in their ability to protect proteins against radical damage according to their own distribution between aqueous and lipid phases (Dean *et al.*, 1991). For instance, the lipid soluble α -tocopherol (vitamin E) has been found not to protect proteins located in the aqueous phase in the absence of lipid, while Trolox (the water-soluble vitamin E-analogue) showed effective protection (Baron *et al.*, 2005; Dean *et al.*, 1991). In the presence of lipid the ability of α -tocopherol to protect proteins increased slightly in an emulsion (Baron *et al.*, 2005) but increased notably in membranes when α -tocopherol was incorporated into the membranes prior to oxidation (Dean *et al.*, 1991). Trolox, however, protected the protein efficiently both in the absence and presence of lipid (Baron *et al.*, 2005; Dean *et al.*, 1991). Furthermore, α -tocopherol protected membrane-bound proteins better than aqueous phase proteins, while Trolox effectively protected both (Dean *et al.*, 1991).

α -Tocopherol is also able to delay both formation of protein hydroperoxides and conjugated dienes in low-density lipoproteins (Gieseg *et al.*, 2003).

A large variety of phenolic compounds and glycosides from plant materials has been found to exhibit antioxidative activity. Plant phenolics may be both hydrophilic and hydrophobic compounds, as antioxidants from plant materials can be extracted from both the lipid and water fraction and thus be located in both the aqueous and lipid phase of a food system (Shahidi & Wanasundara, 1992). In protein-containing liposomes plant phenolics have been found to protect both proteins and lipids from oxidation indicating that addition of plant phenolics to foods may be beneficial for improvement of the oxidative stability of both lipids and proteins. The individual efficiency of the phenolic compounds to protect proteins from oxidation was found to depend on the structure and concentration of the phenolics, the model system used for investigation, and which protein oxidation product was measured (Viljanen *et al.*, 2004b; Viljanen *et al.*, 2004a).

Kinetic and mechanistic studies of the inhibition of protein oxidation by antioxidants are limited as several radicals are formed on one protein simultaneously. The presence of several radicals and radical transfer through a protein makes it difficult to selectively generate a specific radical species of a protein and to estimate the exact concentration in order to characterize and quantify further reaction (Davies & Dean, 2003), e.g. oxidation of myosin causes formation of several radical species on myosin (paper IV). Additionally, the exact position and accessibility of the protein radical to the added antioxidant will almost certainly influence kinetics of such reactions. Thus kinetic data obtained for a particular radical on one protein may be completely erroneous for the same radical on a different protein (Davies & Dean, 2003). Hence, choice of realistic model systems for evaluation of the ability of a potential antioxidant to inhibit protein oxidation is complicated. Traditionally used antioxidants capable of quenching lipid radicals have been studied to some extent in relation to their ability to protect proteins in meat and meat model systems from oxidation during storage. The results obtained in the reported studies are summarized in Table 4.2.

Table 4.2 Effect of various antioxidants on protein and lipid oxidation in meat (upper part) and meat model systems (lower part) during chill storage. + is antioxidative effect, and – is no effect or prooxidative effect of the antioxidant compound. Specific conditions influencing the oxidative stability during storage are given in foot notes. Protein oxidation is determined as carbonyl content and lipid oxidation as TBARS if nothing else is stated in brackets. Thiol groups in proteins (RSH) are determined by UV spectroscopy, Disulfide (RSSR) by SDS-page analysis, hexanal by gas chromatography, and conjugated dienes (CD) by UV spectroscopy. ‘Plant phenolics’ covers both plant extracts and single phenolic compounds. BHT: butylated hydroxytoluene, TBHQ: tert-butylhydroxyquinone, TPP: tripolyphosphate, AA: ascorbic acid, PG: propyl gallate, ND: not determined.

Matrix	Antioxidants	Efficiency of antioxidant towards:		Reference
		Protein oxidation	Lipid oxidation	
Pork patties ^a	Plant phenolics	+	+ (hexanal)	(Vuorela <i>et al.</i> , 2005)
Porcine liver pâté	Plant phenolics BHT	+	ND	(Estevez <i>et al.</i> , 2006)
Chicken ^b	Plant phenolics TBHQ	+	+	(Rababah <i>et al.</i> , 2004)
Beef patties ^c	Plant phenolics AA/citrate	-	+	Paper I
Beef <i>L. lumbrorum</i> ^b	Vitamin E [#]	+	ND	(Rowe <i>et al.</i> , 2004b)
Turkey	Vitamin E [#]	+ (RSH, carbonyl)	+	(Mercier <i>et al.</i> , 1998)
Membranes from turkey muscle ^d	Vitamin E [#]	+ (RSH) - (carbonyl)	+	(Batifoulier <i>et al.</i> , 2002)
Beef heart surimi-like material	TPP AA AA/TPP Vitamin E PG Vitamin E/PG	+ - AA	+ (TBARS, CD)	(Srinivasan <i>et al.</i> , 1996)
Beef heart surimi	PG TPP	+ PG - TPP	+	(Parkington <i>et al.</i> , 2000)
Beef heart surimi-like material	TPP AA PG Vitamin E	+ (RSH, RSSR) - AA	ND	(Srinivasan & Xiong, 1997)
Beef heart surimi	TPP Vitamin E AA PG	+ - AA	+	(Srinivasan & Xiong, 1996)
Chicken myofibrils	AA/PG PG/TPP AA/TPP AA/PG/TPP	-	+ - AA + TPP	(Liu & Xiong, 1996b) (Liu & Xiong, 1996a)*

*no storage, #dietary, ^acooking and storage with exposure to light and atmospheric air, ^birradiation, ^cstorage in high-oxygen atmosphere, ^dincubation with MbFe(III)/H₂O₂

Vitamin E (or α -tocopherol) generally protects proteins in meat from oxidation, but the amount of data available for meat and meat model systems to support this observation is small. In the study by Mercier *et al.* (1998) two turkey muscles were included (*M. pectoralis* and *M. sartorius*), and the effect of vitamin E supplementation on carbonyl content varied between the two muscles. For *M. sartorius* a significant effect on carbonyl content after 9 days of storage was found, while no effect of vitamin E supplementation was found for *M. pectoralis*. In the study by Rowe *et al.* (2004b), dietary vitamin E decreased carbonyl content of sarcoplasmic proteins but not of myofibrillar proteins in irradiated beef steaks. On the other hand, vitamin E has been observed to effectively inhibit protein oxidation in beef heart surimi, which consists mainly of myofibrillar proteins, both in relation to carbonyl formation (Srinivasan *et al.*, 1996; Srinivasan & Xiong, 1996), loss of thiol groups and formation of disulfides in myosin (Srinivasan & Xiong, 1997). In membranes isolated from turkey, vitamin E supplementation only decreased the oxidation of thiol groups in proteins and not the formation of carbonyl groups (Batifoulier *et al.*, 2002).

In general, plant polyphenols seem to exhibit antioxidative activity towards proteins in meat. However, the ability of plant phenolics to inhibit protein oxidation is dependent on the structure and concentration of the phenolic compounds (Vuorela *et al.*, 2005). In the study by Estévez *et al.* (2006) a rosemary extract was found to inhibit protein oxidation in cooked liver pâtés after 90 days of chill storage, while in paper I no significant antioxidative effect of rosemary extract was found on proteins in fresh beef patties after 6 days of storage. However, the amount of antioxidant expressed in gallic acid equivalents was approx. 200 times higher in the liver pâtés compared to the beef patties. Furthermore, the different matrices used in the two studies may influence the development of protein oxidation and hereby the interaction with proteins and antioxidants.

Ascorbic acid has been shown to be a very efficient antioxidant for protein-derived radicals, as the reaction between tyrosine and tryptophan radicals is very rapid (Hoey & Butler, 1984). However, ascorbate is generally reported as being prooxidative (Srinivasan *et al.*, 1996; Srinivasan & Xiong, 1997)(paper I) in meat and meat model systems. The antioxidative effect of ascorbate is known to be concentration dependent and strongly influenced by the presence of transition metal ions, as ascorbate can reduce metal ions and cause formation of reactive radicals through the Fenton reaction, as shown in Figure 4.3.

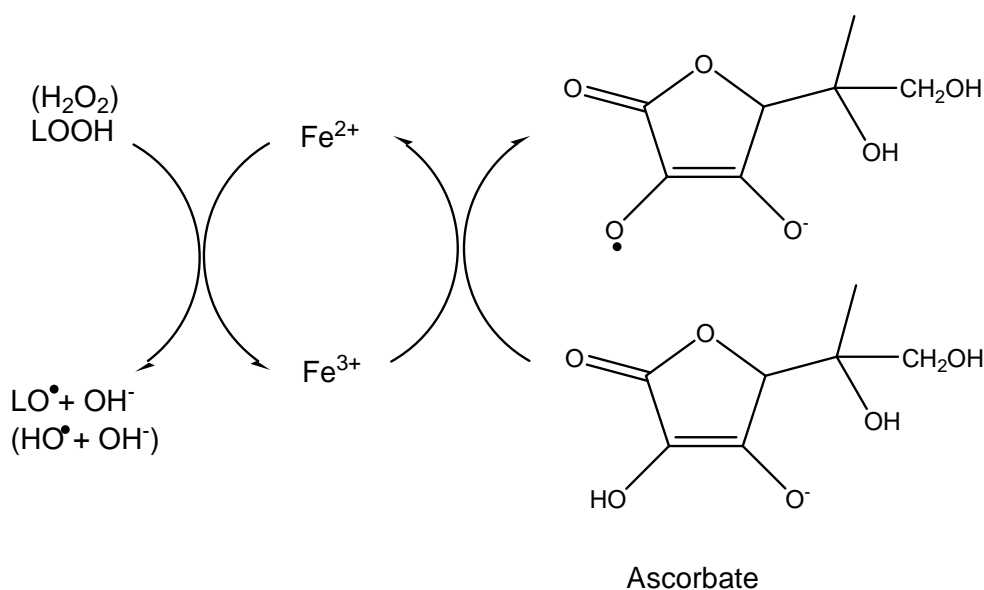


Figure 4.3 The prooxidative role of ascorbate by reduction of transition metal ions (Buettner & Jurkiewicz, 1996).

In Figure 4.3 oxidation of lipid hydroperoxides (or H_2O_2) by Fe^{2+} is shown, but the reaction with protein hydroperoxides and other transition metal ions has also been reported (Davies *et al.*, 1995; Simpson *et al.*, 1992). Oxidation of muscle proteins with Fe^{3+} /ascorbate has been shown to increase protein carbonyl content and cross-linking of myosin as already described in section 2.1 and 2.3.2.

Tripolyphosphate (TPP) and propyl gallate (PG) are synthetic water-soluble and lipid-soluble metal ion chelators, respectively. These compounds have only been investigated in surimi-based matrices, and they were generally found to protect proteins and lipids from oxidation even though a few exceptions have been observed. Synthetic antioxidants are continuously being replaced by natural antioxidants from plant sources due to market requirements, and the antioxidative activity of these compounds are therefore mainly interesting from a mechanistic point of view.

In a few studies, the effect of selected antioxidants on cross-linking of myosin has been investigated using SDS-page. Ascorbic acid was found to increase formation of disulfide and non-disulfide cross-linking in myosin in beef heart surimi (Srinivasan & Xiong, 1997). In contrast, butylated hydroxytoluene (BHT), ascorbic acid and vitamin E have been found to protect myosin from cross-linking during freeze storage of Atlantic mackerel (Saeed & Howell, 2002). However, these results should be interpreted with care as decreased solubility of myosin may have caused the observed loss of myosin band on SDS-page gels, and is not necessarily

due to cross-linking reactions. Additionally, the effect of antioxidants on tenderness of meat has also been studied. No significant effect of dietary vitamin E has been found on tenderness of beef (Harris *et al.*, 2001; Rowe *et al.*, 2004b) or pork (Lahucky *et al.*, 2007), while addition of plant phenolics to chicken has been reported to increase tenderness (Rababah *et al.*, 2004).

Diet can also influence the endogenous antioxidant enzyme activities (e.g. catalase, glutathione peroxidase) of meat and hereby the oxidative stability of meat. In one study lipid oxidation was found to be affected by the diet, while no effect on protein oxidation was observed of beef homogenates exposed to metal-catalyzed oxidation (Mercier *et al.*, 2004). In another study, both lipid and protein oxidation in lamb meat were affected by diet (Petron *et al.*, 2007), but no general conclusion can be made based on these studies as the diets used in the two studies affected the antioxidant enzyme activities differently. Furthermore, the diets may have changed the fatty acid composition of the meat, which also may influence protein oxidation as previously described in section 4.1.

BSA has been found to protect lipids from oxidation by becoming oxidized itself in model systems consisting of BSA and methyl esters or lipid hydroperoxides (Refsgaard *et al.*, 2000). This observation was supported by results showing that prevention of formation of BSA hydroperoxides and deactivation of BSA hydroperoxides by antioxidants effectively protected lipids in oil-in-water emulsions (Baron *et al.*, 2005). BSA has also been shown to prevent membrane lipid oxidation, which has led to the classification of BSA as an antioxidant (Fukuzawa *et al.*, 2005). The ability of BSA to form long-lived radicals upon oxidation with H₂O₂-activated heme proteins (Ostdal *et al.*, 1997) could be a result of an antioxidative mechanism of the protein although these long-lived radicals have proven able to oxidize lipids and other proteins suggesting that BSA radicals are not end products but a reactive intermediate (Ostdal *et al.*, 2002). Hence, the ability of proteins to exhibit antioxidative activity is unclear.

In summary, proteins are generally protected from oxidation by vitamin E, while the antioxidative ability of other phenolic compounds towards protein oxidation has to be further elucidated. The fact that some antioxidants are effective inhibitors for lipid oxidation but not protein oxidation states that the mechanism by which proteins are oxidized is different than the mechanism for lipid oxidation.

5 Methods for Investigation of Protein Oxidation in Meat

Quantification of protein oxidation products in biological samples as meat poses some difficulties because of the heterogeneity of the sample. Advantages and disadvantages of the methods used in the present PhD study are described in this chapter.

5.1 Carbonyl Groups in Proteins

Derivatization of protein carbonyls with 2,4-dinitrophenylhydrazine (DNPH) is one of the most used methods for quantification of protein oxidation and is based on the formation of a hydrazone which is detected spectrophotometrically at 370 nm (Figure 5.1). The method was first described as a semi-quantitative method (Fields & Dixon, 1971), but has later been optimized and used as a quantitative method (Levine *et al.*, 1994; Levine *et al.*, 1990; Oliver *et al.*, 1987; Reznick & Packer, 1994).

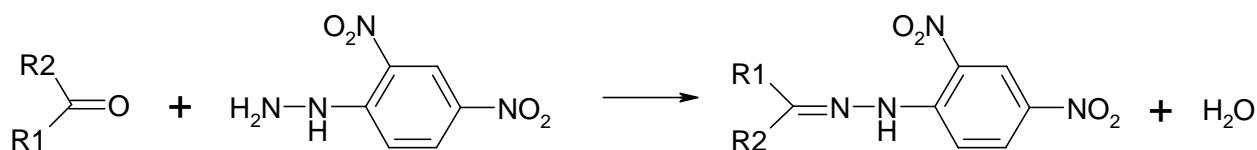


Figure 5.1 The reaction between 2,4-dinitrophenylhydrazine and protein carbonyl groups to form a hydrazone which may be determined spectrophotometrically at 370 nm.

Excess DNPH is required for derivatization of carbonyl groups in the assay and as the unbound DNPH also absorbs around 370 nm it is necessary to wash the sample thoroughly with ethanol/ethyl acetate after derivatization to remove unreacted DNPH. The washing step results in loss of protein (often 10-15%) and causes a large contribution to the standard deviation between and within samples, which reduces the repeatability of the assay. Hence, determination of protein concentration in the sample after washing is necessary and is often performed by measurement at 280 nm. Effective wash of excess DNPH is critical and a five-fold increase of the ethanol/ethyl acetate washing volume as described by Fagan *et al.* (1999) has proven to be advantageous. Excess DNPH may also be removed by HPLC but this has not been attempted in the present study (Levine *et al.*, 1994).

Biological samples of different origin have been tested using this method and some papers describe difficulties and disadvantages of the method (Cao & Cutler, 1995; Fagan *et al.*, 1999; Levine *et al.*, 1994; Reznick & Packer, 1994). Some tissue

samples require removal of nucleic acids (e.g. by streptomycin sulphate treatment) (Cao & Cutler, 1995; Levine *et al.*, 1990), while other samples require removal of chromophores (e.g. by HCl-acetone treatment) (Adams *et al.*, 2001; Fagan *et al.*, 1999; Oliver *et al.*, 1987) prior to derivatization. Background absorption at 370 nm from the sample should as a minimum be determined on a blank sample not treated with DNPH. The nature of biological samples differs greatly and optimization of the procedure for evaluation of the concerned sample is required. Myoglobin is a chromophore present in sarcoplasmic protein homogenates in concentrations that interfere with the carbonyl assay. Washing the sample with HCl-acetone to remove myoglobin prior to derivatization reduces background absorption at 370 nm and thus the standard deviation between samples (Fagan *et al.*, 1999) and personal experience). However, removal of nucleic acids in meat samples was found to be unnecessary (personal experience).

It is unknown to what extent DNPH reacts with carbonyls and if the extent of reaction depends on which amino acid carries the carbonyl group. Generally, lysine, histidine, proline, and arginine are assumed to form carbonyls during oxidation but intermediate products formed during protein oxidation may also contain carbonyl groups. In contrast, non-oxidative formation of carbonyls occurs in biological samples and these carbonyls are also quantified by the DNPH assay giving rise to an erroneous quantification of protein oxidation products (Adams *et al.*, 2001; Reznick & Packer, 1994; Xiong, 2000). Carbonyls are primarily formed during metal-catalysed oxidation, thus if oxidation is initiated by other means another method may be required to evaluate the extent of protein oxidation.

In most studies (including the present) only the sarcoplasmic proteins are investigated using the carbonyl determination. Fagan *et al.* (1999) described a procedure which includes myofibrillar proteins. Immunodetection has also been used successfully to test for qualitative carbonyl formation in myofibrillar proteins (Buss *et al.*, 1997; Levine *et al.*, 1994; Rowe *et al.*, 2004b). Other derivatizing agents as fluoresceinamine and tritiated borohydride may also be used for carbonyl determination but these compounds are not as widely used as DNPH (Climent *et al.*, 1989; Lenz *et al.*, 1989; Levine *et al.*, 1990).

Despite the many factors contributing to problems with the carbonyl determination it is still the most used method for evaluation of protein oxidation in biological samples since this assay is convenient and inexpensive. Hence, the use of this method provides data that can be directly compared to other scientific studies if the above mentioned problems are taken into consideration.

5.2 Free Thiol Groups in Proteins

Quantification of free protein thiol groups in meat samples is a somewhat simple method compared to the carbonyl determination and is based on the reaction between the free protein thiol group and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), which creates a coloured thiolate ion that can be detected spectrophotometrically at 412 nm (Ellman, 1959) (Figure 5.2).

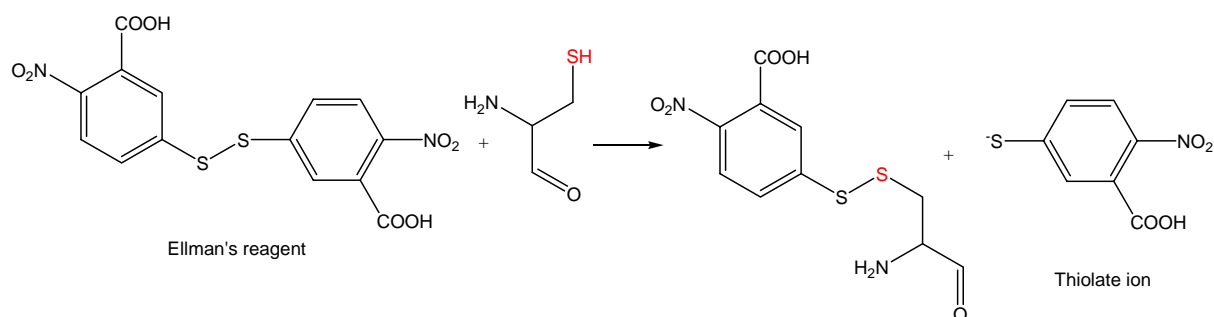


Figure 5.2 The reaction between 5,5'-dithiobis(2-nitrobenzoic acid) and protein thiol group to form a coloured thiolate ion which is determined spectrophotometrically at 412 nm.

The reaction is highly pH dependent and stringent pH control is required (Hu, 1994). Using this method provides data with acceptable standard deviations (< 5% within a triplet determination), which makes the biological variation in the meat the greatest contributor to the standard deviations. The DTNB solution should be prepared freshly every day and a blank without sample measured to compensate for day-to-day variation of this solution.

Myoglobin absorbs around 412 nm, and hence, background absorbance at 412 nm of the meat samples should be measured prior to analysis. The contribution of myoglobin to absorbance at 412 nm in diluted meat homogenates is negligible (Hofmann & Reiner, 1978) and personal experience). In contrast, model systems containing myoglobin may contribute considerably to the absorbance at 412 nm. Therefore, 2,2'-dithiobis(5-nitropyridine) (DTNP) should preferably be used instead as the formed coloured compound has maximum absorbance at 386 nm where the contribution from myoglobin is smaller (Winterbourn, 1990).

One of the major advantages with the thiol determination is that a meat sample containing myofibrillar proteins (obtained e.g. by homogenization with 5% SDS) can easily be tested and the same homogenate used for SDS-page analysis. A

disadvantage is that the method only evaluates oxidation of a single amino acid; cysteine. However, as oxidation of thiol groups to form disulfide bonds has been shown to occur in meat (paper II), the method provides valuable information about the oxidative status of the meat sample.

In most studies the results obtained with the carbonyl determination and thiol determination correlate (Petron *et al.*, 2007)(paper III) but exceptions have been observed (Mercier *et al.*, 1998)

5.3 Radical Formation

EPR (electron paramagnetic resonance, also called electron spin resonance, ESR) spectroscopy is a unique method for detection of radicals formed at the initiation of oxidation and may be a valuable method for mechanistic studies. Basically, a compound containing unpaired electrons (like a radical compound) will change the energy levels of the unpaired electrons when placed in a magnetic field (e.g. an EPR spectrometer). The sample is irradiated with constant electromagnetic radiation while the magnetic field is varied in order to achieve electron resonance. Absorption of the electromagnetic radiation is observed when the energy levels of the sample match the energy of the irradiation. It is the absorption of the electromagnetic radiation as a function of the applied magnetic field, which is detected in the EPR spectrometer and recorded as a first derivative spectrum. The structure of the signal provides information about the nature of the radical, and for more information on this topic the given references should be consulted. The intensity of the spectrum is determined by the concentration of the radicals, and under optimal conditions the minimum detectable concentration of radicals is approximately 10^{-9} M (Andersen & Skibsted, 2002; Eaton & Eaton, 1997).

Radicals can be detected either directly at room temperature, by spin trapping studies, or by freeze quenching (at low temperature using liquid nitrogen). Principally, radicals are too short-lived to accumulate in steady state concentrations above the detection limit of the EPR technique and rarely possible to detect directly at room-temperature. By adding a spin trap to the sample a more stable radical is formed, which is shown by an example in Figure 5.3.

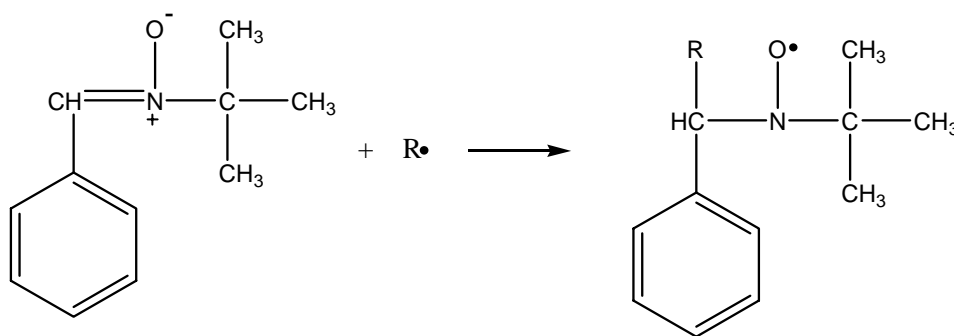


Figure 5.3 Example of the addition of a spin trap (here N-*tert*-butyl- α -phenylnitron, PBN), which is not a radical itself, to a sample consisting of transient radicals ($R\bullet$) to form a more stable radical spin adduct.

It is hereby possible to obtain concentrations of radicals above the detection limit, but often at the expense of the ability to identify the original radical. Another method that is used to increase the stability of a radical is to freeze the sample in liquid nitrogen (freeze quenching); this will often result in anisotropic spectra (broad, poorly resolved signals without distinct features).

The EPR technique can be used on liquid and solid material, which is a great advantage as aggregation and precipitation of proteins due to oxidation does not obstruct the detection of radicals. EPR spectroscopy is mostly used as a qualitative technique but a relative quantification of radicals is possible from an EPR spectrum as long as detectable concentrations of radicals are obtained. As a biological sample contains various molecules that are susceptible to radical formation the EPR spectrum will often contain a mixture of different radicals. Furthermore, anisotropic spectra may be obtained from a biological sample as large molecules rotate slowly and hereby give rise to signals that are poorly resolved.

The EPR technique has been used to detect radical formation in dehydrated chicken (Nissen *et al.*, 2000), cooked pork (Carlsen *et al.*, 2001), dry-cured ham (Andrés *et al.*, 2004), and chicken (Bragagnolo *et al.*, 2006; Bragagnolo *et al.*, 2007), but extremely high amounts of spin trap are required to obtain detectable radical concentrations, which makes the experiments very expensive. No reports on the direct detection of radicals in meat without the incorporation of a spin trap to the sample are available to the author's knowledge. In addition, the EPR technique detects all radicals present in a sample, and does not distinguish between lipid-derived and protein-derived radicals. Radical formation has been shown to correspond to oxygen consumption (Bragagnolo *et al.*, 2007; Carlsen *et al.*, 2001) and correlate with the formation of hexanal (Nissen *et al.*, 2000) in samples, which

are two methods that could be used instead to evaluate the overall oxidative stability of a sample. Therefore, the EPR technique has only been used in model systems containing purified myosin in the present study (paper IV and V) and not for meat samples.

5.4 Protein Cross-linking and Fragmentation

During SDS-page (sodium dodecyl sulphate polyacrylamide gel electrophoresis) the proteins are separated according to molecular weight and the technique is therefore useful for investigating structural changes like cross-linking or fragmentation. By combining SDS-page with identification by mass spectrometry a very powerful qualitative technique is obtained. On the other hand, quantification of proteins by SDS-page should be done with caution, as i) cross-linking of proteins leads to polymerization and precipitation, which makes loading of equal amounts of protein on the gel difficult and high molecular weight compounds unable to enter the gel, ii) quantification of protein fragmentation is difficult as the fragments are often small and difficult to retain during electrophoresis or staining (Davies & Delsignore, 1987), and iii) oxidized proteins or protein fragments may stain differently than the unoxidized protein depending on their composition and degree of oxidation (Davies & Delsignore, 1987).

Numerous staining procedures for the detection of separated protein bands after electrophoresis are available, which differ in sensitivity, reproducibility, and compatibility with subsequent protein identification by e.g. mass spectrometry. The most commonly used staining is Coomassie Brilliant Blue. Modifications of procedures using Coomassie staining to colloidal formulations have been reported to lower the detection limit (Candiano *et al.*, 2004). For detection of cross-linked myosin heavy chain in meat, the fluorescent SYPRO Ruby[®] staining was used (paper II). SYPRO Ruby[®] is more sensitive than colloidal Coomassie and the linear dynamic range extends over three orders of magnitude, which is vastly superior to Coomassie staining, and SYPRO Ruby[®] is fully compatible with mass spectrometry (Berggren *et al.*, 2000; Knight & Chambers, 2003). On the other hand, SYPRO Ruby[®] is quite expensive and requires special equipment for detection of stained protein bands such as a camera with fluorescent detection to photograph the gel or a laser scanner, which may be inconvenient.

6 Discussion

In this chapter the results obtained during the present PhD study will be discussed with special emphasis on: i) the choice of a marker for evaluation of protein oxidation in meat, ii) the significance of long-lived myosin radicals formed during oxidation, iii) the oxidative reactions influencing meat tenderness, iv) the susceptibility to oxidation of beef relative to pork, and v) protection of meat proteins from oxidation.

Markers for evaluation of protein oxidation in meat

It is now known that storage of beef and pork in high-oxygen atmospheres increases oxidation of meat proteins compared to storage without presence of oxygen (paper I, II). Different markers for protein oxidation have been used. However, the determination of carbonyl content, which seems to be the most used method for evaluation of protein oxidation in meat as reported in the literature, can be concluded not to be the best marker of protein oxidation based on different studies.

Myosin heavy chain forms intermolecular disulfide cross-links during storage in high-oxygen atmosphere but not in skin vacuum packaging without oxygen (paper II). This observation clearly demonstrates that meat proteins are in fact oxidized during storage in high-oxygen atmospheres. In the same study, no significant difference was found in carbonyl content between the two packaging atmospheres or during storage for 14 days. On the other hand, a significant difference in content of thiol groups in proteins between the two packaging atmospheres was observed, and notably this significance was observed on the same storage day as the cross-linked myosin heavy chain band could be observed by SDS-page analysis (i.e. day 8 *post-mortem*). These observations show that thiol determination is not only a better marker for protein oxidation in meat stored in high-oxygen atmospheres but also that thiol determination can be used as a marker for disulfide cross-linking of myosin. Of course, this would have to be confirmed by additional studies. Furthermore, the thiol determination is a simpler and faster method for evaluation of protein oxidation compared to the carbonyl determination.

The carbonyl content has only been determined for the sarcoplasmic proteins of meat in the present PhD study, while the thiol determination includes both the sarcoplasmic and the myofibrillar proteins. As myosin, which is part of the myofibrillar protein fraction, has proven to be very susceptible to oxidation during

storage (paper II)(Martinaud *et al.*, 1997), a better approach may be to determine the carbonyl content of the myofibrillar proteins, and not only of the sarcoplasmic proteins. Fagan *et al.* (1999) reported an increased carbonyl content of myofibrillar proteins compared to sarcoplasmic proteins, while the observation was the opposite in the study by Rowe *et al.* (2004b).

Protein carbonyl groups are mainly formed through metal-catalyzed oxidation and only to a smaller extent by enzymatic oxidation induced by hypervalent myoglobin as a pseudoperoxidase (Martinaud *et al.*, 1997). Hence, because of the relatively low contents of protein carbonyl groups formed in meat during storage, it could be speculated that metal-catalyzed oxidation only occurs to a small extent in fresh meat during storage in high-oxygen atmospheres. On the other hand, loss of thiol groups in myofibrillar proteins has previously been found to occur both by metal-catalyzed oxidation and by oxidation with hypervalent myoglobin (Martinaud *et al.*, 1997)(paper IV,V), which again suggests that determination of thiol groups in proteins is a better marker for protein oxidation. However, it should be noted that loss of thiol groups in proteins only concerns the amino acid cysteine, while several amino acids are capable of forming carbonyl groups due to oxidation.

Other markers for protein oxidation in biological samples have been suggested in a comprehensive review by Davies *et al.* (1999), for example, tyrosine-derived oxidation products such as dityrosine and 3,4-dihydroxyphenylalanine (DOPA). Dityrosine formation has been found in systems containing purified myosin (paper IV) and myofibrillar proteins (Bertram *et al.*, 2007), but no reports on formation of dityrosine in meat are available. Several amino acid side chains are also susceptible to hydroperoxide formation, which has been found to be six times more frequent than the formation of protein carbonyl groups in low-density lipoproteins (Giesege *et al.*, 2003). However, protein hydroperoxides are unstable compounds and should therefore only be measured supplementary to other markers.

The significance of long-lived myosin radicals formed during oxidation

The biological significance of protein-to-protein radical transfer reactions and the formation of long-lived protein radicals, as occurring during the reactions between H₂O₂-activated myoglobin and myosin, is not understood at present (Ostdal *et al.*, 1997; Ostdal *et al.*, 1999; Ostdal *et al.*, 2002). Long-lived protein radicals are regarded as reactive intermediates in oxidation processes due to their reactivity with other biomolecules, and are not end products, but their role as intermediates in the oxidation process is not clear. If these protein radicals act as prooxidants, they may

cause damaging reactions in muscle tissue at locations different from where they initially were formed (Ostidal *et al.*, 1997; Ostidal *et al.*, 1999; Ostidal *et al.*, 2002). Alternatively, long-lived radicals may act through a potential protective mechanism by removing reactive compounds and giving rise to long-lived species which can be readily eliminated by antioxidants (Kroger-Ohlsen, 1999; Ostidal *et al.*, 1999).

The impact of long-lived myosin radicals on meat quality is unknown, but it is possible that they have significance during storage of meat in high-oxygen atmosphere. It has been reported that the oxygen penetration depth in muscle rarely exceeds one centimetre even after long periods of storage (Bendall & Taylor, 1972; Brooks, 1938; Kebede *et al.*, 1998; McKenna *et al.*, 2005). However, as myosin and other proteins are able to form radicals with extremely long life-times (paper IV)(Ostidal *et al.*, 1997), and damage transfer can occur between protein radicals (Prutz *et al.*, 1986) in the aqueous phase of muscle, it could be speculated that the oxidative damage can be transferred from the surface to the inside of the meat independently of the oxygen penetration into the meat.

Changes in meat tenderness during storage in high-oxygen atmospheres

Cross-linking of myosin was suggested to have significant influence on the difference in tenderness observed in pork LD stored in high-oxygen atmosphere compared to pork LD stored without the presence of oxygen (paper II). The fact that tenderness of the pork decreased significantly from day 4 to day 8 *post-mortem* supports this hypothesis. Inactivation of μ -calpain would simply halt or delay proteolysis and thereby tenderization, but is not likely to cause decreased tenderness. In the study by Rowe *et al.* (2004a), WBSF was found to decrease during storage for both irradiated and non-irradiated beef samples with a slower decrease in irradiated samples due to μ -calpain inactivation. Of course, it is possible that protein cross-linking also takes place in irradiated meat even though WBSF decreases during storage even in the irradiated samples. On the other hand, it is also possible that the oxidative reactions occurring in meat due to irradiation and high-oxygen atmosphere storage, respectively, have different mechanisms and therefore induce oxidative damage differently. The results showing an effect of storage in high-oxygen atmosphere on the breaking strength of muscle fibres isolated from beef LD confirmed the hypothesis that the structure of the myofibrillar proteins is strengthened in presence of oxygen compared to storage without oxygen (paper VI). However, due to several issues as limited storage period

and the use of only one animal in the study, conclusions based on these results are preliminary.

No activity of μ -calpain could be detected after 3 days of storage (day 4 *post-mortem*) for pork (paper II) and after 2 days of storage (day 3 *post-mortem*) for pork and beef (paper VI) independent of the packaging atmosphere. Therefore, no inactivation of μ -calpain could be observed in beef and pork stored in high-oxygen atmosphere packaging compared to packaging without oxygen as opposed to irradiation of beef (Rowe *et al.*, 2004a). As μ -calpain is normally autolyzed within 1-3 days in meat *post-mortem* (Veiseth *et al.*, 2001), the analysis of μ -calpain activity on day 3 (paper VI) and day 4 *post-mortem* (paper II) may be too late to detect any differences in μ -calpain activity due to packaging atmosphere. However, in a Master thesis undertaken at our department (Miklos, 2007), μ -calpain activity was detected in beef and pork LD stored in high-oxygen atmosphere and 100% nitrogen after 1 and 2 days of storage (day 2 and 3 *post-mortem* for pork and day 3 and 4 *post-mortem* for beef). No difference in μ -calpain activity was observed between the two packaging atmospheres, but only a limited amount of animals was included in the storage experiment. These observations strongly suggest that high-oxygen atmosphere packaging does not create an oxidative environment capable of inactivating μ -calpain. Storage of meat in high-oxygen atmospheres does not cause as extensive protein oxidation as irradiation when comparing the formation of protein carbonyl groups (approx. 2 and 14 nmol/mg protein in beef stored in high-oxygen atmosphere (paper I) and irradiation meat (Rowe *et al.*, 2004b), respectively). Irradiation causes the formation of hydroxyl radicals, which are highly reactive compounds resulting in unselective and uniform initiation of oxidation in the irradiated piece of meat (Garrison, 1987; Rowe *et al.*, 2004a). Storage of meat in high-oxygen atmosphere holds a potential risk for elevated oxidation but the oxidation is dependent on presence of prooxidants in meat or activation of the molecular oxygen by other means. Oxidation in high-oxygen atmospheres is most likely also dependent on the size of the meat sample in relation to oxygen penetration through the muscle.

Myofibrillar fragmentation was determined by surface mean diameter (D(3,2)) of myofibrillar proteins in the storage experiment with pork LD (paper II) as shown in Figure 6.1.

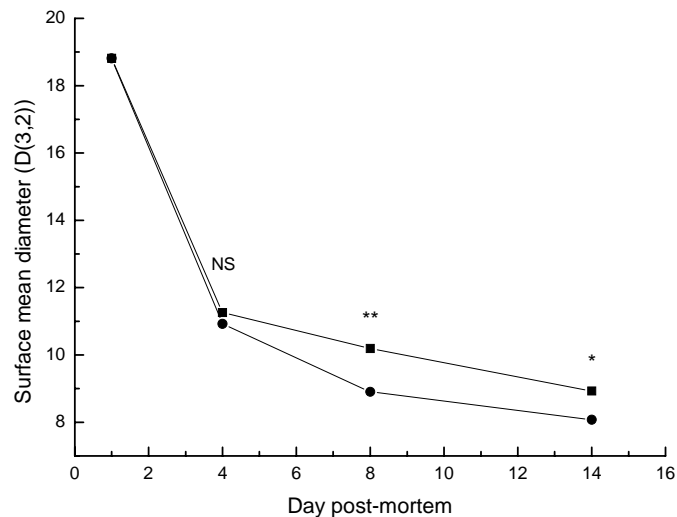


Figure 6.1 Myofibrillar fragmentation determined as surface mean diameter of myofibrillar proteins extracted from pork LD slices stored in high-oxygen atmosphere (70% O₂/30% CO₂) (■) and skin vacuum packaging (●) for up to 14 days at 4°C. Significance levels between packaging atmosphere on each storage day are given by ***($p < 0.001$); **($p < 0.01$); *($p < 0.05$) and NS (non-significant) (paper II).

The surface mean diameter of the myofibrillar proteins in pork LD decreases noticeably in the meat from day 1 to day 4 *post-mortem* showing persistent proteolytic activity in the meat during this storage period. On day 1 *post-mortem* activity of μ -calpain could be detected, while no μ -calpain activity could be detected on day 4 *post-mortem* as previously described. These observations indicate that μ -calpain was indeed activated and hereby autolyzed, which explains why μ -calpain activity in the meat was not detected on day 4 *post-mortem*. On day 8 and 14 *post-mortem* a significantly higher surface mean diameter of the myofibrillar proteins from pork LD stored in high-oxygen atmosphere was found compared to LD stored without oxygen. This observation could be a result of myosin cross-linking, which was observed on day 8 and 14 *post-mortem* by SDS-page analysis. However, the difference in surface mean diameter on day 8 and 14 *post-mortem* may also be caused by altered proteolytic activity due to oxidation. Due to the fact that no μ -calpain activity was detected on day 4-14 *post-mortem*, μ -calpain cannot be responsible for proteolysis after 4 days of storage. Hence, it could be speculated that other proteolytic enzyme systems also influence *post-mortem* meat tenderization and that these enzymatic systems may be affected by oxidation causing the

observed decrease in tenderness. Other cysteine proteases have been shown to be inactivated by protein hydroperoxides (Headlam *et al.*, 2006).

Susceptibility to oxidation of beef relative to pork

The susceptibility to oxidation of meat stored in high-oxygen atmosphere seemed more important for beef than pork, when comparing the formation of carbonyl groups in proteins in the two types of meat (paper I-III). In beef patties protein carbonyl groups were formed during storage for 6 days in the presence of oxygen (paper I), while no significant formation of protein carbonyl groups was found in pork LD and pork patties during 14 and 7 days of storage in high-oxygen atmospheres, respectively (paper II, III). These observations suggest that beef is more susceptible to protein oxidation than pork, which may be due to a three-fold higher content of heme pigment in beef than in pork in combination with a slightly lower content of α -tocopherol in beef. The chemical composition of the beef and pork patties investigated in paper I and III, respectively, is shown in Table 6.1 together with the carbonyl content in the meat patties on the last day of storage. TBARS values are included in the table for comparison.

Table 6.1 Chemical composition of the beef and pork patties stored in high-oxygen atmosphere and oxidation products formed on the last day of storage (paper I, III).

Characteristics	Beef patties (paper I)	Pork patties (paper III)
Heme pigment (ppm)	138 \pm 5.4	45 \pm 2 - 53 \pm 2
α -Tocopherol (μ g/g)	2.5 \pm 0.1	3.2 \pm 0.6 - 3.4 \pm 0.6
Fat content (%)	14.1 \pm 0.7	2.2 \pm 0.6 - 14.6 \pm 0.7
Protein content	18.5 \pm 0.5	18.3 \pm 0.7 - 21.3 \pm 0.5
Carbonyl content (nmol/mg protein)	1.32 \pm 0.02 [#]	0.85 \pm 0.08 [*]
TBARS (μ g/kg meat)	13.4 \pm 0.6 [#]	2.51 \pm 0.01 [*]

[#] Day 6

^{*} Day 7, 2% soybean oil, high fat

High-oxygen atmosphere increased the breaking strength of muscle fibres isolated from beef but had no influence on porcine muscle fibres (paper VI). These observations also indicate that beef is more susceptible to oxidation than pork even though the observed differences in breaking strength of bovine muscle fibres could not be ascribed to protein oxidation.

Protection of meat from oxidation

Protection of foods against oxidative deterioration depends on a combination of optimisation of processing parameters, use of antioxidants, and optimised packaging systems and storage conditions (Skibsted *et al.*, 1998). In the present PhD study focus has been on packaging systems and to a minor extent the use of antioxidants but it should be emphasized that processing parameters may also be of great importance in relation to protection of meat proteins against oxidative damage. It is beyond doubt that packaging of meat without oxygen would increase the quality of meat by increasing tenderness through prevention of protein oxidation, and by decreasing formation of rancid off-flavours formed by lipid oxidation. However, it is also a fact that packaging of meat without oxygen results in a brown or violet surface colour, which is unacceptable to the consumer even though the meat colour has little to do with the eating quality of the meat.

In order to protect meat from oxidation, it is important to know if the lipid part or the protein part of meat is more susceptible to oxidation and which part is oxidized initially and fastest. The formation of both protein and lipid oxidation products have been detected in meat during storage (paper I) and it seems unlikely that one type of oxidation would occur without the other even though the rate of two types of oxidation may differ. In most reported storage experiments with meat, secondary lipid oxidation products are formed at earlier storage times than protein carbonyl groups (paper I, III) indicating that protein oxidation is slower than lipid oxidation in meat or that lipid radicals initiate protein oxidation. Other protein oxidation products than carbonyl groups may be formed at earlier times in meat and since the time dependency of formation of protein oxidation products in meat has not been established, it is not possible to make any final conclusions in relation to the coupling between lipid and protein oxidation. However, the fact that increased unsaturation of the fatty acids in meat only increased oxidation of lipids and not proteins during storage indicates that protein and lipid oxidation in meat are not coupled.

Some antioxidative compounds present in meat have been shown to protect both lipids and proteins as described in Table 4.2. However, two antioxidant systems tested in beef during storage only protected lipids from oxidation and not proteins, indicating that protein and lipid oxidation in meat progress by different mechanisms (paper I). The formation of protein and lipid oxidation products may be affected by the presence of antioxidants differently, and likewise by prooxidants

and unsaturated fatty acids. Therefore, the meat used for storage experiments should be characterized both in relation to content of antioxidants and prooxidants, and fatty acid composition as the content of these compounds may affect the development of lipid and protein oxidation differently.

Observations confirming the importance of oxidation of proteins in fresh meat and meat products are continuously being reported, but the amount of literature available is still sparse. Furthermore, in most studies the carbonyl content has been determined as a marker for protein oxidation, which now has proven insufficient. Even though determination of thiol groups in proteins has proven to be a better marker than determination of carbonyl groups, development of methods with increased sensitivity for quantification of protein oxidation products is still required.

7 Conclusion

Storage of beef and pork in high-oxygen atmospheres increased protein oxidation and decreased meat tenderness compared to storage without oxygen. The reduction of meat tenderness caused by high-oxygen atmosphere packaging was ascribed to oxidative cross-linking of the major myofibrillar protein, myosin, through disulfide formation. No significant effect of packaging atmosphere on the activity of the proteolytic enzyme μ -calpain, which is believed to be responsible for most of the *post-mortem* tenderization taking place in meat, was observed. Determination of the loss of thiol groups in proteins was found to be a better marker than determination of carbonyl groups in proteins for meat stored in high-oxygen atmospheres.

A reaction mechanism for the damage transfer reactions taking place during oxidation of myosin by heme pigments was proposed. Oxidation of myosin included formation of several radical species on myosin; a short-lived thiyl radical, a long-lived tyrosyl phenoxyl radical, and possibly other unidentified radical species. Radical formation on myosin caused formation of intermolecular disulfide and dityrosine cross-linking. The radical formation and oxidation of thiol groups in myosin was found to be fast reactions and of relatively small pH dependency in the pH interval from 5.0 to 7.8.

Even though high-oxygen atmosphere packaging increased protein oxidation in beef and pork, the formation of secondary lipid oxidation products was faster in the studies performed during the present PhD project. Increased unsaturation of fatty acids in pork increased lipid oxidation during storage, but not protein oxidation. However, antioxidants, shown to inhibit lipid oxidation, had no effect or even enhanced protein oxidation. These observations indicate that protein oxidation and lipid oxidation in meat are not coupled.

8 Future Perspectives

From the results obtained in the presented work several new questions have been revealed and the following is suggested for future studies:

- Establishment of the time-dependent development of protein oxidation products in meat (radical formation, formation of protein hydroperoxides, loss of thiol groups, loss of amino acids, formation of protein carbonyl groups, and formation of cross-linked products) in order to characterize primary and secondary oxidation products.
- Study of the coupling between lipid and protein oxidation during storage with the use of other methods than determination of carbonyl content and TBARS and with detailed characterization of the meat samples in relation to content of lipid and proteins, anti- and prooxidants, and fatty acid composition.
- Effect of antioxidants to prevent or inhibit protein oxidation effectively both in meat model systems and meat.
- Creation of different oxidative environments in meat by e.g. irradiation, high-oxygen atmosphere, and high-pressure treatment, to investigate the significance of inactivation of μ -calpain and protein cross-linking in relation to meat tenderization.
- The role of other proteolytic enzymes (e.g. cathepsins, proteasomes, caspases) than μ -calpain for meat tenderization during storage in high-oxygen atmospheres.
- The role of long-lived myosin radicals in relation to initiation and propagation of both lipid oxidation and protein oxidation including the potential transfer of oxidative damage to the inside of meat samples, and in relation to inhibition of oxidation through antioxidative mechanisms.
- The effect of protein oxidation on the water-holding capacity of meat.

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Papers

Due to restrictions from the publisher of the journal in which article I and II has been published, these articles are not available in this PDF. The articles can be found in:

Article I:

Lund, M. N., Hviid, M. S., & Skibsted, L. H. (2007). The combined effect of antioxidants and modified atmosphere packaging on protein and lipid oxidation in beef patties during chill storage. *Meat Science*, 76, 226-233.

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Article II:

Lund, M. N., Lametsch, R., Hviid, M. S., Jensen, O. N., & Skibsted, L. H. (2007). High-oxygen packaging atmosphere influences protein oxidation and tenderness of porcine longissimus dorsi during chill storage. *Meat Science*, 77, 295-303.

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The four remaining articles appear as pre-print editions in this PhD thesis. They are available below. The articles have since been published, and can be found in:

Article III:

Lund, M. N., Hviid, M. S., Claudi-Magnussen, C., & Skibsted, L. H. (2008). Effects of dietary soybean oil on lipid and protein oxidation in pork patties during chill storage. *Meat Science*, 79, 727-733.

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Article IV:

Lund, M. N., Luxford, C., Skibsted, L. H., & Davies, M. J. (2008). Oxidation of myosin by haem proteins generates myosin radicals and protein cross-links. *Biochemical Journal*, 410, 565-574.

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Article V:

Frederiksen, A. M., Lund, M. N., Andersen, M. L., & Skibsted, L. H. (2008). Oxidation of Porcine Myosin by Hypervalent Myoglobin: The Role of Thiol Groups. *Journal of Agricultural and Food Chemistry*, 56, 3297-3304.

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Article VI:

Lund, M., Christensen, M., Fregil, L., Hviid, M., & Skibsted, L. (2008). Effect of high-oxygen atmosphere packaging on mechanical properties of single muscle fibres from bovine and porcine longissimus dorsi. *European Food Research and Technology*, 227, 1323-1328.

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Effects of dietary soybean oil on lipid and protein oxidation in pork patties during chill storage

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Abstract

The effect of dietary soybean oil on lipid and protein oxidation in low and high fat pork patties made from *quadriceps femoris* during chill storage in a high oxygen atmosphere packaging (80% O₂/20% CO₂) in the dark for 7 days was investigated. Pigs were fed either a standard diet or a diet added 2% soybean oil. After slaughter high fat pork patties were prepared for both feeding regimes by addition of back fat from pigs fed the same diet whereas low fat pork patties were prepared without addition of back fat. The 2% soybean diet increased the amount of unsaturated fat in the pork. Secondary lipid oxidation products determined as thiobarbituric acid reactive substances (TBARS) were found to increase in the pork patties with increased unsaturated fat. Increased unsaturated fat in the pork patties had no effect on protein oxidation determined as free protein thiol content and protein carbonyl content. A small, but significant increase in protein oxidation was found in the high fat pork patties independent on dietary fat. In conclusion, protein oxidation is unaffected by dietary fat in pork patties during chill storage for periods normally used in retail trade, and lipid and protein oxidation are not coupled under these conditions.

Keywords: pork, lipid oxidation, protein oxidation, dietary soybean oil, storage.

Introduction

Animal fat is being replaced by vegetable oils in feed for slaughter pigs. Most vegetable oils are more unsaturated than animal fat and feeding with a greater proportion of unsaturated fat has been found to change the fatty acid profile of pork meat (Jensen, Skibsted, & Bertelsen, 1998; Leszczynski *et al.*, 1992a; Miller, Shackelford, Hayden, & Reagan, 1990; Rhee, Ziprin, Ordonez, & Bohac, 1988; Sandstrom, Bugel, Lauridsen, Nielsen, Jensen, & Skibsted, 2000). Increasing the proportion of polyunsaturated fatty acids in meat is recommended as polyunsaturated fatty acids play a favourable role in the prevention of many human diseases (Sandstrom *et al.*, 2000; Solà *et al.*, 1997). However, the increased unsaturation of the fat causes the meat products to be more susceptible to lipid oxidation (Bremner, Ford, MacFarlane, Ratcliff, & Russell, 1976; Daza, Rey, Ruiz, & Lopez-Bote, 2007; Leszczynski *et al.*, 1992b; Rhee *et al.*, 1988; Warnants, VanOeckel, & Boucque, 1996). Limited research has, however, been reported on the effect of animal diets with increased levels of unsaturated fatty acids on protein oxidation in meat (Mercier, Gatellier, Viau, Remignon, & Renerre, 1998; Petron, Raes, Claeys, Lourenco, Fremaut, & De Smet, 2007; Ventanas, Ventanas, Tovar, García, & Estévez, 2007).

Lipid oxidation in meat leads to quality loss by formation of rancid odours and off-flavours and reduction of nutritive value (Bremner *et al.*, 1976; Kanner, 1994). Protein oxidation results in modifications of functional properties of the proteins due to formation of protein carbonyls, hydroperoxides, and sulfoxides, and further to protein fragmentation, cross-linking and aggregation, and to decrease protein solubility (Davies, 1987; Davies & Dean, 2003; Dean, Fu, Stocker, & Davies, 1997; Meucci, Mordente, & Martorana, 1991). Proteolytic enzymes are being inactivated by oxidation, and oxidative modification of proteins has been found to result in reduced tenderness and juiciness of meat (Lund, Lametsch, Hviid, Jensen, & Skibsted, 2007b; Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004a; Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004b).

In most of the studies in which both protein and lipid oxidation have been investigated, oxidation has been initiated chemically by e.g. iron-catalysis (Mercier, Gatellier, & Renerre, 2004; Tokur & Korkmaz, 2007), whereas only a few studies have investigated the correlation between lipid and protein oxidation during storage without addition of prooxidants (Haak, Raes, Smet, Claeys,

Paelinck, & De Smet, 2006; Mercier *et al.*, 1998; Ventanas, Estevez, Tejeda, & Ruiz, 2006). We have previously found that storage of beef patties in a high-oxygen atmosphere increases oxidation with lipid oxidation being faster than protein oxidation (Lund, Hviid, & Skibsted, 2007a). Hence, in the present study, oxidation was initiated by storage of the pork patties in a modified atmosphere with 80% O₂/20% CO₂ as used in retail trade to reduce microbial growth and increase shelf-life during chill storage (Asensio, Ordonez, & Sanz, 1988; Enfors, Molin, & Ternström, 1979; Nissen, Sørheim, & Dainty, 1996).

In the present study it has accordingly been possible to explore the consequences for protein and lipid oxidation of increasing the unsaturation of pork lipids both in low and high fat pork patties during chilled storage in a high oxygen atmosphere.

Materials and Methods

Chemicals

Trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), tris(hydroxyamino)methane (tris), and thiobarbituric acid (TBA) were obtained from Merck, Darmstadt, Germany. Acetone and ethyl acetate were obtained from Lab-scan Ltd., Dublin, Ireland. 2,4-Dinitrophenylhydrazine (DNPH), sodium dodecylsulphate (SDS) and malondialdehyde(diethylacetal) (TEP) were obtained from Bie & Berntsen, Rødovre, Denmark. Sodium pyrophosphate, tris-maleate, propylgallate, and ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) were from Acros Organics, Geel, Belgium. Bovine serum albumin (BSA) and guanidine hydrochloride were from Sigma Chemical Co., St. Louis, USA. Absolute ethanol was obtained from Danish Distillers, Aalborg, Denmark. Water was purified through a Millipore Q-plus system (Millipore Corp., Bedford, MA). All chemicals were of analytical grade.

Pig diets and fatty acid composition

Fourteen female pigs (DxLY, crossbreeds of Duroc males with Landrace x Yorkshire females) from Danbred (The Danish Pig Breeding Programme) were fed a standard diet based on barley without addition of soybean oil (control) and with addition of 2% soybean oil (soybean) using standard conditions for Danish pig farming (seven pigs for each diet). The fatty acid composition of the diets was determined by GC using a Hewlett Packard 6890 series G1530A gas chromatograph (Palo Alto, CA, USA) and a DB-23 capillary column (60 m x 0.25 mm x 0.25 µm) from J&W Scientific, Folsom, CA, USA after conversion to methyl esters using standard measures. The fatty acid composition of the diets is given in Table 1. The amount of vitamin E in the two diets was 58.7 mg/kg (control) and 62.1 mg/kg (soybean). The pigs were slaughtered at a commercial Slaughterhouse in Grindsted, Denmark. The live weight at slaughter was 105-110 kg. The *quadriceps femoris* was used for preparation of patties with two levels of fat content: i) no addition of back fat (low fat), and ii) addition of approx. 15% back fat (high fat), resulting in a total of four different combinations of diet and fat content in the pork patties. The fatty acid composition of the back fat was determined as described above after melting the fat in a microwave oven at 450 W for 3 min (Table 2).

Preparation, Characterization, Packaging, and Storage of Pork Patties

The meat was minced either alone (low fat) or together with approximately 15% back fat (high fat) in a Fleischwolf X70 meat grinder (2 mm knife) (Scharfen, Witten, Germany). After mincing the meat was mixed in a Bjørn Varimixer (A/S Wodshow & Co., Brøndby, Denmark) for 4 min. For each combination of diet and fat content the minced meat was characterised (Table 3) by determination of total fat content according to the NMKL method no. 131 (Nordisk Metodikkomite for Levnedsmidler, 1989), total protein content was determined using the AOAC Official Method 981.10 (AOAC International, 1983), water content was determined using NMKL method no. 23, 2nd ed (Nordisk Metodikkomite for Levnedsmidler, 1974), and total heme pigment concentration was determined according to the method described by Hornsey (Hornsey, 1956). The α -tocopherol content was determined according to the procedure of Jensen et al. (1997) with muscle α -tocopherol extraction procedure described by Butriss and Diplock (1984).

After mixing pork patties of 125 ± 1 g were formed using a circular template ($d = 9.5$ cm). One patty was placed in each polypropylene (PP) tray type 71-43A (Færch Plast, Denmark) and packed with 80% O₂/20% CO₂ on a Multivac T200 (Sepp Haggemüller GmbH & Co., Germany) using TOPSEALTM PP MAP AF 57 film (Færch Plast, Denmark). The packaging gas was from Yara Industrial A/S, Denmark. All combinations of diet and fat content were performed in duplicate giving two trays for each combination of diet and fat content. The preparation and packaging of meat patties were performed in a temperature controlled room at max 7-10°C.

The meat patties were stored at 4°C in the dark for 7 days. The gas composition in the packs was measured with a CheckMate 9900 (PBI Dansensor, Ringsted, Denmark) prior to sample take-out in order to check the packs for leaks. Each patty was divided into four pieces, mixed thoroughly by hand and stored at -80°C until further analysis.

Free protein thiol groups

5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) was used for determination of free thiols in proteins (Ellman, 1959). Briefly, 2.0 g muscle were homogenized in 50 ml 5.0% SDS in 0.10 M tris buffer (pH 8.0) using Ultra Turrax. The

homogenates were placed in a water bath at 80°C for 30 min and centrifuged at 1200 g for 20 min in a Sigma 2-5 centrifuge (Buch & Holm, Herlev, Denmark). The supernatants were filtered through Filtrak filter paper 3w, 100mm, 65g/m² (Spezialpapier FILTRAK GmbH, Niederschlag, Germany). Protein concentration of the filtrate was determined by measuring absorbance at 280 nm using a standard curve prepared from 0-3 mg/ml BSA. The filtrates did not absorb light >300nm and myoglobin was therefore found not to interfere with the assay.

The filtrates were diluted to a concentration of 1.5 mg/ml with the buffer used for homogenization and assayed according to Liu and Xiong (2000) by mixing 0.50 ml sample, 2.0 ml 0.10 M tris buffer (pH 8.0) and 0.50 ml 10 mM DTNB in 0.10 M tris buffer (pH 8.0). Absorbance at 412 was measured after 30 min against an aqueous reference solution of 0.50 ml 5% SDS, 2.0 ml 0.10 M tris buffer (pH 8.0) and 0.50 ml 10 mM DTNB in 0.10 M tris buffer (pH 8.0). Thiol content was calculated as μM thiol/mg protein. Triplicate measurements were made for each meat sample and mean values were used for further statistical analysis.

Protein carbonyl content

Protein carbonyl content was determined by derivatization with DNPH as described by Fagan, Slecka and Sohar (1999). Briefly, muscle samples (3.0 g) were homogenized in 15 ml of pyrophosphate buffer (pH 7.4) (PB) consisting of 2.0 mM Na₄P₂O₇, 10 mM tris-maleate, 100 mM KCl, 2.0 mM MgCl₂ and 2.0 mM EGTA by using an Ultra Turrax. Chromophores from the muscle were removed by washing the sample with HCl-acetone (3:100) (V/V) twice followed by washing with 10% TCA twice. The muscle samples were derivatized for 30 min with 10 mM DNPH in 2.0 M HCl and protein blanks were prepared by adding 2.0 M HCl instead of DNPH solution. Excess DNPH was removed by washing with 5.0 ml 20% TCA once followed by three washings with 5 ml 10 mM HCl in ethanol-ethyl acetate (1:1) (V/V). The pellets were solubilized in 1.0 ml 6.0 M guanidine hydrochloride in 20 mM potassium dihydrogen phosphate (pH 2.3) overnight at 5°C. Absorbance at 280 nm and 370 nm of the samples was measured at room temperature and the carbonyl content in nmol/mg protein was calculated as described by Levine, Williams, Stadtman and Shacter (1994) using an absorption coefficient at 370 nm of 22000 M⁻¹cm⁻¹ for the formed hydrazones (Johnson, 1953). The blank value was subtracted from the

corresponding sample value. Triplicate measurements were made for each muscle sample, and mean values were used for further statistical analysis.

Secondary lipid oxidation products

Secondary lipid oxidation products were evaluated using 2-thiobarbituric acid as described by Vyncke (1970; 1975) and with modifications according to Sørensen and Jørgensen (1995). Briefly, 5.0 g meat was homogenized in 15 ml 7.5% TCA with 0.10% propylgallate and 0.10% EDTA using an Ultra Turrax for 45 s at 13500 rpm and filtered. 5.0 ml of the filtrate was mixed with 5.0 ml 0.020 M thiobarbituric acid (TBA) and incubated at 100°C in a waterbath for 40 min. Absorbance was measured at 532 nm and 600 nm at room temperature. Results are expressed as 2-thiobarbituric reactive substances (TBARS) in μmol malondialdehyde/kg dry matter using a standard curve prepared from TEP, and mean values of two independent determinations are given. Dry matter was determined by incubating 2.0 g meat at 104°C for 4 hours. The water content of the sample was determined as the weight loss after 4 hours. Triplicate measurements were made for each meat sample and mean values were used for further statistical analysis.

Statistical Analysis

Statistical analysis were performed using the SAS[®] 9.1 package, SAS Institute, Inc., USA. Data was analysed by analysis of variance using proc glm. Diet, fat content, and interaction between diet and fat content were included as fixed effects. Means were used to compare differences and LSD was applied to compare the mean values of diet and fat content.

Results

Pigs fed an experimental diet with 2% soybean oil added to the standard feed had reduced level of saturated and monounsaturated fatty acids and an increased level of polyunsaturated fatty acids ($p < 0.001$) in the back fat compared to the back fat of the pigs fed a standard diet without soybean oil (Table 2). In Denmark, additional fat is often added to pork prior to mincing to produce a minced meat product sold in the supermarkets with a higher fat percentage in order to reduce the price. Hence, in this study additional back fat obtained from pigs fed the same of the two diets was added to obtain a product that closely resembles products sold Danish supermarkets in addition to the low fat product obtained by mincing the meat. Minced pork with 2% (low fat) and approx. 14% fat (high fat) was accordingly obtained for each of the two diet groups (Table 3).

The effect of diet and fat content on protein oxidation in pork patties was investigated by determination of loss of free protein thiol groups (Figure 1). No difference between the four treatments was found within the first four days of storage. After 7 days of storage a significant decrease ($p = 0.0424$) in the thiol content of high fat pork patties was found indicating that a higher fat content in pork patties increases protein oxidation. This decrease in thiol content was independent on feeding regime, which implies that feeding diets with increased content of unsaturated fat does not affect protein oxidation within 7 days of chill storage.

Determination of protein carbonyl content is generally used to describe the extent of protein oxidation in biological samples and the pork patties stored for 0 and 7 days were further investigated using this method (Table 4). Results showed a slight increase in carbonyl content over storage time but no significant difference between experimental diets or fat content.

Lipid oxidation in the pork patties was evaluated by TBARS analysis, which clearly showed that a diet containing 2% soybean oil increased lipid oxidation in pork patties during storage (Figure 2). A similar observation has previously been reported for pork chops (Leszczynski *et al.*, 1992b). An increase in fat content in the pork patties did not influence lipid oxidation after 7 days of storage for pork from both feeding regimes. In contrast, on day 4 the pork patties with a low fat content showed more lipid oxidation than pork patties with a high fat content.

Discussion

Dietary soybean oil in the pig feed increased the level of polyunsaturated fat in the pork patties produced and increased lipid oxidation but did not affect protein oxidation in the product. Analysis of free thiol groups in the proteins showed that oxidation increased in high fat pork patties independent on the feeding regime but the observed differences in thiol oxidation are rather small. Analysis of proteins carbonyls showed no significant effects of dietary fat and fat content. The level of carbonyls was < 1 nmol/mg protein which is consistent with un-oxidized tissue showing that only limited protein oxidation has taken place in the pork patties (Reznick & Packer, 1994). These low values of carbonyl content have previously been reported for chill stored fresh lamb (Petron *et al.*, 2007) and fresh pork (Lund *et al.*, 2007b). TBARS values in the present study reached a maximum value of $3.6 \mu\text{mol/kg}$ dry matter, which can be converted into 0.3 mg/kg meat and is similar to the values reported by Haak *et al.* (2006) for fresh pork fed with 2% soybean oil. The limiting TBARS value from where off-flavours in pork can be detected has been reported to be $0.5\text{-}1.0 \text{ mg/kg}$ meat (Tarladgis, Watts, & Younathan, 1960), which indicates that the observed TBARS values are not relevant from a sensory perspective.

Dietary soybean oil has previously been shown to increase the TBARS and protein carbonyl content in two different turkey muscles stored for 9 days compared to dietary tallow and dietary rapeseed oil, which both are less polyunsaturated than soybean oil. However, contradictory results were obtained when free thiol content in the protein was measured as a lower thiol content was found for dietary rapeseed and tallow compared to soybean oil (Mercier *et al.*, 1998). Furthermore, in a later study where oxidation was induced by Fe^{3+} /ascorbate in the microsomal fraction from turkey fed similar diets, dietary soybean oil enhanced lipid oxidation (measured as TBARS), but reduced protein oxidation (measured as protein carbonyls and thiols) compared to dietary tallow and rapeseed oil (Mercier, Gatellier, Vincent, & Rennerre, 2001). The results show that the actual initiation of oxidation has a strong impact on the correlation between lipid and protein oxidation. The nature of the initiation of oxidation has previously been shown to have influence on the interaction between lipid and protein oxidation. A NADH-Fe enzymic system has been shown to oxidize lipids in cod sarcoplasmic reticulum to a greater extent than

protein sulfhydryls, while in a non-enzymic ascorbate-Fe system the opposite effect was observed (Soyer & Hultin, 2000). Furthermore, the concentration of each component in the system used to initiate oxidation has been shown to influence the interaction between lipid and protein oxidation. A hydroxyl radical generating system (10 μM FeCl_3 , 0.1 mM ascorbic acid, and 0.05-5.0 mM H_2O_2) with 1.0 mM H_2O_2 resulted in the maximum development of TBARS while a metmyoglobin-oxidizing system (0.05-5.0 mM) with 0.5 mM metmyoglobin resulted in the highest development of protein carbonyls. Changing the concentrations of the reactants of the system used to initiate oxidation, however, altered this conclusion (Park, Xiong, Alderton, & Ooizumi, 2006). Hence, the choice of system used to initiate oxidation and even the concentrations of the reactants together with which components of meat from which species are investigated, and which methods are selected for detection of oxidation should be carefully considered when investigations of the interaction between lipid and protein oxidation are compared.

The fact that protein oxidation in the present study seems to be unaffected by an increase in polyunsaturated fat in the pork patties indicates that i) there is no simple correlation between lipid oxidation and protein oxidation and that ii) the rate of lipid oxidation is increased more than the rate of protein oxidation in meat stored in high oxygen atmosphere. The latter conclusion may be altered when more sensitive methods used for detection of protein oxidation are becoming available; however, lipid oxidation occurs more rapidly than protein oxidation. A faster development of secondary lipid oxidation products (measured as TBARS) compared to protein carbonyls has previously been observed in beef patties stored in high oxygen atmospheres (Lund *et al.*, 2007a). Development in TBARS and carbonyl content was much more rapid for the beef compared to the pork patties analyzed in the present study, even though the concentration of oxygen in the packaging atmosphere was the same for the two storage experiments. The faster development of oxidation products is consistent with higher pigment content in the beef patties previously studied (138 ± 5 ppm) compared to pigment content of the pork patties used for the present study where a content of 45-53 ppm was found in agreement with a pivotal role of heme pigments as initiator of lipid oxidation in muscle systems.

In general, the level of oxidation was rather limited in the pork patties as the maximum TBARS level was only 3.6 $\mu\text{mol/kg}$. Modified atmosphere packaging

with a high level of oxygen has previously been shown to cause higher levels of TBARS in pork without α -tocopherol supplementation to the animals (Jensen, Flensted-Jensen, Skibsted, & Bertelsen, 1998). The α -tocopherol status of the meat is highly dependent on α -tocopherol content of the feed (Jensen *et al.*, 1998). In the present study the α -tocopherol content in the pork meat prior to storage was approximately 3 $\mu\text{g/g}$ meat, which is high compared to pork from pigs raised on a diet without α -tocopherol supplementation (1.6 $\mu\text{g/g}$ meat) (Jensen *et al.*, 1998). α -Tocopherol supplementation has been shown to reduce protein oxidation in meat (Batifoulier, Mercier, Gatellier, & Renerre, 2002; Mercier *et al.*, 1998; Rowe *et al.*, 2004a), and thus it is possible that the high level of α -tocopherol in the pork patties studied has caused a reduced protein oxidation. Dietary α -tocopherol has shown to decrease the carbonyl content of sarcoplasmic proteins but not of myofibrillar proteins in irradiated beef steaks (Rowe *et al.*, 2004a), and the effect of α -tocopherol supplementation has also been shown to vary between turkey muscles (Mercier *et al.*, 1998). The mechanism behind protection of proteins against oxidation by the lipophilic α -tocopherol is not obvious and deserves further attention.

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Figure Legends

Figure 1 Free protein thiol groups in pork patties stored for up to 7 days at 4°C in the dark in modified atmosphere packaging with 80% O₂/20% CO₂. High fat (circular symbols) and low fat (square symbols) pork patties were obtained from pigs fed a standard diet without soybean oil addition (closed symbols) and the same diet added 2% soybean oil (open symbols).

Figure 2 Secondary lipid oxidation products measured as TBARS in pork patties stored for up to 7 days at 4°C in the dark in modified atmosphere packaging with 80% O₂/20% CO₂. High fat (circular symbols) and low fat (square symbols) pork patties were obtained from pigs fed a standard diet without soybean oil addition (closed symbols) and the same diet added 2% soybean oil (open symbols).

Table 1

Table 1 Fatty acid composition (%) of the experimental diets. SFA: Saturated fatty acids, MUFA: Mono unsaturated fatty acids, PUFA: poly unsaturated fatty acids.

Fatty acid	Standard diet	Standard diet with 2% soybean oil
Total SFA	24.4	20.05
C14:0	0.24	0.14
C15:0	0.11	0.06
C16:0	21.63	16.08
C18:0	1.89	3.03
C20:0	0.21	0.31
C22:0	0.30	0.42
C24:0	0.03	0.01
Total MUFA	13.98	19.33
C16:1	0.18	0.13
C18:1	13.13	18.73
C20:1	0.53	0.40
C22:1	0.14	0.08
Total PUFA	61.50	60.58
C18:2	55.47	54.31
C18:3	5.68	6.09
C20:2	0.12	0.07
C22:5	0.005	0.008

Table 2

Table 2 Average fatty acid composition (% weight/weight of total fatty acid in the sample) in the back fat of the pigs fed with a standard diet without soybean oil and the same diet with 2% soybean oil added (tr: traces (<0.1)). Significance levels: ns (non-significant), * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$).

Fatty acid	Standard diet	Standard diet with 2% soybean oil	Significance
Total SFA	40.9	37.6	***
C14:0	1.3	1.2	*
C16:0	25.1	23.0	***
C18:0	14.1	12.9	***
C20:0	0.2	0.2	ns
Total MUFA	46.1	41.2	***
C16:1	2.3	1.8	***
C18:1 (n-9)	40.2	36.3	***
C18:1 (n-7)	2.8	2.3	***
C20:1	0.8	0.8	ns
Total PUFA	11.6	20.0	***
C18:2	10.3	17.3	***
C18:3	0.9	1.6	***
C20:2	0.5	0.8	***
C20:3	tr	0.2	***
C22:4	tr	0.1	*

Table 3

Table 3 Chemical composition of the minced pork from pigs fed with a standard diet without soybean oil and the same diet with 2% soybean oil added.

Feeding	Standard diet	Standard diet	Standard diet with 2% soybean oil	Standard diet with 2% soybean oil
Fat content	Low fat	High fat	Low fat	High fat
Fat content (%)	2.3 ± 0.4	13.1 ± 0.7	2.2 ± 0.6	14.6 ± 0.7
Protein content (%)	21.2 ± 0.5	18.6 ± 0.5	21.3 ± 0.5	18.3 ± 0.7
Water content (%)	76.1 ± 0.4	67.8 ± 0.4	76.0 ± 0.4	66.7 ± 0.4
Pigment (ppm)	47.8 ± 2	47.3 ± 2	52.8 ± 2	44.6 ± 2
α-Tocopherol (µg/g)	3.2 ± 0.6	-	3.4 ± 0.6	-

Table 4

Table 4 Protein carbonyl content in high fat and low fat pork patties stored for 7 days at 4°C in the dark in modified atmosphere packaging with 80% O₂/20% CO₂. Pork was obtained from pigs fed with a standard diet without soybean oil and the same diet with 2% soybean oil added.

Diet and fat content	Day 0	Day 7
0% soybean oil, low fat	0.81 ± 0.04	0.86 ± 0.17
0% soybean oil, high fat	0.72 ± 0.04	0.77 ± 0.12
2% soybean oil, low fat	0.93 ± 0.02	0.95 ± 0.02
2% soybean oil, high fat	0.79 ± 0.03	0.85 ± 0.08

Figure 1

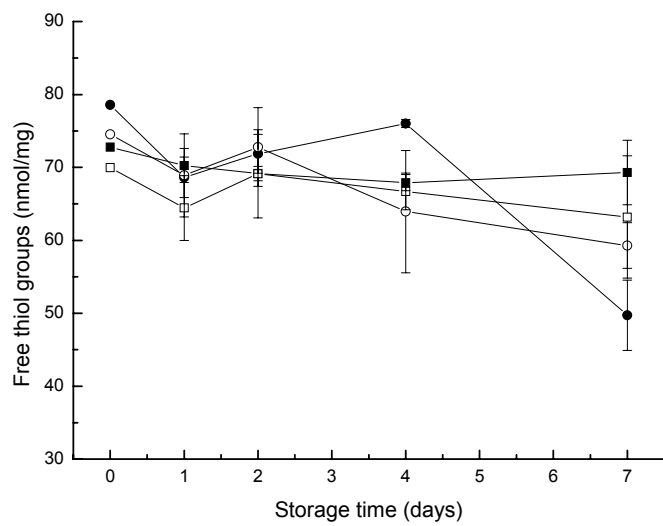
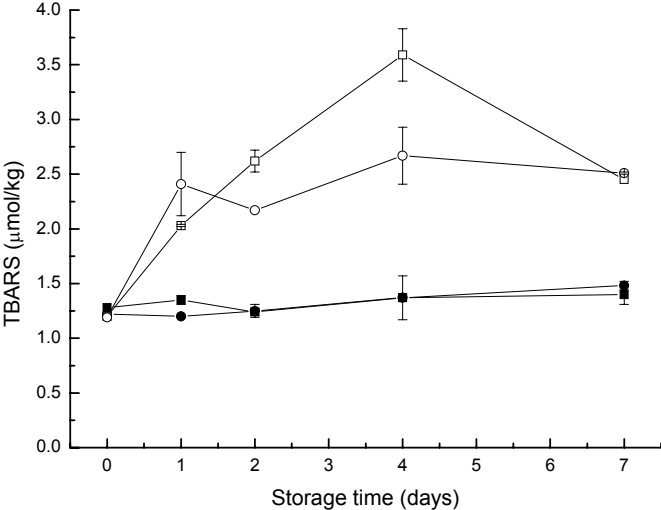


Figure 2



Oxidation of myosin by heme proteins generates myosin radicals and protein cross-links

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Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTNP, 2,2'-dithiobis(5-nitropyridine); DTT, dithiothreitol; HRP, horseradish peroxidase; Mb, myoglobin in its +3 (met) oxidation state; MNP, 2-methyl-2-nitrosopropane; NEM, N-ethylmaleimide; RS[•], thiyl radicals; TEMPO, 2,2,6,6-tetramethyl-piperidine-1-oxyl radical; tris, tris(hydroxymethyl) aminomethane.

SYNOPSIS

Previous studies have reported that myosin can be modified by oxidative stress and particularly by activated heme proteins. These reactions have been implicated in changes in the properties of this protein in food samples (changes in meat tenderness and palatability), in human physiology (alteration of myocyte function and force generation), and disease (e.g. cardiomyopathy, chronic heart failure). The oxidant species, mechanisms of reaction and consequences of these reactions are incompletely characterized. In this study the nature of the transient species generated on myosin as a result of reaction with activated heme-proteins (horseradish peroxidase/H₂O₂ and metmyoglobin/H₂O₂) has been investigated by EPR spectroscopy, amino acid consumption and product formation has been characterized by HPLC, and changes in protein integrity by SDS-PAGE. Multiple radical species have been detected by EPR in both the presence and absence of spin traps. Evidence has been obtained for the presence of thiyl, tyrosyl and other unidentified species on myosin as a result of damage transfer from oxidized myoglobin or horseradish peroxidase. The generation of thiyl and tyrosyl radicals is consistent with the observed consumption of Cys and Tyr residues, the detection of di-tyrosine by HPLC, and detection of both reducible (disulphide bond) and non-reducible cross-links between myosin molecules by SDS-PAGE. The time course of radical formation on myosin, product generation and cross-link induction are consistent with these being interlinked processes. These changes are consistent with the altered function and properties of myosin in muscle tissue exposed to oxidative stress, arising from disease or food processing.

Keywords: myosin, protein oxidation, radicals, myoglobin, horseradish peroxidase, EPR spectroscopy

INTRODUCTION

Oxidation of proteins by radicals, in the presence of O_2 , can result in major alterations to the physical and chemical nature of the protein, with these changes including oxidation of side-chain groups, backbone fragmentation, cross-linking, unfolding, changes in hydrophobicity and conformation, altered susceptibility to proteolytic enzymes, and formation of new reactive groups (e.g. 3,4-dihydroxyphenylalanine (DOPA), carbonyls, and hydroperoxides). These alterations can result in loss of structural and enzymatic activity of the protein and hence biological perturbations [1-3].

Myosin is the most abundant protein in muscle tissue and is located in the myofibrillar part of the muscle. Several studies show that myosin is susceptible to oxidation causing intermolecular cross-linking and aggregation of the protein [4-7]. The type of cross-linking seems to be highly dependent on how oxidation on myosin is initiated. Oxidation with myoglobin/ H_2O_2 has been reported to result in non-disulfide bond formation whereas oxidation with a metal-catalyzed (Fenton-like) system results, primarily, in the formation of disulfide bonds. Evidence has been presented for the generation of thiyl radicals (RS^\cdot) on myosin on reaction with the mild oxidant Ce(IV) in the presence of the spin trap *N-tert-butyl- α -phenylnitrone* (PBN) [8]. However the relevance of such radical formation to damage induced by heme proteins is unclear.

Initiation of oxidation of proteins by myoglobin/ H_2O_2 and horseradish peroxidase/ H_2O_2 has attracted considerable attention due to high abundance of these heme proteins in many cell types and tissues and the (likely) continuous formation of hydrogen peroxide *in vivo* as a result of the plethora of sources of superoxide radicals within, and on the surface of, cells (e.g. via electron leakage from mitochondrial and endoplasmic reticulum electron transport chains, NADPH oxidases, lipoxygenases, xanthine oxidase [9]). Enzymatic (catalysed by the multiple forms of superoxide dismutase) and spontaneous dismutation of such radicals yields H_2O_2 and O_2 . H_2O_2 -activation of peroxidases and metmyoglobin (a pseudo-peroxidase) is known to form radicals on these proteins, as a result of electron transfer from the protein to the oxidized heme group [10]. A number of sites have been reported for these species (e.g. Tyr, Trp, Cys), with this being species- and sequence-dependent [11-15]. It has been demonstrated that such protein-derived radicals are able to transfer damage to other proteins via radical transfer reactions [16-19]. Thus long-lived radicals have been detected on BSA and a number of other

proteins incubated with H₂O₂-activated heme proteins [17]. The structural and functional consequences of these radical transfer reactions, and their role in disease have yet to be fully elucidated.

It is well established that oxidation of skeletal muscle myosin occurs in a number of human diseases, including chronic heart failure [20], in animal models of diabetes [21] and as a result of muscle ageing [21,22]. Evidence has also been presented for modification of myosin light chains during myocardial “stunning” as a result of short periods of ischaemia and subsequent reperfusion in rabbits [23,24]. Myosin oxidation has also been detected in intact myofibrils exposed to myoglobin and H₂O₂ [25], and with isolated myosin [6,20,26]. A number of other oxidizing systems, in addition to H₂O₂-activated heme proteins, have been proposed to play a role in myosin damage including peroxynitrite [20,22], hydroxyl radicals [27] and reactive aldehydes [22].

Damage to skeletal muscle components is also of considerable significance to the food industry as oxidative modifications that occur during food processing and storage can lead to changes in the physical and functional properties of myosin. Alterations in protein conformation and the formation of protein aggregates through intermolecular cross-linking as a result of oxidation decrease gel-forming ability, protein solubility, and water-holding capacity, which negatively influence sensory characteristics (e.g. juiciness and tenderness) and palatability [5,28-30]. Biochemical properties such as the ATPase activity of myosin, which plays a role in muscle contraction, has also been found to change with oxidation of myofibrillar proteins [31].

In the study reported here the mechanism of myosin oxidation by two enzymatic systems (myoglobin/H₂O₂ and horseradish peroxidase/H₂O₂) has been examined using EPR spectroscopy (both direct and using spin trapping), amino acid consumption and product analysis by HPLC and SDS-PAGE. Comparative studies were also carried out using a range of other oxidant systems. It is shown that a number of different radicals are generated on myosin (thiyl, tyrosyl and unidentified species) by H₂O₂-activated heme proteins, that these reactions result in the depletion of Cys and Tyr residues, the formation of di-tyrosine, and the generation of both reducible and non-reducible protein cross-links. These reactions may contribute to the loss of meat palatability and tenderness in food samples, and the loss of function of myosin in certain pathologies and ageing.

EXPERIMENTAL

Materials

Myoglobin from horse heart (> 90%), adenosine triphosphate (ATP), N-ethylmaleimide (NEM), dithiothreitol (DTT), mercaptoacetic acid, methanesulphonic acid (packed under Ar), o-phthaldialdehyde (with 2-mercaptoethanol added) and 2-methyl-2-nitrosopropane dimer (MNP) were obtained from Sigma (St. Louis, USA). Hydrogen peroxide 30%, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 2,2'-dithiobis(5-nitropyridine) (DTNP) were obtained from Merck (Darmstadt, Germany). Horseradish peroxidase (HRP) was obtained from Roche Diagnostics (Basel, Switzerland). 2,2,6,6-tetramethyl-piperidine-1-oxyl radical (TEMPO) was obtained from Molecular Probes (Leiden, Netherlands). Ethylenbis(oxyethylenenitrilo) tetraacetic acid (EGTA) was purchased from ACROS Organics (New Jersey, USA). Nitrilotriacetic acid and $(\text{NH}_4)_2\text{Ce}(\text{SO}_4)_2$ were obtained from BDH (Poole, UK). Buffer solutions were all of analytical grade. HPLC solvents were purchased from EMD Chemicals (Merck, Kilsyth, Vic, Australia).

Myosin preparation

Myosin was prepared from porcine *longissimus dorsi* muscle which had been frozen immediately in liquid nitrogen after slaughter, followed by storage at $-18\text{ }^\circ\text{C}$. Myosin was isolated as described previously [32] with minor modifications. Thus 50 g of muscle, trimmed of adhering fat and connective tissue, was homogenised using a Ultra-Turrax apparatus and 3 volumes (v/w) of a modified Guba-Straub solution (0.3 M KCl, 0.1 M KH_2PO_4 , 0.05 M K_2HPO_4 and 1 mM EGTA, pH 6.4). The crude myofibrillar extract was diluted with 3 volumes of water (v/v) and filtered through a 400 μm plastic mesh. Water (6.5 volumes (v/v)) was added to the filtrate and the samples allowed to precipitate on ice for > 2 hr. The clear supernatant was decanted and the remaining precipitate was centrifuged at 2000 g for 45 min (HiCen21 centrifuge, Herolab, Wiesloch, Germany). The precipitate was subsequently dissolved in 100 ml 0.5 M KCl, 1 mM EGTA, 10 mM Tris (pH 7.5), treated with 2 mM ATP and 5 mM MgCl_2 , and centrifuged at 70000 g for 30 min (Beckmann Optima TM LE-80IC Ultracentrifuge, Beckman Coulter, Inc., Fullerton, USA) in order to remove residual actomyosin. Myosin present in the supernatant was precipitated by ammonium sulphate. The fraction precipitating between 38% and 50% saturation

was collected and re-suspended in the minimum possible volume of 0.5 M KCl, 1 mM EGTA, 10 mM Tris (pH 7.5), and dialyzed overnight against three changes of the same buffer, without EGTA, using a 6-8000 g/mol cut-off dialysis tube (Spectra/Por membrane, Spectrum, Rancho Dominguez, USA). Purified myosin stocks were stored at $-80\text{ }^{\circ}\text{C}$ in small portions. On the day of use, stock myosin was thawed, diluted with 2 ml phosphate buffer (5 mM, pH 7.0, $I = 1.0$), and centrifuged at 2100 g (Beckman Coulter, Inc., Fullerton, USA) for 5 min. The resulting solution was kept at $4\text{ }^{\circ}\text{C}$ until used. The myosin concentration was determined by measuring absorbance at 280 nm and using $A_{280(1g/l)} 0.496$.

Oxidation reactions

MNP (1.0 M) was prepared daily in acetonitrile and protected from light. The final concentrations of MNP in the reaction solutions did not cause precipitation of myosin. All other solutions were prepared in a 5 mM phosphate buffer (pH 7.0, $I = 1.0$) using water passed through a four-stage Milli Q system (Millipore Corp., Bedford, MA) equipped with a $0.2\text{ }\mu\text{m}$ pore-size final filter. Stock solutions of myoglobin, horseradish peroxidase and hydrogen peroxide were prepared daily. Myoglobin was purified using a PD-10 column (Pharmacia, Uppsala, Sweden) and the concentration determined by its optical absorbance at 525 nm using $\epsilon_{525\text{nm}} 7700\text{ M}^{-1}\text{ cm}^{-1}$ [33]. H_2O_2 concentrations were determined from their absorbance at 240 nm using $\epsilon_{240\text{nm}} 39.4\text{ M}^{-1}\text{ cm}^{-1}$ [34]. All concentrations quoted in the text are final concentrations after mixing.

Blocking of thiol groups on myosin

Thiol groups in myosin were blocked by reaction with 5 mM N-ethylmaleimide (NEM) for 90 min at $4\text{ }^{\circ}\text{C}$. The extent of blocking was measured using DTNB [35]; under these conditions $> 95\%$ of the thiols were blocked.

EPR spectroscopy

Samples for EPR spectroscopy were prepared by mixing 10-13 μM myosin, 300 μM myoglobin (Mb)/300 μM H_2O_2 or 100 μM horseradish peroxidase (HRP)/10 mM H_2O_2 in this order at $22\text{ }^{\circ}\text{C}$. Reaction times refer to the time after H_2O_2 addition. Additional oxidation systems were used consisting of i) 0.10 mM FeCl_3 , 1.0 mM sodium

ascorbate, 20 mM H₂O₂, ii) 0.50 mM Ce(IV)-solution prepared from 0.01 M nitrilotriacetic acid and 0.05 M (NH₄)₂Ce(SO₄)₂ [8], and iii) 10 mM peroxyxynitrite prepared according to [36]. For experiments at 22 °C, the incubation mixture was subsequently transferred to an EPR flat cell (Wilmad, Buena, New Jersey, USA). For low temperature EPR analysis, the reaction solution was frozen in liquid nitrogen in cylindrical EPR cells 40 s after addition of H₂O₂. EPR spectra were recorded on a Bruker EMX X-band spectrometer equipped with 100 kHz modulation and a standard 4103TM/9702 cavity. Typical spectrometer settings for 22 °C analysis were: gain, 1.0 x 10⁵; modulation amplitude, 2 G; time constant, 163.840 ms; sweep time, 83.886 s; center field, 3480 G; field sweep width, 100 G; microwave power, 2.5 mW; frequency, 9.7 GHz, with four acquisitions averaged. Typical spectrometer settings for 77 K analysis were: gain, 1.0 x 10⁵; modulation amplitude, 2 G; time constant, 163.840 ms; sweep time, 83.886 s; center field, 3360 G; field sweep width, 160 G; microwave power, 2.5 mW; frequency, 9.4 GHz, with eight acquisitions averaged. Spectral manipulations and signal integrations were performed using the program WINEPR.

Determination of thiol oxidation

Thiol concentrations in samples containing 13 μM myosin with varying concentrations of Mb and H₂O₂ (ratio 1:1) or HRP and H₂O₂ (ratio 1:1 and 1:100) were determined at 22 °C, after incubation for 2 min, using DTNP as described in [37]. Absorbance at 386 nm was measured 5 min after addition of DTNP to the sample. Blanks where individual components were omitted were measured in parallel. The thiol concentration was calculated by using ϵ_{386} 14.0 mM⁻¹.

Quantification of amino acid consumption

Amino acid analysis of parent and oxidized protein samples was carried out, by HPLC with fluorescence detection after derivatisation with *o*-phthaldialdehyde, on amino acid hydrolysates generated by methanesulphonic acid as described previously [35]. Data are reported as moles of amino acid lost per mole of Ala to compensate for any loss of material during processing.

Quantification of tyrosine oxidized products

Myosin was incubated with myoglobin (Mb) or horseradish peroxidase (HRP), and H₂O₂ at 22 °C, with aliquots (100 µl) withdrawn after 0, 0.3, 10, 60 min, and 24 hours and stored at –80 °C until analysis. Hydrolysis of the protein samples to free amino acids was performed according to [38]. Forty microliters of sample were loaded onto a Shimadzu HPLC system equipped with a Zorbax ODS column with a Pelliguard guard column and eluted using a gradient solvent system [38]. The eluate was monitored in series by UV (set at 280 nm to quantify parent *p*-tyrosine) and fluorescence detectors (λ_{ex} 280 nm / λ_{em} 320 nm for DOPA; λ_{ex} 280 nm / λ_{em} 410 nm for di-tyrosine) and compared to standard curves prepared using authentic materials. Data are reported as mmoles of oxidized product per mole of parent Tyr to compensate for any loss of material during processing.

SDS-PAGE

Samples for SDS-page were prepared and incubated as described above, with 2.5 µg of protein loaded directly onto the gel at the appropriate time points. NuPAGE[®] Novex Tris-Acetate Gels (Invitrogen, Carlsbad, CA, USA) were used according to the protocol supplied by the manufacturer. DTT was added directly to the samples when reducing conditions were required. Gels were stained with Colloidal Coomassie blue.

Statistical analysis

All experiments were performed in triplicate or greater, and results are expressed as means \pm SD except where indicated otherwise. Statistical analysis was carried out by 2-way ANOVA with Dunnett's *post-hoc* test. Significance was assumed at $P < 0.05$.

RESULTS

Detection of radical intermediates on myosin by direct EPR

Incubation of myosin with either HRP (Figure 1, panel A) or Mb (Figure 1, panel B) in the presence of H₂O₂ at 22 °C, pH 7.0 and subsequent examination by direct EPR at 22 °C spectroscopy resulted in the detection of signals assigned to myosin-derived radicals (Figure 1). These signals were long-lived and could be detected for up to 120 mins (Figure 1). The g-value of these signals was determined, for both oxidation

systems, to be ca. 2.005 by reference to TEMPO. The species generated with Mb/H₂O₂ decayed less rapidly than those generated by HRP/H₂O₂. Control experiments with the Mb/H₂O₂ system, in the absence of myosin, gave less intense signals (Figure 1, panel B), which have been assigned to Mb-derived tyrosyl radicals as observed previously [11,16]. These features decayed more rapidly than those detected in the presence of myosin. No signals were observed with the HRP/H₂O₂ system in the absence of myosin (Figure 1, panel A).

The EPR signal detected with the HRP/H₂O₂/myosin system consisted of a single broad absorption (peak-to-peak line width 8.22 G), with poorly resolved fine structure, as evidenced by the presence of weak inflections on the signal envelope. No significant changes in line shape were detected over time. The long-lived nature of this signal, its *g* value, line shape and peak-to-peak line width are consistent with an assignment to (one or more) tyrosine-derived phenoxyl radicals present on myosin [18]. The initial EPR signal detected with Mb/H₂O₂/myosin system was much broader than that from the HRP/H₂O₂/myosin system (peak to-peak line width 11.10 G), with this becoming considerably sharper as the signal decayed (Figure 1, panel B). These changes are consistent with the presence of multiple species. The broader component has been assigned to Mb-derived tyrosyl radicals (cf. previous data [39,40]) and the narrower component to myosin-derived species (cf. the absence of this component in spectra from incubations carried out in the absence of myosin and the similarity of this narrower signal to that detected with HRP/H₂O₂/myosin). This second, myosin-derived, species becomes more distinct on decay of the Mb-derived species (i.e. at longer reaction times; Figure 1, panel B). The different line widths of the proposed Mb- and myosin-derived tyrosyl radicals is believed to arise from different conformations of the aromatic ring of these radicals relative to the methylene hydrogens through which the ring is attached to the backbone of the protein. It is well established [39,41,42] that the hyperfine couplings constants from these hydrogens is the major determinant of the overall line width and line shape of tyrosine phenoxyl radicals, with these values varying dramatically with the angle between the methylene C-H bonds and the plane of the tyrosine ring.

Analogous oxidation experiments carried out with other oxidizing systems (Fe²⁺/ascorbate/H₂O₂, Ce(IV), and peroxyxynitrite, see Experimental section) in the presence of myosin did not result in the (direct) detection of any signals that could be assigned to myosin-derived radicals.

The role of thiol groups on myosin in the generation of the observed EPR signals (cf. previous evidence for the formation of disulfide bonds on oxidised myosin

[5,28-30]) was probed by pre-treating the myosin with NEM (5 mM) to block these sites. Subsequent oxidation with Mb/H₂O₂ or HRP/H₂O₂ resulted in a decrease in the intensity of the EPR signals when compared to the unmodified protein (Figure 2); for the HRP system the signal intensity was decreased to 38% of that detected with the unmodified protein after 2 min of reaction (Figure 2, spectrum 1 vs 2), and with Mb the signal was reduced to 80% of the original intensity (Figure 2, spectrum 3 vs 4). At longer incubation times the intensity differences in the EPR spectra between the NEM-treated myosin and the native myosin was greater with both oxidizing systems (data not shown). The smaller decrease with the Mb system at 2 min is consistent with the presence of significant concentrations of Mb-derived radicals at this time point. These data are consistent with some of the observed signals requiring the presence of thiol groups for their formation. Whilst thiyl radicals have been previously detected on myosin (e.g. using Ce(IV) and spin trapping [8]), the observed EPR signals are not consistent with the direct detection of these species, as thiyl radicals are typically short-lived and yield highly anisotropic EPR signals [43]. Thus the effect of NEM is interpreted in terms of a requirement for thiol groups in the generation of some of tyrosine phenoxyl radicals detected. The incomplete inhibition observed on use of NEM (e.g. with the HRP/H₂O₂ system) is consistent with the additional occurrence of thiol-independent pathways to myosin radical formation.

Detection of radical intermediates on myosin by EPR spin trapping

Inclusion of the nitroso spin trap MNP (100 mM) in both the HRP/H₂O₂/myosin and Mb/H₂O₂/myosin systems resulted in the detection of spin adduct signals. With the Mb/H₂O₂ system identical EPR signals were detected, at 22 °C, in both the presence and absence of myosin, indicating that the majority of the radicals trapped were present on Mb (data not shown). The signals detected were similar to those reported previously for this system [14,44]. Addition of MNP 20 or 60 min after the addition of H₂O₂ resulted in similar EPR spectra, though of lower intensity, indicating that some Mb radicals were present at these time points (data not shown); this is consistent with previous reports of long-lived radicals on H₂O₂-treated Mb [44]. Blocking of the thiol groups on myosin did not have any effect on the spin adduct signals, consistent with the observed species being Mb-derived.

Inclusion of MNP in the HRP/H₂O₂ system, in the absence of myosin, did not result in the detection of any protein-derived species under the conditions employed. When myosin was present, multiple signals were detected with the ratio of these

species dependent on the reaction time, and whether the thiol groups on myosin had been blocked. With unmodified myosin, 2 min after the initiation of oxidation, a highly anisotropic spectrum was detected (Figure 3, spectrum 1) with the distance between the outermost features of this spectrum, $2A'_{zz}$, being ca. 63.1 G. On the basis of the observed anisotropy, this signal is assigned to a slowly-tumbling, myosin-derived radical adduct. The signal from this species decayed rapidly indicating that this adduct is short-lived (Figure 3, spectrum 2). In both the initial and subsequent spectra (e.g. at 45-60 min; Figure 3, spectrum 3) an isotropic triplet signal, with $a(N)$ 15.35 G, was also observed with no discernable fine structure. This signal was observable for many hours. These data are consistent with the presence of an adduct species with no beta-hydrogen couplings. This signal has been assigned, as previously, to a radical species arising from the trapping of a Tyr-derived phenoxyl radical (through the ortho position) and the subsequent rearrangement of this species to the observed tertiary radical species [45]. Treatment of this long-lived adduct species with the proteolytic enzyme pronase (from *Streptomyces griseus*, 0.1 mg/ml to 10 μ M myosin) resulted in an increase in signal intensity consistent with the release of highly-mobile radical adducts from initial myosin adduct species of more limited mobility (data not shown). A similar isotropic triplet signal ($a(N)$ 15.6 G) has been detected previously in a HRP/H₂O₂/tyrosine/MNP system, with this signal assigned to a long-lived tyrosine-derived radical [46]. The signal detected in the current study is therefore assigned to a trapped Tyr-derived radical present on myosin, consistent with the direct EPR data.

Repetition of the above experiments with thiol-blocked myosin resulted in the immediate detection of an isotropic triplet signal (Figure 3, spectrum 4) with an identical hyperfine coupling to the species described above. This adduct decayed rapidly (Figure 3, spectrum 5). At longer time points (e.g. 60 mins, Figure 3, spectrum 6) a further persistent adduct species with a triplet spectrum ($a(N)$ 15.6 G) identical to that detected with the native protein was observed. The identical signals detected at long time points with both unmodified and modified myosin is consistent with these being assigned to Tyr-derived radical adducts generated by reactions that are thiol-independent. In contrast the highly anisotropic, short-lived adduct detected with native myosin, but not the thiol-blocked protein, is consistent with the trapping of a thiyl radical. The second short-lived triplet signal detected with the thiol-blocked protein may be due to a different population of Tyr-derived species, or an additional trapped tertiary carbon-centered radical.

Detection of radical intermediates by low temperature direct EPR

Previous studies have demonstrated that Trp-derived radicals are readily formed on proteins due to their low oxidation potentials [1-3]. Thus Trp-14 peroxy radicals have been detected on H₂O₂-treated Mb by low temperature EPR [13,16,47]. The formation of analogous species on myosin as a result of damage transfer from H₂O₂-activated HRP or Mb was therefore examined. Examination of rapidly frozen (in liquid nitrogen) samples of HRP/H₂O₂/myosin incubations by direct EPR at 77 K resulted in the detection of a similar signal to that detected at room temperature (c.f. Figure 1, panel A), consistent with the presence of identical radicals under both conditions; no evidence for Trp-derived peroxy radicals was obtained. As with the room temperature studies, omission of the myosin resulted in the loss of these spectral features.

Examination of the Mb/H₂O₂ system at 77 K, in the absence of myosin, resulted in the detection of anisotropic EPR signals which have been assigned, as previously, to a mixture of the Trp-14 peroxy radical and (one or more) Tyr-derived phenoxyl radicals on Mb [16] (Figure 4). The inclusion of myosin in these incubations decreased the intensity of the Mb-derived signals in a concentration-dependent manner (Figure 4); no additional myosin-derived signals were detected. Blocking the thiol groups on myosin had no effect on either the nature, or the intensity, of the Mb-derived Trp-14 peroxy radical signal (data not shown). These data are consistent with reaction of the Mb-derived Trp-14 peroxy radical with myosin, with the site(s) of reaction on the myosin not including the thiol residues. The incomplete loss of these observed signals does not preclude the formation of either Tyr-phenoxyl or Trp-derived species on myosin as a result of these reactions (i.e. the Mb species may be being replaced by species with similar EPR features on the myosin).

Quantification of amino acid side-chain oxidation

Oxidation of the thiol (Cys) residues on myosin was quantified with DTNP. Both the Mb/H₂O₂ and HRP/H₂O₂ systems gave rise to rapid thiol loss; this was quantified after 2 min of oxidation at room temperature with a range of oxidant concentrations (Figure 5). Increasing the concentration of either Mb/H₂O₂ or HRP/H₂O₂ (with fixed ratios of heme protein to H₂O₂) added to a fixed concentration of myosin resulted in a significant loss of myosin thiol groups. Use of the HRP/H₂O₂ system with a ratio of HRP:H₂O₂ of 1:100 resulted in particularly rapid thiol loss (Figure 5, panel A). Lower

extents of loss were observed with HRP/H₂O₂ and Mb/H₂O₂ at heme:H₂O₂ ratios of 1:1. Control experiments with Mb and HRP alone gave, as expected on the basis of their amino acid composition, zero free thiol levels indicating that all of the thiols consumed were on the myosin. Control experiments in the absence of heme protein, but with 25 μM-10 mM H₂O₂ did not result in significant loss of thiols on the myosin (data not shown).

Quantification of the possible loss of other amino acids on myosin was assessed by HPLC after hydrolysis to free amino acids. Only data for the HRP/H₂O₂/myosin system are considered as the lower concentrations of HRP protein relative to myosin in this system employed allowed changes on the myosin to be discerned after subtraction of the levels seen with HRP/ H₂O₂ alone. The higher concentration of Mb used in the Mb/ H₂O₂/myosin system prevented the attribution of any loss to a particular protein. Of all the amino acids analysed for (Arg, Asp/Asn, Glu/Gln, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val), significant changes were only detected for Tyr (Figure 5, panel B), though there was a (non-significant) trend for loss of Trp residues. These data are standardised to the levels of Ala present in the samples to compensate for any losses during processing. Ala was chosen as this amino acid would not be expected to be oxidized to a significant extent [1-3].

Quantification of oxidized amino acid side-chains on proteins

The formation of the Tyr oxidation products di-tyrosine and DOPA were quantified by HPLC after acid hydrolysis of the reaction mixtures. With both the Mb/H₂O₂/myosin and HRP/H₂O₂/myosin systems significant levels of DOPA were detected relative to reaction mixtures in the absence of H₂O₂ (data not shown). However omission of myosin from the complete reaction systems resulted in similar levels of DOPA consistent with DOPA formation occurring predominantly, or completely, on the heme protein. In contrast with di-tyrosine significantly greater levels of this product were detected with both complete reaction systems, than in the absence of either H₂O₂ or myosin, consistent with significant di-tyrosine formation on the myosin (Figure 6). These increases were apparent when the data was expressed both as absolute values (for the myosin/HRP/H₂O₂ system) and when expressed relative to parent Tyr. Non-oxidised myosin contained insignificant levels of di-tyrosine ($< 0.014 \pm 0.009$ mmol di-tyrosine/mol Tyr, 0.122 ± 0.009 pmol absolute concentration; $n = 15$). The formation of di-tyrosine was rapid with the maximum yield of these products observed

at the first time point examined (30 s) after addition of H₂O₂. At longer time points a small decrease in di-tyrosine was detected (Figure 6); this may be due to further oxidation of the di-tyrosine to higher aggregates (e.g. tri-tyrosine). The yield of di-tyrosine detected on myosin induced by the Mb/H₂O₂ system was not significantly affected by the use of thiol-blocked myosin, whilst that induced by the HRP/H₂O₂ system was, with significantly higher levels of dityrosine detected on the thiol-blocked protein compared to native myosin (Figure 6).

The formation of the oxidation product methionine sulphoxide from methionine was also assessed by HPLC after acid hydrolysis. As expected from the insignificant decrease in the level of the parent amino acid (see above), no significant increase in the level of this species was detected in the systems under study (data not shown).

Gross structural changes induced by oxidation of myosin

SDS-page analysis was employed to investigate the potential formation of both fragmented and cross-linked products. Oxidation of myosin with 100 µM Mb/100 µM H₂O₂ resulted in a reaction time-dependent decrease in the band intensity of the myosin heavy chain on both non-reducing and reducing gels (Figure 7), and the formation of higher, but not lower, molecular mass material. These observations are consistent with the formation of intermolecular cross-linkings, but not extensive protein fragmentation. The mass of these higher molecular mass bands, as judged from the molecular mass markers, indicates that these cross-linked species are predominantly myosin-myosin species and not myosin-Mb dimers. The extent of loss of the parent myosin heavy chain was greater in the non-reducing gels than the reducing, though both were observed to decrease, consistent with the formation of both reducible and non-reducible cross-links (Figure 7, panel A). A similar pattern was observed on oxidation of myosin by HRP/H₂O₂ (gel not shown) though the extent of modification was less extensive as judged by densitometric analysis of the parent myosin heavy chain band (Figure 7, panel B).

Blocking of the thiol groups on myosin prior to oxidation caused a greater reduction in the intensity of the myosin heavy chain band as assessed by densitometric analysis, compared to native myosin with both the Mb/H₂O₂ and HRP/H₂O₂ systems (Figure 8). These data are consistent with an increased formation of non-reducible cross-linkings in myosin with both oxidizing systems.

DISCUSSION

Previous studies have provided evidence for the modification of the heavy and light chains of myosin in both human and animal muscle and heart tissue. These alterations to protein structure have been associated with both human and animal pathologies and the loss of positive characteristics in food samples (e.g. tenderness and palatability). It has been demonstrated that the myosin undergoes aggregation and, in some cases, loss of ATPase activity on exposure to a number of oxidizing (activated heme proteins, metal-ion / ascorbate systems, high valence metal ions, peroxyxynitrite) or modifying agents (reactive aldehydes) (reviewed in Table 1). The mechanisms by which these changes arise, and the nature of the reactive intermediates involved, have not been examined previously in detail.

In the study reported here, various forms of EPR spectroscopy (direct studies at both ambient- and low-temperature and spin trapping) have been used to examine radical formation on myosin on exposure to two systems of physiological relevance – myoglobin (Mb)/H₂O₂ and horseradish peroxidase (HRP)/H₂O₂. The former system is likely to be a major source of oxidant stress in muscle tissue due to the high concentrations of Mb present in muscle cells, and peroxidase/H₂O₂ systems (for which HRP/H₂O₂ is a commonly used surrogate) are believed to be of importance at sites of inflammation due to the release of myeloperoxidase and eosinophil peroxidase from activated neutrophils/monocytes/ macrophages and eosinophils respectively. It is shown that both these systems give rise to multiple radicals on myosin as a result of damage transfer from the H₂O₂-activated Mb or HRP. In the case of activated Mb evidence has been provided for myosin oxidation by both Tyr-phenoxy and Trp-peroxy radicals. With activated HRP, the nature of the species that damage the myosin is less clear; this may involve reactions of the heme edge, or via undetected protein-derived radicals.

These reactions generate multiple potential radicals on the myosin, with evidence having been obtained for Tyr-phenoxy and thiyl radicals; data supporting the formation of additional unidentified species has also been obtained from spin trapping studies. These additional species are possibly alternative Tyr- or Trp-derived radicals, as both Tyr and Trp residues are known to be readily oxidised. No evidence has been obtained for the oxidation of Met residues, as evidenced by either loss of the parent amino acid, or formation of Met sulphoxide. Previous studies have reported the formation of long-lived radicals, probably Tyr-phenoxy radicals on

bovine serum albumin, beta-lactoglobulin and casein [17,19], but no previous studies have been reported for myosin.

Previous studies on the oxidation of myosin using SDS-PAGE, have indicated that the type of cross-links formed are dependent on the nature of the oxidising agent. Thus earlier studies have proposed that oxidation of myosin by hydroxyl radical generating systems (i.e. metal ion / ascorbate systems) results primarily in disulphide bonds whilst heme proteins cause formation of dityrosine or other non-reducible cross-linkings. This is surprising in the light of indiscriminate reactivity of hydroxyl radicals with protein side-chains and the considerable evidence for the formation of di-tyrosine and DOPA from Tyr by this species (reviewed [1-3]), and the known reactivity of activated heme proteins with thiol residues to generate thiyl radicals (e.g. [48-51]).

In the present study, oxidation of myosin by both the Mb/H₂O₂ and HRP/H₂O₂ systems has been shown to result in di-tyrosine formation, oxidation of Tyr and Cys residues and the formation of both reducible (believed to be disulphide) and non-reducible (possibly di-tyrosine) cross-links. The occurrence of these processes indicates that the formation of thiyl and Tyr-phenoxy radicals on myosin are alternative competing processes, with the yield of each intermediate species dependent on the system used. Furthermore modulation of the flux through one route appears to increase flux through the alternative pathway. Thus the experiments with thiol-blocked myosin have provided evidence for increased formation of non-reducible cross-linkings and di-tyrosine with both oxidizing systems. Increased formation of non-reducible cross-linkings in myosin when thiol groups have been blocked by NEM has been observed previously [7].

It is possible that Tyr oxidation proceeds via Cys oxidation based on the studies using MNP, where signals assigned to Tyr-derived radicals were only detected at time periods after the formation of thiyl radicals; this may however be an artifact of the time required to examine the reaction samples by direct EPR (typically > 1 min, as a result of the time required to tune the spectrometer) compared to that by spin trapping, where trapping of radicals should occur immediately due to the presence of the spin trap in the reaction system. The reaction of Tyr phenoxy radicals with Cys to give thiyl radicals has been shown previously to be a reversible (equilibrium) reaction; conclusive evidence has also been obtained for the interaction of Tyr and Trp species (e.g. [52,53]). Thus Trp-derived radicals may also be formed on myosin; this is supported by the trend towards a loss of Trp in the amino acid

analysis experiments, and the detection of additional short-lived adduct species with MNP on myosin; these may be trapped Trp-derived radicals.

A proposed reaction mechanism for the oxidation of myosin by activated heme proteins is suggested in Figure 9. This reaction mechanism includes two reaction pathways for the formation of myosin radicals: one involving direct oxidation of thiol groups to give thiyl radicals – a process that appears to be favored under the reaction conditions employed in the present study – and a second that generates the (long-lived) Tyr-derived radicals detected by direct EPR. This second pathway may involve the mediation of other short-lived radicals (X^*) that may be another population of (transient) Tyr-derived phenoxyl radicals or possibly Trp-derived species. The identification of these short-lived radicals requires further study, although the hyperfine splitting constant of 15.35 G obtained for the MNP adduct would be consistent with a Tyr-derived radical located on the surface of myosin. The formation of a population of reactive Tyr-derived phenoxyl radicals would also be consistent with the rapid formation of di-tyrosine observed in the product studies.

Overall these studies indicate that multiple reaction pathways can result in radical transfer from an activated heme protein to myosin, and that these reactions result in the generation of both thiyl and Tyr-phenoxyl radicals on the target protein. Other radicals may also be formed (e.g. on Trp residues). The formation of these species results in the formation of both reducible (believed to be disulphide) and non-reducible (di-tyrosine) cross-links between myosin molecules. The significance of the reactions examined in the current study for more complex systems remains to be fully established. In particular it might be expected that alternative reactions of the initial Mb- and HRP-derived species, particularly those involving low molecular mass thiols (e.g. GSH) and ascorbate, may diminish radical transfer to myosin. Thus it has been shown that both of these reductants can react with Mb-derived species with corresponding oxidation of the GSH and ascorbate [16,39,50,51]. However there is also extensive evidence for the modification of myosin in intact tissues and myofibrils [20,22-25,30] suggesting that such antioxidant systems may be inefficient or overwhelmed under certain circumstances. Thus we believe that the generation of these radical intermediates and intermolecular cross-links may account for the altered and dysfunctional myosin observed in human pathologies and degraded meat samples.

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Figure Captions

Figure 1 EPR spectra of myosin-derived radicals detected by direct EPR.

EPR spectra were recorded at 22 °C from reaction mixtures containing: panel A) 12 μM myosin, 100 μM HRP, 10 mM H₂O₂; and panel B) 12 μM myosin, 300 μM Mb, and 300 μM H₂O₂. Spectra were recorded at the indicated time points after the addition of H₂O₂. The control spectra presented are those where myosin was excluded from the reaction mixture. Spectrometer settings are described in the Experimental section.

Figure 2 Effect of blocking of the thiol groups on myosin on the nature of the myosin-derived radicals detected by direct EPR.

EPR spectra were recorded at 22 °C from reaction mixtures containing: spectrum 1) 12 μM unmodified myosin, 100 μM HRP, 10 mM H₂O₂; spectrum 2) As spectrum 1) except with 12 μM thiol-blocked myosin; spectrum 3) 12 μM unmodified myosin, 300 μM Mb, and 300 μM H₂O₂; spectrum 4) As spectrum 3) except with thiol-blocked myosin. All spectra were recorded 2 min after initiation of oxidation at 22 °C. The thiol groups on myosin were blocked by incubation with NEM prior to oxidation, as described in the Experimental section. Spectrometer settings are described in the Experimental section.

Figure 3 Detection of myosin-derived radicals by EPR spin trapping

EPR spectra were recorded at 22 °C from reaction mixtures containing 12 μM myosin, 100 μM HRP, and 10 mM H₂O₂ in the presence of 100 mM MNP at the following time points after the addition of H₂O₂: 2 min (spectra 1, 4), 8 min (spectra 2, 5) and 60 min (spectra 3, 6). Spectra 1-3 were obtained from native myosin, spectra 4-6 from thiol-blocked myosin. The thiol groups on myosin were blocked by incubation with NEM prior to oxidation, as described in the Experimental section. Spectrometer settings are described in the Experimental section. The anisotropic features (indicated by $2A'_{zz}$ in spectrum 1, and which are also present in the other spectra although at lower concentrations) are assigned to a short-lived Trp (or X') radical adduct. The asterisked features are assigned to a long-lived Tyr-derived species. See text for further details.

Figure 4 Detection of radicals by low temperature EPR

EPR spectra, recorded at 77 K, of reaction mixtures containing 300 μM Mb and 300 μM H_2O_2 without (control) or with added myosin (5 or 13 μM). Samples were frozen in liquid nitrogen 40 s after initiation of oxidation and subsequently examined by EPR. Features arising from the Trp-14 peroxy radical of myoglobin: $g(\text{parallel}) \sim 2.035$ and $g(\text{perpendicular}) \sim 2.006$, are indicated. Spectrometer settings are described in the Experimental section. Inset data are mean \pm SD of triplicate determinations of the EPR signal area in the presence of the indicated concentration of myosin.

Figure 5 Consumption of thiol and Tyr residues on oxidation of myosin by activated heme proteins

Panel A: Myosin (13 μM) was incubated with the indicated concentrations of Mb/ H_2O_2 (1:1 ratio; black bars), HRP/ H_2O_2 (1:1 ratio; grey bars), or HRP/ H_2O_2 (1:100 ratio; white bars) for 2 min at 22 $^\circ\text{C}$, before assessment of residual thiols on myosin using DTNP as described in the Experimental section. Data are mean \pm SD of triplicate determinations after incubation with the stated concentration of oxidant system.

Panel B: Myosin (13 μM) was incubated with 100 μM HRP and 10 mM H_2O_2 for the indicated period before removal of aliquots for the quantification of remaining amino acids after acid hydrolysis of the protein. Dark bars indicate myosin samples indicated with the oxidant system, white bars are corresponding incubation controls of myosin in the absence of oxidant. Data for HRP/ H_2O_2 alone have been subtracted from the reported values. Tyr levels are expressed per mole of Ala present in the protein to compensate for any losses during processing. For further details see text and Experimental Section. Data are mean \pm SEM of $n = 3 - 6$ determinations. No significant change in concentration was detected for any of the other amino acids examined (see text).

Figure 6 Formation of di-tyrosine on oxidized myosin

Di-tyrosine formation on myosin was quantified by HPLC after oxidation with 300 μM Mb/300 μM H_2O_2 (grey bars) or 100 μM HRP/10 mM H_2O_2 (white bars) with either native (no hatching) or thiol-blocked myosin (hatched bars) for the indicated time periods. Control samples of myosin gave insignificant levels of di-tyrosine (see text). The contributions of Mb/ H_2O_2 alone, and HRP/ H_2O_2 alone, to di-tyrosine formation were determined in parallel, and subtracted from the values detected in the presence of myosin. Levels of di-tyrosine are given as mmol di-tyrosine/mole parent Tyr to

compensate for any losses during processing. For further details see text and Experimental Section. Data are mean \pm SD of n = 4-6 determinations.

Figure 7 Effect of oxidation on the structural integrity of myosin exposed to activated heme proteins as assessed by SDS-PAGE

Panel A: Myosin (13 μ M) was incubated at 22 °C with 100 μ M Mb and 100 μ M H₂O₂ for 0 (control without Mb/H₂O₂), 2, 10, 30 or 60 min, or 24h before analysis by SDS-PAGE as described in the Experimental section. DTT was added directly to samples when reduced conditions were employed. Myosin heavy chain (MHC) bands and cross-linked MHC (CL MHC) are indicated by arrows.

Panel B: Quantification of the loss of the myosin heavy chains determined by densitometric analysis of SDS-PAGE gels (reducing or non-reduced) of native and oxidized myosin. Samples were incubated with the Mb/H₂O₂ (square symbols) as described in panel A, or under identical conditions with 100 μ M HRP and 10 mM H₂O₂ (circle symbols) with the samples then run in the absence of reduction (open symbols) or after treatment with DTT (closed symbols). Representative data from multiple experiments with similar trends are shown.

Figure 8 Effect of thiol-blocking on the structural integrity of myosin exposed to activated heme proteins as assessed by SDS-PAGE

Quantification of the loss of myosin heavy chain bands determined by densitometric analysis of SDS-PAGE gels of oxidized myosin. Native (closed symbols) or thiol-blocked myosin (open symbols) was oxidized with 100 μ M Mb and 100 μ M H₂O₂ (square symbols), or 100 μ M HRP and 10 mM H₂O₂ (circle symbols), for 0 min (control without Mb/H₂O₂ or HRP/H₂O₂), 2, 10, 30, or 60 min, or 24 h at 22°C. Samples were subsequently analysed by SDS-PAGE as described in the Experimental section after reduction with DTT.

Figure 9 Proposed damage transfer reactions from H₂O₂-activated heme proteins to myosin that result in radical formation on myosin, and the consequences of these processes. The myosin-X[•] species indicated may be an alternative population of myosin Tyr-phenoxy radicals (myosin-TyrO[•]), or Trp-derived radicals.

Table 1 Intermolecular cross-links formed from myosin on treatment with various oxidizing systems.

Oxidizing system	Type of cross-link	Reference
Fe ³⁺ /ascorbate/H ₂ O ₂	Disulphide ^a	[7]
Fe ³⁺ /ascorbate	Disulphide ^a	[5]
Cu ²⁺ /ascorbate		
Mb/H ₂ O ₂	Non-disulphide ^a	[4]
Mb/H ₂ O ₂	Disulphide ^a	[4]
HRP/H ₂ O ₂	Dityrosine ^{a,b}	[6] ^c
Mb/H ₂ O ₂		
MbO ₂ /H ₂ O ₂	Dityrosine ^{a,b}	[26] ^c
Mb/H ₂ O ₂		

^a As determined by SDS-PAGE

^b As determined by fluorescence spectroscopy.

^c Disulphide formation not investigated.

Figure 1

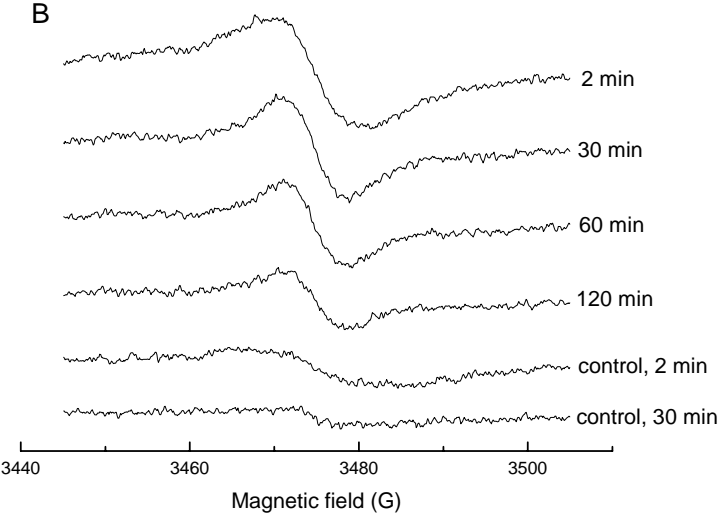
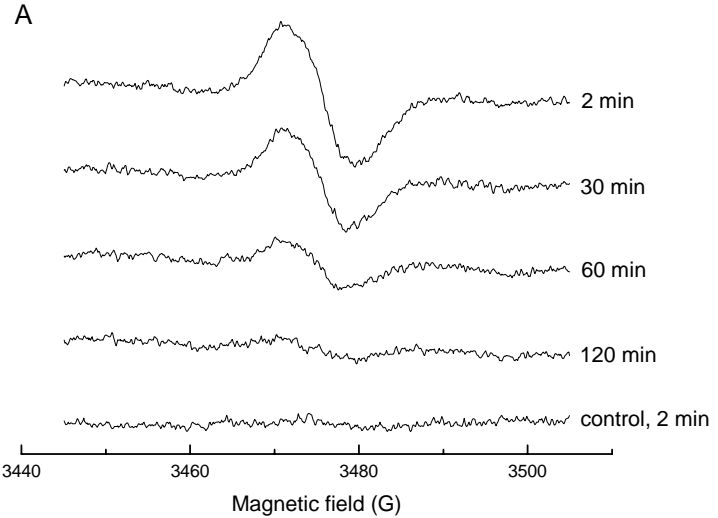


Figure 2

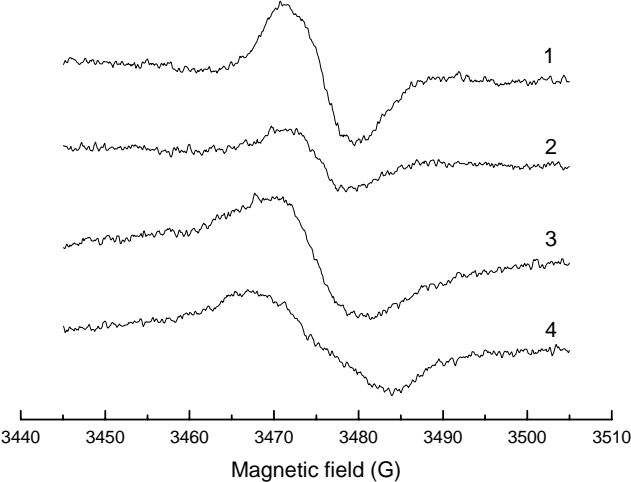


Figure 3

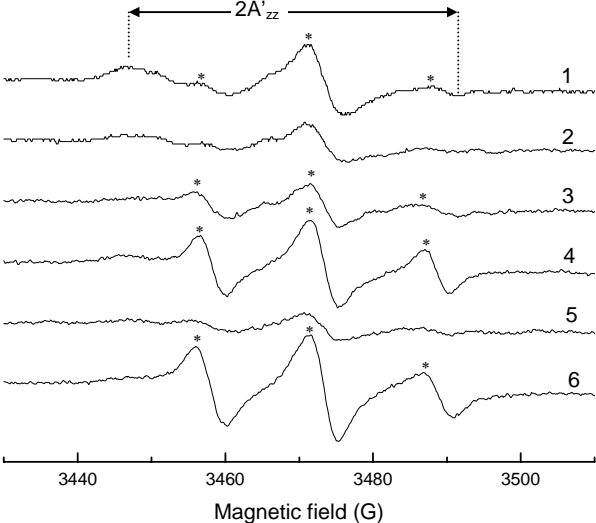


Figure 4

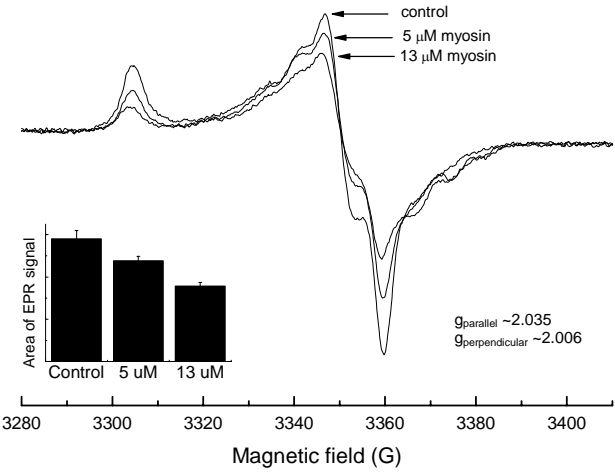


Figure 5

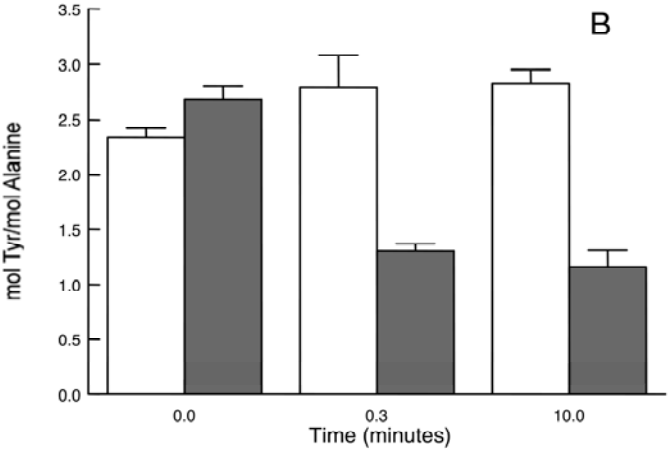
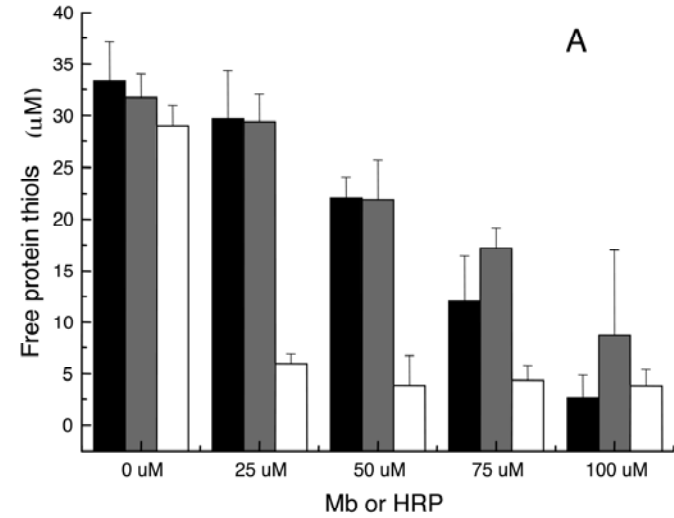


Figure 6

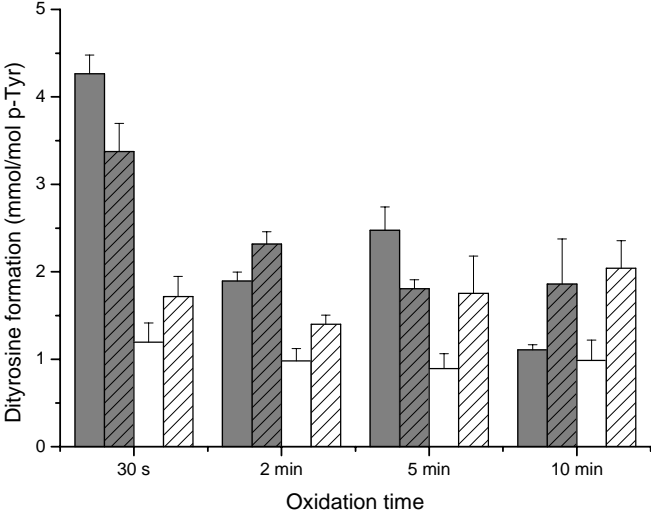


Figure 7

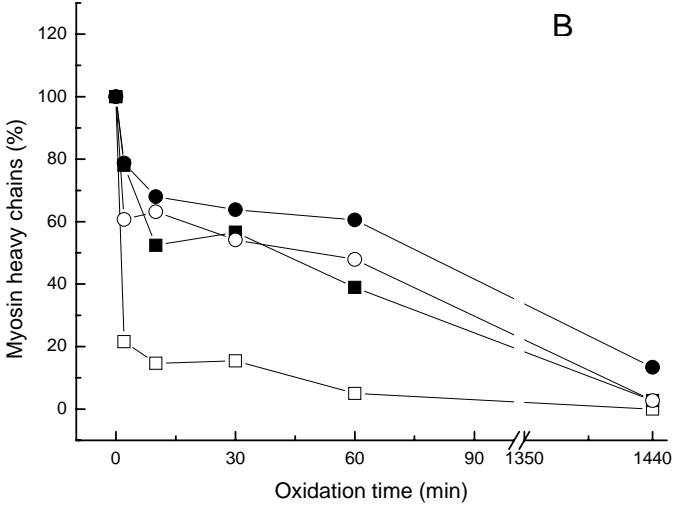
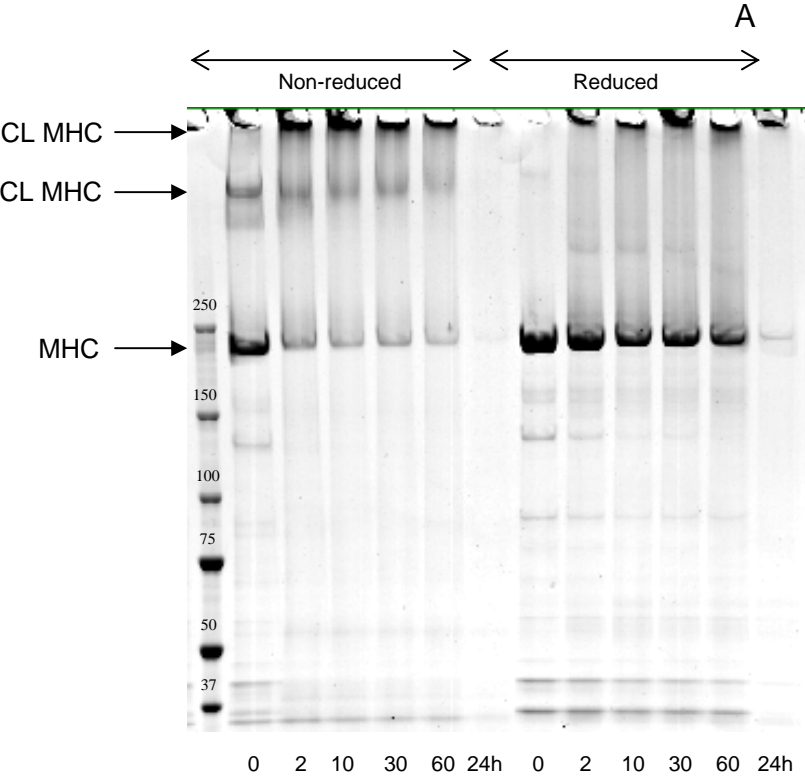


Figure 8

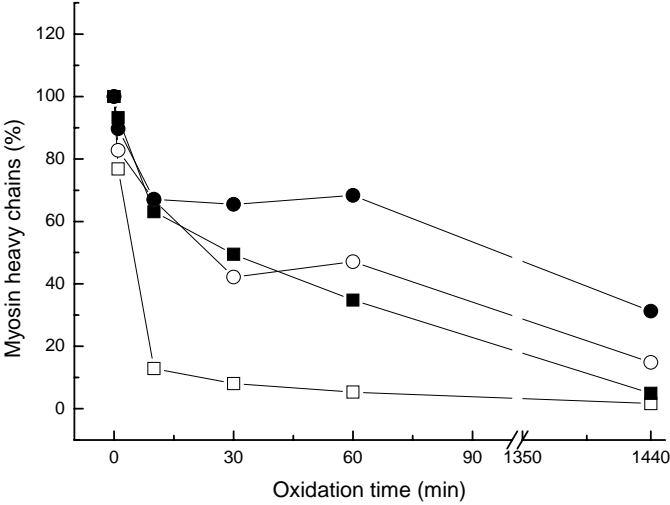
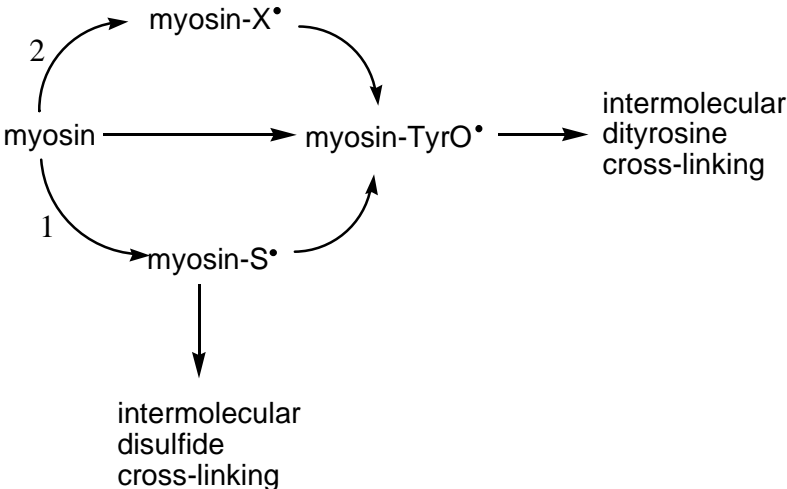


Figure 9



Oxidation of porcine myosin by hypervalent myoglobin: The role of thiol groups

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ABSTRACT

Oxidation of the myofibrillar muscle protein myosin from pork by hypervalent myoglobin species (MbFe(III)/H₂O₂ radical generating system) was investigated in aqueous solution in the pH range 5.0-7.8 by electron spin resonance (ESR) spectroscopy using PBN as spin trap and indirectly by determination of the rate of reduction of hypervalent myoglobin species by UV spectroscopy. Cross-linking of myosin was examined by SDS-PAGE. The target for oxidative modification of myosin was studied by thiol blocking by N-acetylmaleimide (NEM-treatment) and by determining oxidative modification of myosin thiols. The reaction between myosin and hypervalent myoglobin was fast and showed little dependence on pH. The formed myosin radicals were observed to be short-lived. Myosin thiols are suggested to be the main target for oxidative modification, as NEM-treated myosin did not form radicals in the presence of hypervalent myoglobin. A significant decrease in thiol content was demonstrated already 25 s after initiation of oxidation of myosin. The majority of myosin heavy chain (MHC) was demonstrated to be cross-linked through intermolecular disulfide bonding 1 h after initiation of oxidation. This together demonstrates that thiols are important for radical formation and cross-linking of myosin during oxidation with hypervalent myoglobin at pH of meat products.

Keywords: myosin, hypervalent myoglobin, protein oxidation, radical formation, thiol groups, cross-linking, pH.

INTRODUCTION

Oxidative reactions are together with microbial growth important for the quality and shelf life of meat. Reactive oxygen species (ROS) accumulate during the conversion of muscle to meat and pro-oxidative species such as hypervalent myoglobin are formed in parallel with depletion of the antioxidative defence of the muscle cells (1-3). Most studies on oxidation in meat and other food systems have focused on the effects of lipid oxidation (4-7). However, proteins, which except from water are the major constituents of meat, are also susceptible to oxidative modification (8). Oxidation of proteins has been widely studied in the field of pathology and in relation to human diseases, but the importance of protein oxidation on food systems has been less examined.

The oxidation of proteins in meat has previously been suggested to reduce the eating quality of meat by decreased tenderness and juiciness (9-11). Recently, intermolecular disulfide cross-linking of myosin was found to take place in pork chops packed in a high-oxygen atmosphere (70% O₂/30% CO₂), and the disulfide cross-linking of myosin was suggested to be responsible for reduced meat tenderization of the pork chops as evaluated by a sensory panel (12). One major consequence of protein oxidation is the formation of polymers through intermolecular protein cross-linking, which includes disulfide bonds formed by oxidation of cysteine thiol groups, dityrosine formation, and reactions between protein carbonyls (formed through oxidation of protein side chains) and the ε-amino group of lysine side chains (13-18). The intermolecular cross-linking of meat proteins has mainly been studied in model systems and based on oxidative modification of myofibrillar protein fractions by a variety of radical generating systems but without considering reaction mechanisms and pH dependency (19-26).

We have accordingly undertaken a mechanistic study of oxidation of purified myosin at varying pH in an aqueous model system with special emphasis on sulphur centred myosin oxidation and using hypervalent myoglobin species, ferrylmyoglobin (MbFe(IV)=O) and perferrylmyoglobin (•MbFe(IV)=O) as an oxidant inherently occurring in meat following reaction between the major meat pigment myoglobin and H₂O₂ accumulating in meat during storage (27-29).

MATERIALS AND METHODS

Chemicals

Adenosine triphosphate (ATP), Coomassie Brilliant Blue G, ethylenediaminetetraacetic acid (EDTA), N-ethylmaleimide (NEM), 90% horse heart metmyoglobin type III, 2-[N-morpholino]ethanesulfonic acid (MES) and 98% N-tert-butyl- α -phenylnitron (PBN) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane (tris) and L-cysteine were purchased from Merck (Darmstadt, Germany). Standardized 0.2000M aqueous HCl and 99% sodium dodecyl sulfate (SDS) were purchased from Bie & Berntsen (Rødovre, Denmark). 2,2'-Dithiobis(5-nitro-pyridine) (DTNP) (96%) was purchased from Aldrich (St. Louis, MO, USA). 2,2,6,6-Tetramethylpiperidine 1-oxyl (TEMPO) was purchased from Molecular Probes (Leiden, Holland). Hydrogen peroxide (30%) was purchased from Sigma-Aldrich (Steinheim, Germany). Ethylenbis(oxyethylenitrilo) tetraacetic acid (EGTA) was purchased from ACROS Organics (New Jersey, USA). N-[tris(hydroxymethyl)methyl]glycine (tricine) was purchased from AppliChem (Darmstadt, Germany). Pure ethanol (96%) was purchased from Danish Distillers (Aalborg, Denmark). Precision Plus Protein Standard was purchased from Bio-Rad Laboratories (CA, USA). Sample reducing agent (DTT) (10X) and LDS sample buffer (4X) were purchased from NuPAGE, Invitrogen (Carlsbad, USA). All chemicals were of analytical grade or of highest purity available. Water was purified through a Millipore Q-Plus purification train (Millipore Corp., Bedford, MA).

Purification of myosin

Myosin was isolated as described previously by Nauss *et al.* (30) and Wang & Smith (31) with minor modifications. Porcine *longissimus dorsii*, supplied by The Danish Meat Research Institute, Roskilde, Denmark, was immediately frozen in liquid nitrogen after slaughter, followed by storage at -18°C. Fifty grams of muscle, trimmed of adhering fat and connective tissue, was homogenised using Ultra-Turrax and 3 volumes (v/w) of a modified Guba-Straub solution (0.30 M KCl, 0.10 M KH₂PO₄, 0.05 M K₂HPO₄ and 1.0 mM EGTA, pH 6.4). The crude myofibrillar extract was diluted with 3 volumes of water (v/v) and filtered through a 400 μ M plastic mesh. Water (6.5 volumes (v/v)) was added to the filtrate and the samples were allowed to precipitate on

ice for at least two hours. The clear supernatant was decanted and the remaining precipitate was centrifuged at 2000 *g* for 45 min (HiCen21 centrifuge, Herolab, Wiesloch, Germany). The precipitate was subsequently dissolved in 100 ml 0.50 M KCl, 1.0 mM EGTA, 10 mM Tris (pH 7.5), treated with 2.0 mM ATP and 5.0 mM MgCl₂, and centrifuged at 70000 *g* for 30 min (Beckmann Optima TM LE-801C Ultracentrifuge, Beckman Coulter, Inc., Fullerton, USA) in order to remove residual actomyosin. Myosin present in the supernatant was salted out by ammonium sulphate precipitation. The fraction precipitating between 38% and 50% saturation was collected and re-suspended in a minimal volume of 0.50 M KCl, 1.0 mM EGTA, 10 mM Tris (pH 7.5), and dialyzed overnight against three changes of the same buffer, without EGTA, using a 6-8000 *g*/mol cut-off dialysis tube (Spectra/Por membrane, Spectrum, Rancho Dominguez, USA).

The purity of the obtained myosin solution was evaluated by reducing SDS-PAGE applying a NuPAGE[®] Electrophoretic System. A 3-8 % tris-acetate gel (NuPAGE, Invitrogen, Carlsbad, USA) was used in combination with a tris-acetate discontinuous buffer system (50 mM tricine, 50 mM tris and 0.1 % (w/v) SDS). Following electrophoresis, the gel was stained with Coomassie Brilliant Blue, allowing visualisation of the separated protein bands in comparison to a protein standard (Precision Plus Protein standard, Bio-Rad Laboratories, CA, USA). The myosin solution contained primarily myosin heavy chain but also smaller molecular weight compounds to a minor extent, e.g. the presence of a protein band between 37-50 kDa assigned as actin was observed.

Purified myosin stock solution was stored at -18°C. On the day of use, stock myosin was thawed and kept on ice until application. The myosin concentration was determined by measuring absorbance at 280 nm by use of $A_{280(1g/l)} = 0.496$ (32).

Purification of MbFe(III) and preparation of H₂O₂

MbFe(III) was dissolved in a minimum volume of buffer, filtered by use of a 0.45 μ M filter (Minisart, Satorius AG, Uppsala, Sweden) and purified on a PD-10 Shepadex G-25 column (Amersham Biosciences, Uppsala, Sweden). A concentration of approximately 20 mM of the eluted MbFe(III) was determined spectrophotometrically at 525 nm, $\epsilon = 7700 \text{ M}^{-1}\text{cm}^{-1}$ (33) against a blank consisting of the same buffer. MbFe(III) solutions were stored at 2-5°C in the

dark and used within three days. H_2O_2 was diluted in buffer and concentration was determined spectrophotometrically at 240 nm, $\epsilon = 39.4 \text{ M}^{-1}\text{cm}^{-1}$ (34) against a blank consisting of the same buffer. A concentration of approximately 20 mM H_2O_2 was prepared, used within a week and stored at 2-5°C. Choice of buffer for MbFe(III) purification and H_2O_2 preparation was in agreement with specifications of analysis (pH and ionic strength). A Me6.0234.100 combination glass electrode connected to a Methrom 713 pH meter was used for pH measurements (Methrom, Herisau, Switzerland), and a HP8453 UV-VIS diode array spectrophotometer (Hewlett Packard Co., Palo Alto, CA, USA) was applied in the spectrophotometric analysis.

Determination of myosin cross-linking by SDS-PAGE

Oxidized and non-oxidized myosin samples were analyzed by gel electrophoresis (SDS-PAGE) using NuPAGE® Novex 3-8% tris-acetate gels (Invitrogen, Carlsbad, CA, USA). Oxidized myosin sample was prepared by incubating 13 μM myosin with 75 μM MbFe(III) and 75 μM H_2O_2 (pH 5.0, pH 6.4, and 7.8, $I = 0.50$, 5% SDS) for 1 h at 25°C. MES buffer (20 mM, pH 5.0, $I = 0.50$, 5% SDS) and phosphate buffer (20 mM, pH 6.4 and pH 7.8, $I = 0.50$, 5% SDS) were used as solvent for all reactants in order to obtain pH 5.0, 6.4, and 7.8 of the oxidized myosin solution. As a control, myosin was incubated for 1 h at the respective pH at 25°C without addition of MbFe(III) and H_2O_2 . Incubations were carried out in Eppendorf tubes, allowing three volumes headspace of air. Samples with 5 μg of protein were loaded directly onto the gel after 1 h of oxidation. DTT was added directly to samples when reducing conditions was employed during electrophoresis. Myosin samples were run in duplicate.

Protein thiol groups

Thiol oxidation of myosin was measured spectrophotometrically by a modification of Ellman's method using the thiol sensitive reagent 2,2'-dithiobis(5-nitropyridine) (DTNP) as modification of the methods described by Winterbourn (35) and Morzel *et al.* (36). Free thiol content (nmol/mg myosin) of i) non-oxidised myosin, ii) oxidized myosin and iii) MbFe(III)/ H_2O_2 radical generating system was determined at high ionic strength ($I = 0.50$).

Preparation of oxidized and non-oxidized myosin sample was done in accordance with samples prepared for SDS-PAGE analysis. MES buffer (20

mM, 5 % SDS, I = 0.50) was applied for incubation of myosin at pH 5.0 and 5.7, while phosphate buffer (20 mM, 5 % SDS, I = 0.50) was used as solvent for higher pH values (6.4, 7.5, 7.8). Briefly, thiol determination was carried out by adding 1.00 ml phosphate buffer (20 mM, 5 % SDS, pH 8.0, I = 0.50) to the 0.50 ml thawed oxidized or non-oxidized myosin sample, mixed forcefully (whirl) and heated at 70-80 °C for 10 min in order to get myosin into solution. Timing was started as soon as the myosin sample was thawed. The heated oxidized myosin sample (120 µl) was mixed with 860 µl phosphate buffer (20 mM, 5 % SDS, pH 8.0, I = 0.50). Absorbance was measured at 386 and 700 nm prior to addition of DTNP in order to subtract contribution of myosin and myoglobin species to absorbance at 386 nm. Five min after heat treatment of the reaction mixture, 20 µl 5 mM DTNP in absolute ethanol was added and absorbance was recorded at 386 and 700 nm 5 min after addition (that is 20 min after thawing). Phosphate buffer (20 mM, 5% SDS, pH 8.0, I = 0.50) was used as blank. pH was measured in the reaction mixture instantly after addition of DTNP and adjusted with HCl/NaOH if the pH deviated from 8.0 with more than ± 0.1 . A standard curve was established using 1 mM L-cysteine in 20 mM 5 % SDS phosphate buffer (pH 8.0, I = 0.50) in the concentration range 0-90 µM, with a reference solution consisting of the same buffer (37). All samples were measured in triplicate. Thiol content was calculated as nmol cysteine/mg myosin.

Detection of myosin oxidation by spin trapping

Myosin oxidation was carried out at room temperature (pH 7.0, I = 0.16). Equimolar amounts of MbFe(III) and H₂O₂ were mixed prior to addition of myosin and PBN, reaching final concentrations of 300 µM MbFe(III), 300 µM H₂O₂, 0, 11.8, 23.5 and 46.8 µM myosin and 30 mM PBN. Phosphate buffer (5.0 mM, pH 6.9, I = 0.16) was used as solvent for all reactants, and the pure buffer solution was used as blank. The samples were whirled and the ESR spectra of samples (50 µl) in micropipettes (Brand GMBH, Wertheim, Germany) were recorded at room temperature 30s after mixing of MbFe(III) and H₂O₂. A MiniScope MS 200 ESR spectrometer (Magnettech, Berlin, Germany) was used with the following settings: Microwave power: 10 mW, sweep width 97.76 G, sweep time 120 s, modulation amplitude 2 G, and time constant 0 s. An aqueous solution of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) (2 µM) was

used as a reference sample in order to calculate the absolute radical concentration measured in the sample by double integration of the ESR spectra. The TEMPO standard was measured as the first and last sample of the day, and all samples were measured in triplicate. Bruker Win-EPR software (version 2.11, Bruker-Franzen, Analytik GmbH, Germany) was applied for data treatment of ESR spectra.

Ionic strength (0.16, 0.25 and 0.50, respectively) and pH effect ($5.0 < \text{pH} < 7.8$) on myosin oxidation was determined at room temperature. The ionic strength of the myosin stock solution were obtained by dialysis in 100 times volume 20 mM MES buffer (pH 5.0) or 20 mM phosphate buffer (pH 6.5 and 7.5) depending on choice of ionic strength and pH of the myosin solution and using a dialysis tube with a 6-8,000 g/mol cut-off (Spectra/Por membrane, Spectrum, Rancho Dominguez, USA) at 2-5°C during more than three hours against three changes of the same buffer. Stock solution of MbFe(III), H₂O₂ and PBN were prepared using a buffer matching the desired pH and ionic strength of investigation.

Blocking of free thiols by N-ethylmaleimide (NEM)

Chemical modification of thiol groups in myosin was carried out by use of NEM, according to a modification of the methods by Smyth *et al.* (38, 39) and Wada & Kitabatake (40). Accessible thiol groups of 23.5 μM myosin were blocked by incubation with 0, 1.0, 2.5 and 5.0 mM NEM for 90 min on ice and protected from light. Subsequently, oxidation of NEM-treated and untreated myosin was carried out at room temperature (pH 7.0, I = 0.50) and ESR spectra recorded under the conditions previously mentioned. Myosin sample and PBN were added simultaneously to MbFe(III) and H₂O₂, reaching final concentrations of 300 μM MbFe(III), 300 μM H₂O₂, 30 mM PBN and 23.5 μM myosin. Phosphate buffer (5.0 mM, pH 7.0, I = 0.50) was used as solvent for all reactants. Radical formation by the MbFe(III)/H₂O₂ radical generating system was performed as a control. A final concentration of 300 μM MbFe(III) was treated with 0, 1.0, 2.5 and 5.0 mM NEM for 90 min kept on ice. Measurements in the presence of 30 mM PBN were started 30 s after activation of NEM-treated MbFe(III) by H₂O₂ in a final concentration of 300 μM H₂O₂. A blank consisting of phosphate buffer (5.0 mM, pH 7.0, I = 0.50) was measured. All measurements were done in triplicate.

Reduction of hypervalent myoglobin species by myosin

Reduction of hypervalent myoglobin species was investigated in presence and absence of myosin at 25°C (pH 7.5, I = 0.16) by recording spectral changes between 450-700 nm for up to 60 min. Upon mixture of equal concentrations of MbFe(III) with H₂O₂ perferrylmyoglobin (\bullet MbFe(IV)=O) are formed together with ferrylmyoglobin (MbFe(IV)=O). Reduction of both hypervalent myoglobin species by myosin was measured by addition of myosin 5 s after mixing MbFe(III) and H₂O₂, to yield final concentrations of 100 μ M MbFe(III), 100 μ M H₂O₂ and 0, 1.9, 3.9, 7.8 and 11.6 μ M myosin in the reaction mixture. Reduction of MbFe(IV)=O by myosin in the absence of \bullet MbFe(IV)=O was obtained by allowing the reaction mixture to age 8 minutes at room temperature prior to addition of myosin. Stock solutions of MbFe(III) and H₂O₂ were prepared using 5.0 mM phosphate buffer (pH 7.5, I = 0.16) as solvent. The sample was whirled and recorded 100 s after addition of myosin. Blanks were prepared by substitution of the myosin solution with buffer. A HP8453 UV-VIS diode array spectrophotometer (Hewlett Packard Co., Palo Alto, CA, USA) equipped with a temperature controlled cuvette compartment adjusted to 25°C (Hetrofrig thermo, Birkerød, Denmark) was used. The concentration of hypervalent myoglobin species ([MbFe(IV)=O] + [\bullet MbFe(IV)=O]) was determined according to the following expression {Miller, 1993 885 /id}:

$$[\text{Hypervalent myoglobin species}] = -62(A_{490}-A_{700}) + 242(A_{560}-A_{700}) - 123(A_{580}-A_{700}) \quad [1]$$

pH of the sample mixtures was measured after completion of the reaction. All samples were run in duplicate.

Statistical Analysis

Statistical analysis was performed using the SAS[®] 8.2 package, SAS Institute, Inc., USA. Data was analysed by analysis of variance in order to determine the significance of main effects (e.g. oxidation time, pH). Significant (p < 0.05) differences between means were identified by the least significant difference (LSD) procedure.

RESULTS

Intermolecular cross-linking of myosin

Purified myosin was oxidized with hypervalent myoglobin species for 1 h at 25°C at pH 5.0, 6.4 and 7.8. The hypervalent myoglobin species were generated in situ by mixing hydrogen peroxide and metmyoglobin. Analysis of the reaction mixtures by SDS-PAGE under non-reducing conditions demonstrated that the intensity of the protein band from myosin heavy chain (MHC) was reduced in comparison to controls consisting of non-oxidized myosin (Figure 1A). A protein band appeared at high molecular weights in the oxidized samples (indicated by an arrow in Figure 1). This band was not observed when the SDS-PAGE analysis was made with reducing conditions, where also only a small reduction in intensity of the MHC protein band of oxidized myosin was observed in comparison to non-oxidized myosin (Figure 1B). This suggests that primarily disulfide bonding was responsible for the cross-linking of oxidised MHC. However, polymerization of MHC occurred to a small extent via other covalent bonds than disulfide bonding (e.g. dityrosine or Schiff base cross-linking), since not all the cross-linked MHC was dissociable under the SDS-PAGE reducing conditions. The cross-linking of myosin heavy chain through both disulfide and non-disulfide bonding was more pronounced for myosin that had been incubated with hypervalent myoglobin for 24 h (data not shown). The cross-linking of myosin was not clearly affected by pH as no difference in the myosin heavy chain band intensities were found in the pH interval 5.0-7.8. However, a tendency of more myosin cross-linking at pH 7.8 compared to pH 6.4 and 5.0 was observed under non-reducing conditions. Oxidation of myosin in the absence of SDS resulted in precipitation of myosin at pH 5.0 making a study of the effect of pH in more acidic solutions on myosin cross-linking impossible. Myosin did not precipitate in the absence of SDS during oxidation for pH > 6.0; but no difference was observed between pH 6.1 and pH 7.6 on myosin cross-linking in a control experiment performed without presence of SDS (data not shown).

Effect of oxidation on myosin thiol content

The role of thiol groups during oxidation of myosin by hypervalent myoglobin was further investigated by studying the amount of free thiol groups. The determination of myosin thiols was performed with myosin samples oxidized

from 25 s to 24 h (pH 5.0-7.8, I = 0.50) and in presence of 5% SDS in order to avoid myosin precipitation at the lower pH value. The thiol determination showed a significantly higher amount of free thiols in non-oxidized myosin (approx. 30 nmol thiols/mg myosin) in comparison to oxidized myosin (Figure 2). The major change in the amount of free thiol groups took place within the first 25 s, and subsequent changes (after 1 h and 24 h) were small and only observed at the highest and lowest pH values. Myosin oxidized for 25 s showed a minor pH tendency with a significant larger decrease in the thiol content during the oxidation at pH 7.2 in comparison to pH 5.7 ($p < 0.05$). The greatest decrease in thiols of approx. 19 nmol thiols/mg myosin was observed at pH 7.8 after 24 h of oxidation, equivalent to a 65% decrease in myosin thiols. A control experiment with oxidized myosin at pH 6.1 and pH 7.6 in the absence of SDS confirmed that oxidation of thiols in myosin is a fast reaction with a similar pH dependency as in the presence of SDS.

Detection of radicals by ESR

Radicals were detected during oxidation of myosin by hypervalent myoglobin using the spin trapping technique with detection by ESR spectroscopy (Figure 3). The ESR spectra of the PBN spin adducts had features that closely resembled nitroxide centered powder-spectrum, which suggests the PBN adducts have a low rotational mobility and therefore most likely are formed by trapping of protein centered radicals. Due to the lack of resolvable hyperfine coupling constants in the obtained ESR spectra, the exact identity of the initial protein radical can not be determined. However, in the study by Könczöl *et al.* (42) a similar myosin-PBN spin adduct was formed by oxidation with Ce(IV), which was assigned to a thiyl radical. The concentration of PBN spin adducts increased linearly as a function of myosin concentration ($R^2 = 0.999$) at room temperature (pH 7.4, I = 0.50) (Figure 5), which demonstrates that the formation of spin adducts are closely linked to the oxidation of myosin.

The role of myosin thiol groups on the oxidation of myosin was examined by blocking the cysteines in myosin with NEM. A significant decrease ($p < 0.001$) in the amount of PBN spin adducts was observed when NEM-treated myosin was oxidized in comparison to oxidation of untreated myosin (Figure 4). At high concentrations of NEM the same concentrations of PBN spin adducts were formed by the hypervalent myoglobin species in the presence and in the

absence of myosin ($p > 0.05$). This demonstrates that the thiol groups on myosin play an important role for the formation of radicals during the oxidation of myosin. NEM-treatment had only a small effect on the MbFe(III)/H₂O₂ radical generating system as a minor decrease in radical concentration of NEM-treated spin adducts was observed in comparison to radical concentrations without addition of NEM. These myoglobin-derived spin adducts are most likely due to reactions with histidine or α -amino groups of MbFe(III) (38, 39). •MbFe(IV)=O radical species are known to be either tyrosine- or tryptophan centred (43), and the pro-oxidative effect of the MbFe(III)/H₂O₂ radical generating system are therefore not expected to be strongly affected by NEM.

Effect of pH and ionic strength on myosin radical formation

The ionic strength (0.16, 0.25 and 0.50) and pH (5.1-7.4) affected the amount of detected spin adducts during the oxidation of myosin (Figure 5A). The highest amounts of myosin-PBN spin adduct were detected at the high ionic strength, where myosin exists primarily as free monomers in contrast to lower ionic strength where the majority of myosin is bound as dimers and synthetic filaments (44-47). The PBN spin adduct concentration of myosin derived radicals increased linearly with increasing pH at all three ionic strengths. Control studies where the hypervalent myoglobin species were generated in the absence of myosin gave significantly lower amounts of spin adducts at all ionic strengths and at all pH values except at pH 6.25 and I = 0.16 (Figure 5B).

Reduction of hypervalent myoglobin species by myosin

The hypervalent myoglobin species used in the present study to oxidise myosin are a mixture of •MbFe(IV)=O and MbFe(IV)=O together with unreacted MbFe(III) as generated by the activation of MbFe(III) with H₂O₂ (27, 48, 49). •MbFe(IV)=O and MbFe(IV)=O are both able to oxidise proteins, however their reactivities and the pH-dependency are very different (50). Kinetic studies of •MbFe(IV)=O reactions are complicated since the formation of •MbFe(IV)=O is slow compared to the reactions by which it decays. MbFe(IV)=O is long-lived, and kinetic studies of the reactions of this species are therefore more reliable. The reactions between i) •MbFe(IV)=O/MbFe(IV)=O and myosin, and between ii) MbFe(IV)=O and myosin were studied by UV-VIS spectroscopy at 25°C (pH

7.45, $l = 0.16$). It is well known that the reactions of MbFe(IV)=O are pH dependent (49). However, determination of the effect of pH on the reduction of MbFe(IV)=O by myosin was not possible as myosin precipitates at lower pH values. The product spectra showed that MbFe(IV)=O was reduced to MbFe(III) both in the absence (auto-reduction) and presence of myosin (Figure 6). Reduction of MbFe(IV)=O was clearly found to be accelerated by the presence of myosin in comparison to the auto-reduction of MbFe(IV)=O as evidence by the faster decrease in absorbance at 525-625 nm and faster increase around 500 nm and 650 nm in the presence of myosin.

The amount of MbFe(IV)=O reduced after 1 h increased with the concentration of myosin (Figure 7). This experiment could only be carried out with myosin concentrations up to 12 μ M due to limited solubility of myosin. Hence, an excess of hypervalent myoglobin species relative to myosin had to be used for these studies. A similar experiment was carried out by having myosin present during the activation of metmyoglobin with H₂O₂. However, in this experiment the concentration of hypervalent myoglobin species after 1 h was nearly independent of the myosin concentration. As •MbFe(IV)=O has the same spectral characteristics as MbFe(IV)=O it is not possible to separate the two species by UV-Vis spectroscopy. In the presence of both hypervalent myoglobin species, myosin reacted primarily with •MbFe(IV)=O to form MbFe(IV)=O, as no change in the total concentration of hypervalent myoglobin species was observed. On the other hand, in the experiment containing only MbFe(IV)=O, a decrease in the concentration of hypervalent myoglobin species was observed, which is caused by the reduction of MbFe(IV)=O to MbFe(III) in the presence of a sufficient concentration of myosin when no •MbFe(IV)=O is present.

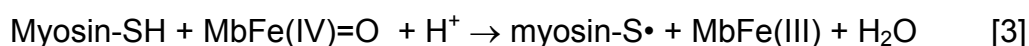
DISCUSSION

The role of thiol groups in myosin oxidation

Thiols are sensitive to oxidation and are generally believed to be a major target for oxidation in proteins (8). The present study has shown that thiol groups play an important role when myosin is oxidized by hypervalent myoglobin species. The oxidation has been shown to lead to the formation of disulfide cross-linked polymers of myosin and a corresponding major reduction of the amount of free thiol groups. The amount of radicals detected by the spin trapping technique during the oxidation of myosin was proportional to the myosin concentration indicating that the detected spin adducts were myosin-derived radicals. Additionally, blocking of thiol groups with NEM reduced the amount of spin adducts indicating that the formed myosin radical species were thiol-derived. Thiol centred radical formation on myosin is consistent with the study of Könczöl *et al.* (42), which demonstrated that myosin thiols are involved in the oxidation of myosin by reaction with the oxidizing agent Ce(IV). The proposed thiol-derived myosin radicals, initially generated by hypervalent myoglobin species, were observed to be short-lived, as evaluated by ESR spectroscopy. Myosin might be less effective in stabilizing radicals due to its rod-shaped structure, and application of a spin trap to the myosin reaction solution was required in order to obtain a detectable steady-state concentration of myosin radicals. However, using another type of ESR cell for larger sample volumes has previously been found to increase the sensitivity of ESR measurements and make a direct detection of myosin radicals without addition of spin traps possible (51). This effect may be due to the formation of long-lived myosin radicals upon reaction of the initially formed radical with myoglobin species or simply by an increased steady-state concentration of the myosin radicals. In the previous study (51), the radicals formed on myosin upon oxidation with hypervalent myoglobin species were found to be both tyrosyl- and thiol-derived radicals. Due to the reactivity of the formed myosin radicals and the rapid loss of thiol groups in myosin, as demonstrated in the present study, cross-linking of myosin is likely to occur immediately upon radical formation.

When a radical generating system consisting of exclusively MbFe(VI)=O was applied, reduction of MbFe(IV)=O to MbFe(III) was found to depend on myosin concentration indicating that MbFe(IV)=O reacts directly with myosin. The

reactions between hypervalent myoglobin species and the thiol groups in myosin may be summarized in two separate reactions:



Reaction 2 shows the fast reaction between $\bullet\text{MbFe(IV)=O}$ and myosin thiol groups. MbFe(IV)=O formed in reaction 2 may be further reduced to MbFe(III) if myosin is present in excess concentrations relative to $\bullet\text{MbFe(IV)=O}$ (reaction 3). The reaction between $\bullet\text{MbFe(IV)=O}$ and myosin has previously been found to be concentration dependent, where myosin at higher concentration was found by low temperature ESR spectroscopy to react with a similar higher concentration of $\bullet\text{MbFe(IV)=O}$ (51).

It is noteworthy, that the ESR study of myosin radical formation and thiol determination, both reflecting the early oxidative events, and the study of intermolecular disulfide cross-linking upon myosin oxidation, reflecting the late events (end products formation), support the same conclusion of thiols as the reactive site in myosin.

The observed intermolecular disulfide cross-link of myosin, formed as the major oxidation product after treatment of myosin with hypervalent myoglobin species, has also been demonstrated in model systems exposing myofibrillar protein fractions or purified myosin to hydroxyl radical generating systems (19, 22, 24-26, 52, 53). In contrast, hypervalent myoglobin species has previously been reported to mainly cause formation of intermolecular dityrosine bonds in myosin, but notably, formation of disulfide cross-linking was not investigated (20, 21). However, in the study by Morzel *et al.* (36), dityrosine formation and loss of thiol groups was observed after oxidation of myofibrillar proteins with a hydroxyl radical generating system indicating that both disulfide and dityrosine formation might have occurred. In the present study and in the previous study (51) both reducible (disulfides) and non-reducible (assigned to dityrosine) cross-links were observed in oxidized myosin samples indicating oxidation of other amino acid side chains apart from the cysteine.

pH dependency of myosin oxidation

During the first 25 s of reaction (referred to as early oxidation events in the following), myosin was concluded to be more sensitive towards oxidation by hypervalent myoglobin species at physiological pH (~7.4) than at pH representative for meat products (pH 5.5 - 6.0). This conclusion was based on the increased formation of myosin-PBN spin adducts and a significantly higher loss of thiol groups in myosin at pH 7.2 in comparison to 5.7 ($p < 0.05$) 25 s after initiation of oxidation. Increased oxidative modification of myosin at physiological pH is consistent with myosin thiols to be of importance regarding initiation of oxidation by hypervalent myoglobin species. The thiol anion (RS^-) is the most reactive form of sulphur compounds (54), and conversion of myosin thiols into its deprotonated form (RS^-) is more pronounced at physiological pH in comparison to pH representative for meat systems (pK_a of cysteine in proteins is 9-9.5) (55). In contrast, $MbFe(IV)=O$ was expected to be a stronger oxidant towards myosin at pH representative for meat systems in comparison to physiological pH (pK_a of $MbFe(IV)=O$ is 5.2 as determined in a study based on 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonate); (56)), as the protonated form of $MbFe(IV)=O$ is more reactive towards reducing substrates in comparison to the non-protonated $MbFe(IV)=O$ (50, 57, 58). However, increased oxidative modification of myosin at pH 5.5-6.0 compared to pH 7 was not observed during the early oxidation events, as evaluated by myosin-PBN spin adduct formation and decrease in thiol concentration 25 s after initiation of oxidation. The reaction between $\bullet MbFe(IV)=O$ and myosin is fast and the reactivity of $\bullet MbFe(IV)=O$ towards other proteins has previously been found to be less pH dependent compared to the reactivity of $MbFe(IV)=O$ (50). The lack of increased oxidation of myosin at lower pH values may be due to the fact that both hypervalent myoglobin species are present in the reaction solution with $\bullet MbFe(IV)=O$ being more reactive than $MbFe(IV)=O$ as discussed previously.

As for the late oxidation events, additional loss of thiol groups in myosin was observed after 1 h and 24 h at pH 5.0 and pH 7.8, but not at the intermediary pH values studied ($p < 0.001$). At pH representative for meat systems (5.7), a 28% decrease of myosin thiols was observed 25 s after initiation of oxidation, in comparison to a 38% decrease obtained after 24 h. This suggests that thiol centered myosin oxidation occurs shortly after initiation of oxidation and only increases slightly up to 24 h. This indicates that the early oxidation events are

more pH dependent than the late oxidation events for myosin. Consistently, no difference in extent of intermolecular myosin disulfide bonding was observed 1 h after oxidation of myosin at pH 5.0, 6.4 and 7.8 by SDS-PAGE analysis. Hence, choice of pH of the model system to simulate conditions of meat was demonstrated to be of minor importance for the oxidative sensitivity of myosin.

Dependency of ionic strength for oxidation of myosin

Ionic strength of the model system was found to be important for the oxidative sensitivity of myosin. At $I = 0.50$ the majority of myosin is present as monomers (59), which were demonstrated to be more sensitive towards oxidation in comparison to the synthetic myosin filaments (mainly present at $I = 0.16$) as higher concentrations of myosin radical spin adducts were detected at $I = 0.50$ than at $I = 0.16$. This difference could be explained by better access of hypervalent myoglobin species to the myosin molecule at higher ionic strength where the majority of myosin is present as monomers.

Based mainly on ESR spectroscopy it became possible to identify two steps in myosin oxidation by hypervalent myoglobin formed in meat during storage. The initial step including radical formation is less dependent on pH but strongly dependent on ionic strength. The late oxidation event results in disulfide cross-linking of myosin and is related to reduced tenderness of meat.

Early oxidation events regarding myosin are more pH dependent than late oxidation events. Hence, myosin thiols were suggested to be the main target of oxidative modification by hypervalent myoglobin species resulting in intermolecular cross-linking through disulfide bonding.

ABBREVIATIONS USED

ATP, adenosine triphosphate; DTNP, 2,2'-dithiobis(5-nitropyridine); DTT, dithiothreitol; EDTA, ethylenediamine-tetraacetic acid; EGTA, ethylenbis(oxyethylenenitrilo) tetraacetic acid; MbFe(III), metmyoglobin; MbFe(IV)=O, ferrylmyoglobin; •MbFe(IV)=O, perferrylmyoglobin; MES, 2-[N-morpholino]ethanesulfonic acid; NEM, N-ethylmaleimide; PBN, N-tert-butyl- α -phenylnitron; SDS, sodium dodecyl sulphate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TEMPO, 2,2,6,6-tetramethylpiperidine 1-oxyl; tricine, N-[tris(hydroxymethyl)methyl]glycine; tris, tris-(hydroxymethyl) aminomethane.

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FIGURE CAPTIONS

Figure 1 SDS-page of myosin oxidized by hypervalent myoglobin species. Myosin (13 μM) was incubated 1 h at room temperature with MbFe(III) (75 μM) and H_2O_2 (75 μM) at pH 5.0 (lane 1), pH 6.4 (lane 2), and pH 7.8 (lane 3) and without MbFe(III) and H_2O_2 at pH 5.0 (lane 4), pH 6.4 (lane 5), and pH 7.8 (lane 6) at non-reducing conditions (gel A) and reducing conditions (gel B). Buffers (20 mM, $I = 0.50$) contained 5% SDS during the oxidation. Five μg myosin was loaded in each lane. Lane 7 contained molecular weight standard. MHC: myosin heavy chain.

Figure 2 Free thiol groups on myosin after oxidation with hypervalent myoglobin species. Myosin (46.8 μM) was oxidized with 75 μM MbFe(III) and 75 μM H_2O_2 for 25 s (\bullet), 1 h (\blacktriangle) and 24 h (\blacktriangledown). Control experiments with myosin, but without MbFe(III) and H_2O_2 , after 1 h (\blacksquare). All incubations were carried out at 25 $^\circ\text{C}$ (pH 5.0-7.8, $I = 0.50$).

Figure 3 (I) Radical formation (μM PBN spin adducts) demonstrating oxidation of myosin by hypervalent myoglobin species. Myosin (0-46.8 μM) was oxidized by 300 μM MbFe(III) and 300 μM H_2O_2 at room temperature (pH 7.4, $I = 0.50$). (II) ESR spectra of control with MbFe(III)/ H_2O_2 /PBN (A) and myosin/MbFe(III)/ H_2O_2 /PBN (B) were recorded 25 s after initiation of oxidation at room temperature.

Figure 4 The effect of thiol blocking by NEM on the formation of PBN spin adducts during oxidation of myosin by hypervalent myoglobin species. Myosin (23.5 μM) was treated with NEM (0-5 mM) before mixing with MbFe(III) (300 μM), H_2O_2 (300 μM), and PBN (30 mM) and ESR detection of spin adducts (■). Control experiments without myosin (●). ESR spectra were recorded 25 s after initiation of oxidation at room temperature (pH 7.0, $I = 0.50$) by presence of 30 mM PBN.

Figure 5 The effects of pH and ionic strength on the formation of spin adducts during myosin oxidation by hypervalent myoglobin species. Spin adducts detected by ESR 25 s after mixing myosin (46.8 μM) with MbFe(III) (300 μM), H_2O_2 (300 μM), and PBN (30 mM) in buffers with ionic strengths $I = 0.50$ (■), 0.25 (●) and 0.16 (▲) (Figure A). Control experiments without myosin (Figure B).

Figure 6 Spectral change (450-700 nm) of MbFe(IV)=O in A) absence of myosin (auto-reduction) and B) presence of 11.2 μM myosin. The reactions were followed at 25 °C (pH 7.5, $I = 0.16$) for 60 min. MbFe(IV)=O was prepared by mixing equal concentrations of MbFe(III) and H_2O_2 corresponding to final concentrations of 100 μM . The direction of spectral change in relation to the initial spectrum is indicated by an arrow. The insert shows the relative absorbance (588-700 nm) of MbFe(IV)=O as a function of time (min) in absence of myosin (●) and presence of 11.2 μM myosin (○).

Figure 7 Concentration of hypervalent myoglobin species after mixing MbFe(IV)=O (30 μM) with myosin (■), and after mixing MbFe(III) (100 μM) with H_2O_2 (100 μM) in the presence of myosin (●). Concentrations are calculated on the basis of equation 1 after 60 min of reaction in the presence of 0-12 μM myosin at 25 °C (pH 7.5, $I = 0.16$).

FIGURES

Figure 1

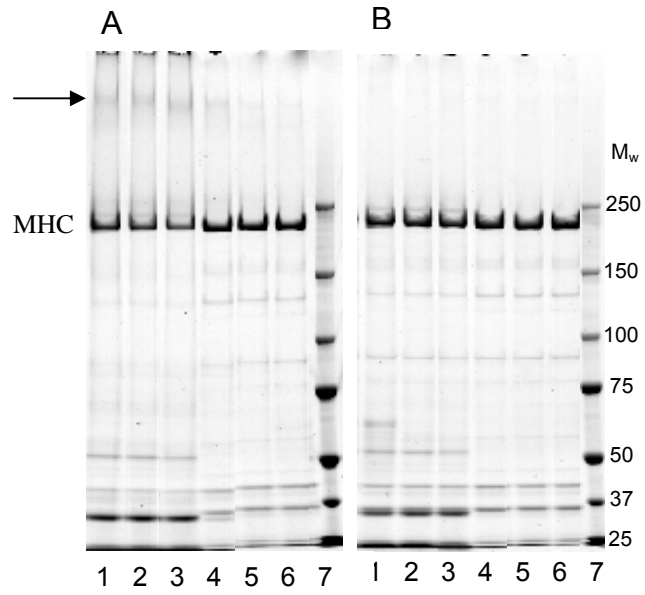


Figure 2

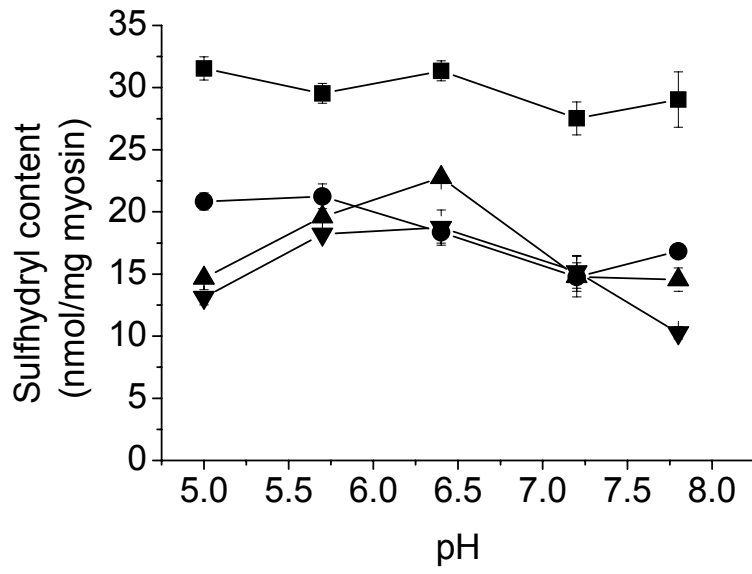


Figure 3

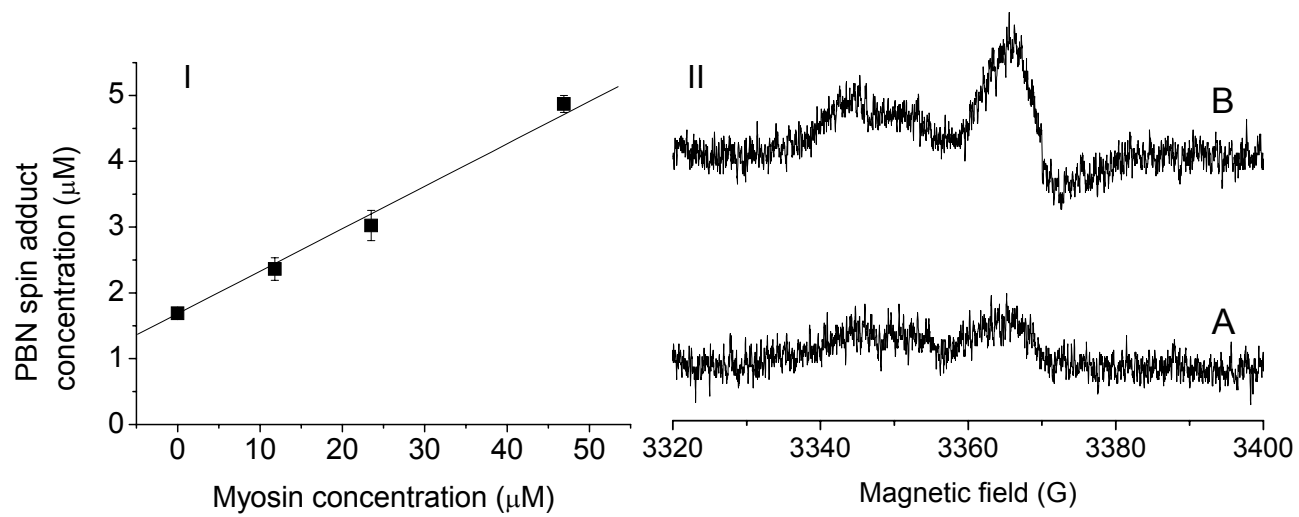


Figure 4

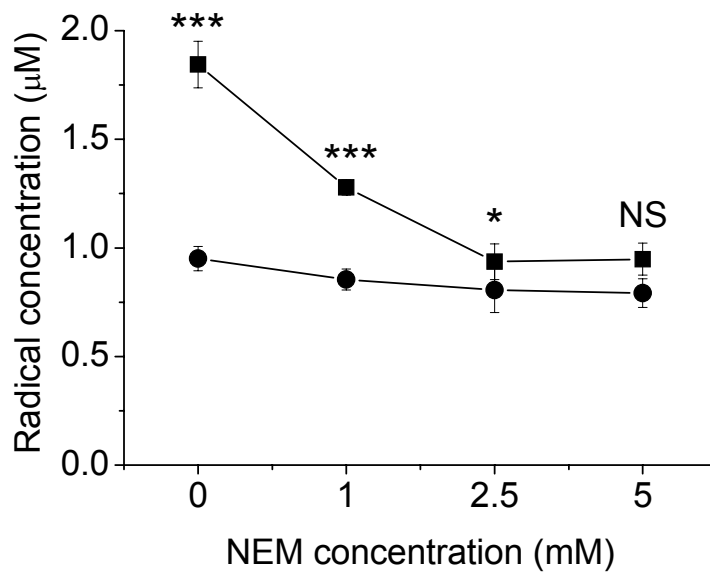


Figure 5

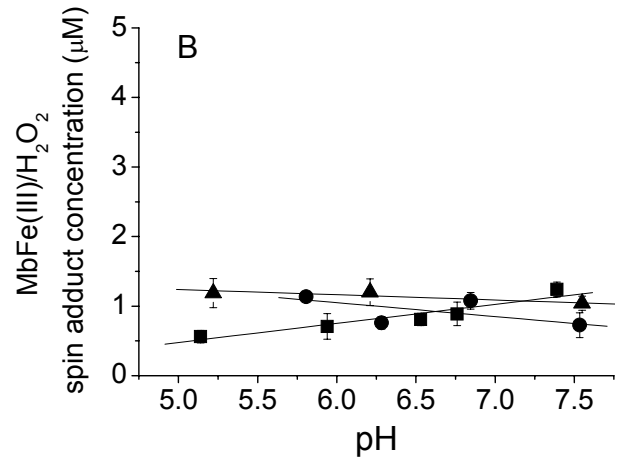
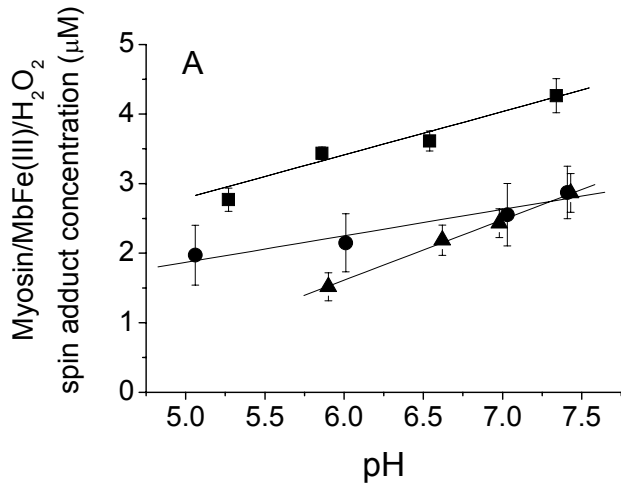


Figure 6

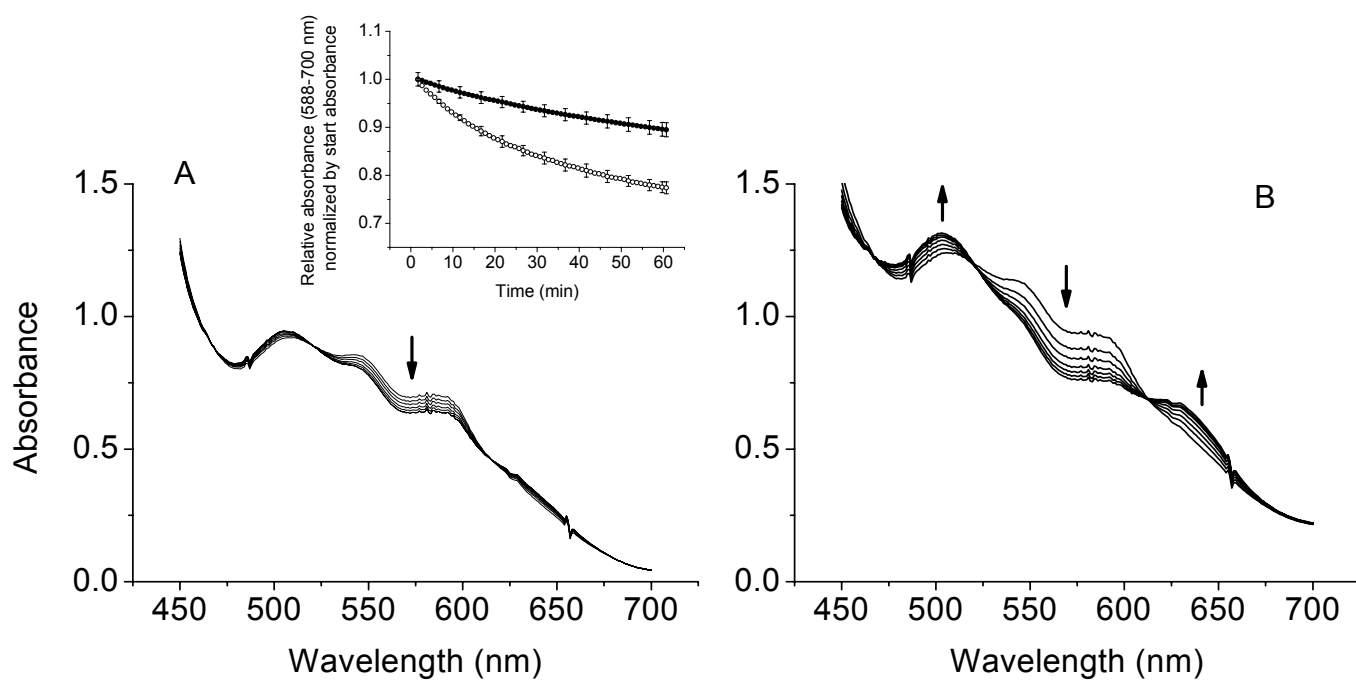
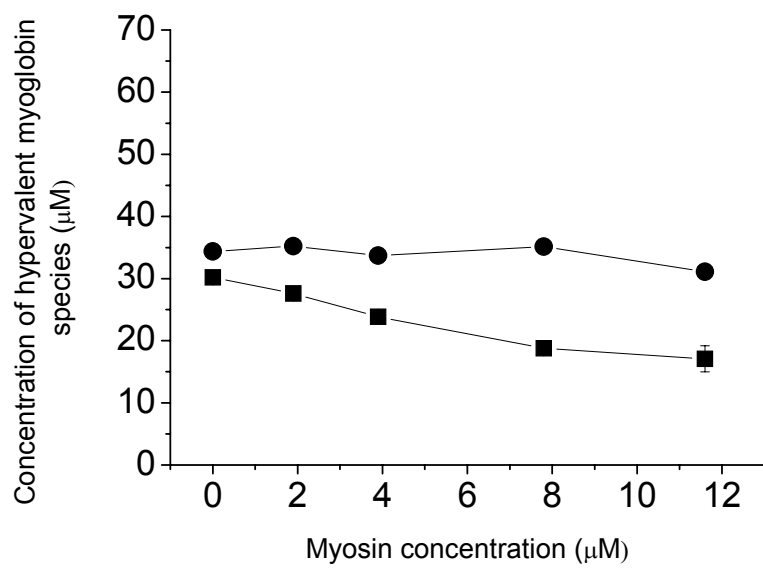


Figure 7



Effect of high-oxygen atmosphere packaging on mechanical properties of single muscle fibres from bovine and porcine *longissimus dorsi*

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Abstract

The effect of storage in high-oxygen atmosphere packaging for 48 h at 4°C on the myofibrillar component of meat toughness were studied by performing tensile tests on single muscle fibres isolated from beef and pork *longissimus dorsi* (LD). Storage of bovine LD for 48 h in presence of oxygen significantly increased the breaking strength of single muscle fibres compared to storage in a 100% nitrogen atmosphere. In contrast, the breaking strength of porcine LD stored for 2 days *post-mortem* in presence of oxygen in the packages was not influenced.

Keywords: beef, pork, modified atmosphere packaging, breaking strength, tensile test.

Introduction

Modified atmosphere packaging with high concentrations of oxygen of beef (Lund, Hviid, & Skibsted, 2007; Seyfert *et al*, 2005) and pork (Lund, Lametsch, Hviid, Jensen, & Skibsted, 2007) or irradiation of beef (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004b) have been found to reduce subjectively assessed tenderness and increase Warner-Bratzler shear force (WBSF). The decrease in tenderness has been assigned to oxidative processes although the mechanism is yet to be described in detail. Sensory analysis and WBSF are performed on large meat samples, which complicate conclusions on the effect of oxygen on the individual myofibrillar and connective tissue components. However, tensile tests performed on single muscle fibres isolated from large meat samples would provide unique information of direct effects on the myofibrillar component.

Post-mortem proteolysis and the resulting meat tenderization is believed mainly to depend on the calpain system (Koochmaraie & Geesink, 2006). Calpains (m-calpain and μ -calpain) are calcium-activated proteases containing an oxidizable cysteine residue at the active site (Guttmann, Elce, Bell, Isbell, & Johnson, 1997). Oxidative conditions resulting from irradiation have been shown to influence beef tenderness negatively through inactivation of μ -calpain (Rowe *et al.*, 2004b). A positive correlation has been found between myofibrillar protein carbonyl content (a general measure of protein oxidation) and WBSF (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004a). Furthermore, myosin, which constitutes approx. 50% of the myofibrillar proteins (Pearson & Young, 1989), is very susceptible to *in vitro* oxidation and forms intermolecular cross-links which cause polymerization and aggregation of the protein (Decker, Xiong, Calvert, Crum, & Blanchard, 1993). Oxidative cross-linking of myosin has been found to reduce gel-forming ability and protein solubility which is important for the functional performance of muscle proteins in processed meat products (Liu, Xiong, & Butterfield, 2000; Srinivasan & Hultin, 1997). The cross-linking of myosin due to oxidation has also been suggested to influence tenderness of fresh porcine *longissimus dorsi* (LD) stored in high-oxygen atmospheres negatively (Lund *et al.*, 2007). Two mechanisms could accordingly explain that modified atmosphere packaging with a high concentration of oxygen affects the mechanical properties of the myofibrillar component and thereby tenderness: i) inactivation of μ -calpain due to oxidation, and ii) oxidative cross-linking of myosin.

The aim of the present study was to investigate the effect of presence of oxygen in the packaging atmosphere (100% nitrogen, atmospheric air, and 80% oxygen/20% nitrogen) on the breaking strength of single muscle fibres isolated from pork and beef LD. The

activity of μ -calpain, the degree of myosin cross-linking, and content of free thiol groups in proteins were also investigated.

Materials and methods

Chemicals

5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), ethylenediaminetetraacetic acid (EDTA), mannitol, and tris(hydroxyamino)methane (tris) were purchased from Merck, Darmstadt, Germany. Glycine and sodium dodecylsulphate (SDS) were purchased from MP Biomedicals, Inc., Ohio, USA. Bovine serum albumin (BSA) and Coomassie Brilliant Blue were obtained from Sigma Chemical Co., St. Louis, USA. Monothioglycerol was obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Dithiothreitol (DTT) was obtained from Acros Organics, Geel, Belgium. Bromophenol blue, ethyleneglycolbis(β -aminoethylether)-N,N,N',N'-tetracetate (EGTA), glycerol, and 2(N-morpholino)ethanesulfonic acid (MES) were obtained from AppliChem GmbH, Darmstadt, Germany. Water was purified through a Millipore Q-plus system (Millipore Corp., Bedford, MA). All chemicals were of analytical grade.

Packaging of meat

Longissimus dorsi (LD) from left and right side of one animal of each species were obtained from The Danish Meat Trade College (Roskilde, Denmark) at 1 day *post-mortem* (day 1). The LD muscles were cut into four pieces each of 10 cm (along the fibre direction) x 10 cm (width) x 3 cm (height) and trimmed to obtain the same headspace in each package. The four pieces were used as i) control (immediately frozen and stored at -80°C until analysis), ii) packed in 100% N₂, iii) packed in atmospheric air (21% O₂/79% N₂, and iv) packed in high-oxygen atmosphere (80% O₂/20% N₂). Left and right sides of each species were used as duplicates. Each LD piece was placed in a polypropylene (PP) tray type 71-43A (Færch Plast, Denmark). For packaging in 100% N₂ and 80% O₂/20% N₂ a Multivac T200 (Sepp Hagenmüller GmbH & Co., Germany) was used with TOPSEAL™ PP MAP AF 57 film (Færch Plast, Denmark) with oxygen transmission rate: <100 cm³/m²/24 h/atm, and packaging gas from Yara Industrial A/S, Denmark. For packaging in atmospheric air the trays containing a LD piece was wrapped with a plastic film (Borden Packaging Ltd., South Glamorgan, UK). The LD pieces packed in three different packaging atmospheres were stored at 4°C in the dark for 2 days. The gas composition in the packs was measured with a CheckMate 9900 (PBI Dansensor, Ringsted, Denmark) prior to sampling of meat in order to check the packs for leaks. pH of the LD pieces was measured with a electrode (Knick Portamess 751 calimatic, Berlin, Germany), which had been calibrated at 4°C prior to measurement. Each LD piece was cut into one 7 cm piece for

tensile testing and the remaining 3 cm was cut into smaller pieces for chemical and SDS-page analysis. All samples were vacuum packed, frozen, and stored at -80°C until further analysis.

Isolation of single muscle fibres

The procedure for isolation of single muscle fibres broadly followed the methodology described by Christensen, Young, Lawson, Larsen, and Purslow (2003). Prior to isolation of fibres, the LD sample was thawed at 5°C for 24 hours and cooked in a circulating waterbath (75°C) until the centre of the sample obtained 70°C. The sample was subsequently cooled under running water until a centre temperature of 20°C. Approximately 40 single muscle fibres were isolated from muscle samples packed in each of the three packaging atmospheres in a dissection buffer containing 50 mM MES (pH 5.60), 100 mM KCl, 280 mM mannitol and 0.2 mM EGTA. The diameter and sarcomere length of the fibres were measured using a Leica DMIRB microscope equipped with a 40X/0.55 objective (Leica Microscopie und Systeme GmbH, Bensheim, Germany). Photos of the fibres were taken with Photometric Cool SNAP™ camera (Roper Scientific Inc., Tucson, USA) and the software program RSIImage (Roper Scientific Inc.). Measurements of fibre diameters (n = 9) and sarcomere length (n = 6) were performed using Image Pro Plus software (Media Cybernetics, Silver Spring, Maryland, USA). The fibre cross-sectional area ($\pi \times (\text{fibre diameter}/2)^2$) were estimated and used for calculation of breaking strength and the free length of the fibre was measured using vernier callipers and used for calculation of breaking strain.

Tensile tests

The specimen chamber of the mechanical testing device was similar to that described by Lewis and Purslow (1989). The aluminium template with the fibre glued onto it was attached between two screw-up clamps, one side attached to a motor and the other to an isometric force transducer (model UF1, Pioden Controls Ltd, Canterbury, England). The side-pieces of the aluminium template were carefully cut leaving the fibre hanging between the two plate ends. Fibres were stretched at a constant rate of 13.8 $\mu\text{m/s}$ until fracture. Breaking loads and extensions were monitored using LabView data acquisition software (National Instruments, Kokkedal, Denmark). Breaking strength was calculated as maximum force per unit cross-sectional area of each fibre and breaking strain was taken

as the strain at maximum strength divided by the initial length of the fibre and expressed as percentage.

Free protein thiol groups

5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) was used for determination of free thiols in proteins (Ellman, 1959). Briefly, 2.0 g muscle were homogenized in 50 ml 5.0% SDS in 0.10 M tris buffer (pH 8.0) using Ultra Turrax (Ika, Labortechnik, Staufen, Germany). The homogenates were placed in a water bath at 80°C for 30 min and centrifuged at 1200 g for 20 min in a Sigma 2-5 centrifuge (Buch & Holm, Herlev, Denmark). The supernatant was filtered through Filtrak filter paper 3w, 100mm, 65g/m² (Spezialpapier FILTRAK GmbH, Niederschlag, Germany). Protein concentration of the filtrate was determined by measuring absorbance at 280 nm and using a standard curve prepared from 0-3 mg/ml BSA. The filtrates did not absorb light >300nm and myoglobin was therefore not present and did not interfere with the assay.

The filtrates were diluted to a concentration of 1.5 mg/ml with homogenization buffer and assayed according to Liu and Xiong (2000) by mixing 0.50 ml sample, 2.0 ml 0.10 M tris buffer (pH 8.0) and 0.50 ml 10 mM DTNB in 0.10 M tris buffer (pH 8.0). Absorbance at 412 was measured after 30 min against an aqueous reference solution of 0.50 ml 5% SDS, 2.0 ml 0.10 M tris buffer (pH 8.0) and 0.50 ml 10 mM DTNB in 0.10 M tris buffer (pH 8.0). Thiol content was calculated as μM thiol/mg protein. Triplicate measurements were made for each meat sample and mean values were used for statistical analysis.

Protein cross-linking by SDS-page

The extent of myosin cross-linking was determined SDS-page analysis using the diluted filtrates prepared for determination of free protein thiol groups. Samples were analysed twice with gel electrophoresis using NuPAGE® Novex Tris-Acetate Gels (Invitrogen, Carlsbad, CA, USA). The amount of protein loaded in each lane was 1 μg and DTT was added directly to samples when reducing conditions was employed during electrophoresis. Fluorescence staining of proteins was used (Molecular Probes SYPRO® Ruby Protein Gel Stain from Invitrogen, Carlsbad, CA, USA), and stained gels were photographed by a Charge-Coupled Device (CCD) camera (Raytest, Camilla II, Straubenhardt, Germany).

Casein zymography

Casein zymography was conducted to measure μ - and m-calpain activity (Raser, Posner, & Wang, 1995). Frozen meat (2.0 g), minced by a coffee grinder (Braun AromaGourmet KSM2, Naucalpan de Juárez, Mexico), were homogenized in cold extraction buffer (50 mM tris, 5.0 mM EDTA, 10 mM monothioglycerol, pH 8.0) by Ultra Turrax T25 equipped with a S25N-18G dispersing element (Ika, Labortechnik, Staufen, Germany) at 13500 rpm for 3 x 20 s. The homogenate was left on ice for 10 min and subsequently centrifuged at 15000 g for 30 min at 4°C (HiCen21 centrifuge, Herolab, Wiesloch, Germany). Glycerol (33% final concentration) was added to the supernatant and samples were frozen at -80°C until analysis.

The casein gel (Criterion™ Zymogram Gel, 12.5% zymogram, casein, Bio-Rad Laboratories, Hercules, CA, USA) was prerun at 80 V with running buffer containing 25 mM tris, 192 mM glycine, and 1 mM EDTA for 15 min. Gel samples were made by diluting the sample with ice-cold sample buffer (75 mM tris, 10% glycerol, 50 mM DTT, and bromophenol blue, final concentration) at a ratio of 1:3 ((V/V) sample: sample buffer). Samples were then loaded onto the casein gel and the electrophoresis was run at 80 V for 3 hours on ice. Subsequently, the gel was incubated in 50 mM tris, 4 mM CaCl₂, 10 mM monothioglycerol (pH 7.5) for 60 min. Incubation was terminated by transferring the gel to a solution of 20 mM tris and 10 mM EDTA for 30 min. Finally, the gel was stained with Coomassie blue.

Statistical analysis

Statistical analysis were performed using the SAS® 9.1 package, SAS Institute, Inc., USA. Data was analysed by analysis of variance using proc mixed. Packaging atmosphere (100% N₂, atmospheric air, and 80% O₂/20% N₂) was included as a fixed effect and packaging replicate (each side of the animal was used as replicate) was included as a random effect. Least square means were used to compare differences, and differences of least square means (LSD) were applied to compare the mean values of packaging atmosphere and storage time.

Results

Single muscle fibres isolated from beef LD packed and stored in an oxygen atmosphere (either 21% or 80% oxygen) had significantly higher breaking strength compared to storage in 100% N₂ (Figure 1A). The breaking strength of single muscle fibres isolated from day 1 beef LD (control) did not differ significantly from the breaking strength of single muscle fibres isolated from beef LD packed and stored in 100% N₂ or atmospheric air. However, single muscle fibres from beef LD was found to be significantly stronger ($P < 0.01$) when stored in a high-oxygen atmosphere compared to the control indicating that packaging in high-oxygen atmosphere increases the strength of the myofibrillar component from day 1 to day 3 *post-mortem*. Breaking strain of single muscle fibres isolated from beef LD were significantly lower ($P < 0.01$) when beef was stored in 100% nitrogen compared to the control and the other packaging atmospheres (Figure 1B).

The breaking strength of porcine single muscle fibres did not differ significantly between the different gas compositions. However, porcine single muscle fibres isolated from day 1 (control) were significantly weaker ($P < 0.05$) than fibres isolated from pork samples stored for 2 days in different atmospheres (Figure 1A). For breaking strain no difference was found between the four different packages (Figure 1B).

Figure 2 shows that the protein pattern did not differ between the different packages for neither beef nor pork samples. Hence, the extent of myosin cross-linking is limited and identical in all samples. Additionally, determination of free protein thiol groups did not show any differences (data not shown).

To determine the effect of the gas composition in the packaging atmosphere on calpain activity in beef or pork LD, casein zymography was employed. From day 1 (control) to day 3 *post-mortem* the μ -calpain activity decreased in both beef and pork regardless of the gas composition (data not shown). After 2 days of storage the μ -calpain activity was too low to determine any differences in beef or pork LD stored in the three different packaging atmospheres.

Discussion

Effect of gas composition on breaking strength of bovine single muscle fibres

Single muscle fibres isolated from beef LD stored in 100% nitrogen for 2 days at refrigerated temperatures were weaker than fibres isolated from the un-aged control (day 1 *post-mortem*). This indicates that proteolysis had occurred and induced weakening of the myofibrillar component in the absence of oxygen. However, single muscle fibres isolated from beef stored in a high-oxygen atmosphere for 2 days was stronger than fibres isolated from the un-aged control indicating that even though proteolysis had occurred during the two days of storage the presence of 80% oxygen in the packaging atmosphere induced strengthening of the myofibrillar component.

The increased strength of bovine muscle fibres stored in high-oxygen packaging atmosphere may be due to i) inactivation of μ -calpain leading to reduced proteolysis of myofibrillar proteins and hereby reduced tenderization or ii) cross-linking of myofibrillar proteins and formation of aggregates.

According to Veiseth, Shackelford, Wheeler, and Koochmaraie (2001) μ -calpain undergoes autolysis in meat *post-mortem* and is thereby inactivated typically within 1 to 3 days after slaughter. However, when beef steaks are irradiated, which is a powerful method to initiate oxidation, μ -calpain is inactivated and unable to autolyze, and μ -calpain activity can be detected even after 15 days *post-mortem* (Rowe *et al.*, 2004b). No significant difference in μ -calpain activity between the three packaging atmospheres was found in the present study. Hence, i) either the oxidation taking place in a high-oxygen atmosphere does not cause reversible oxidation of μ -calpain, or ii) the oxidation is not powerful enough to inactivate μ -calpain and prevent the enzyme from autolysis, or iii) other enzymatic systems (as e.g. cathepsins or the 20S proteasome) are involved in development of tenderness *post mortem* as suggested by others (Dutaud, Aubry, Guignot, Vignon, Monin, & Ouali, 2006; Sentandreu, Coulis, & Ouali, 2002).

In the present study differences in protein oxidation by measurement of free protein thiol groups (data not shown) and myosin cross-linking by SDS-page analysis (Figure 2) could not be detected. In order to increase the sensitivity of the methods, muscle samples were homogenized in a buffer containing 5% SDS to extract as much protein as possible from the samples, and a highly sensitive fluorescent staining was used to stain the proteins after SDS-page. However, the presence of highly diverse proteins might have disguised any effect of packaging atmosphere in the chemical and electrophoretic analyses which may not occur in single muscle fibres used for tensile tests. Another explanation could be that the methods used are still not sensitive enough to detect any

differences between the samples, or that the proteins are simply not oxidized in the muscle samples.

According to Lund et al. (2007) oxidation of protein thiol groups and myosin cross-linking in porcine LD was only detectable after 8 days of storage in high-oxygen atmosphere, whereas decreased tenderness was observed after 4 days of storage. This observation may also explain why it was not possible to detect any differences in protein oxidation and myosin cross-linking after only 2 days of storage in the present study.

Hence, to what extent inactivation of μ -calpain or myosin cross-linking influence the breaking strength of single muscle fibres is not possible to conclude from the present study.

Effect of gas composition on breaking strength of porcine single muscle fibres

Single muscle fibres isolated from porcine LD is much weaker than bovine muscle fibres. This difference in strength probably reflects the variations in the rate of tenderness development between the two species. Meat tenderization differs greatly in rate between animal species with tenderization rate being faster in pork than beef (Dransfield, Jones, & Macfie, 1981; Etherington, Taylor, & Dransfield, 1987; Koohmaraie, Whipple, Kretchmar, Crouse, & Mersmann, 1991). The involvement of the calpain system in tenderization has been extensively investigated. Although, no difference between μ -calpain levels in different species have been found, the calpain inhibitor, calpastatin, are found in much greater amount in bovine muscle compared to porcine muscle (Ouali & Talmant, 1990), which might also explain why single muscle fibres isolated from porcine LD is weaker than single muscle fibres isolated from beef LD. In contrast to the results obtained on beef, comparison of the three packaging atmospheres showed that the breaking strength of porcine muscle fibres is not affected by presence of oxygen in the packages. Surprisingly, the breaking strength of single muscle fibres isolated from control samples (day 1 *post-mortem*) is lower than day 3 samples (all packaging atmospheres) (Figure 1). Sensory and objectively assessed tenderness have found variation along the porcine longissimus muscle (Hansen, Hansen, Aaslyng, & Byrne, 2004; Møller & Vestergaard, 1986), and it can not be ruled out that sample location affected the breaking strength of fibres.

Bovine LD consist of more myoglobin than porcine LD (Hazell, 1982; Lawrie, 1952). Myoglobin is known to interact and promote oxidation by formation of hypervalent myoglobin species under exposure of reactive oxygen species (Kanner & Harel, 1985). Hence, increased presence of myoglobin in beef muscle fibres may cause higher susceptibility to oxidation of beef and explain the obtained effects of oxygen presence

during storage on breaking strength of bovine muscle fibres. In the present study, fibre type characterisation of the single muscle fibres used for tensile tests was not performed. However, it is likely that more type I fibres has been included in the tensile test for beef LD as compared to porcine LD and hence it can not be excluded that this may partly explain the different results for the two species.

In conclusion, high-oxygen packaging atmosphere results in strengthening of single muscle fibres isolated from beef LD, whereas the strength of single muscle fibres is not affected by packaging atmosphere in porcine LD. Protein oxidation by measurement of free protein thiol groups, myosin cross-linking, and inactivation of μ -calpain could not be detected.

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Figure Captions

Figure 1 Breaking strength (A) and strain at maximum strength (B) of single muscle fibres isolated from fresh beef and pork *longissimus dorsi* 1 day after slaughter (control; black bars) and stored in modified atmosphere for 2 days in 100% nitrogen (dark grey bars), atmospheric air (21% oxygen/79% nitrogen) (light grey bars), and 80% oxygen/20% nitrogen (white bars). Bars bearing different letters are significant different within species of animal ($p < 0.01$).

Figure 2 SDS-page of homogenates obtained from beef (A) and pork (B) *longissimus dorsi* 1 day after slaughter (lanes 1,8) and stored in modified atmosphere for 2 days in 100% nitrogen (lanes 2,3), atmospheric air (21% oxygen/79% nitrogen) (lanes 4,5), and 80% oxygen/20% nitrogen (lanes 6,7).

Figure 1

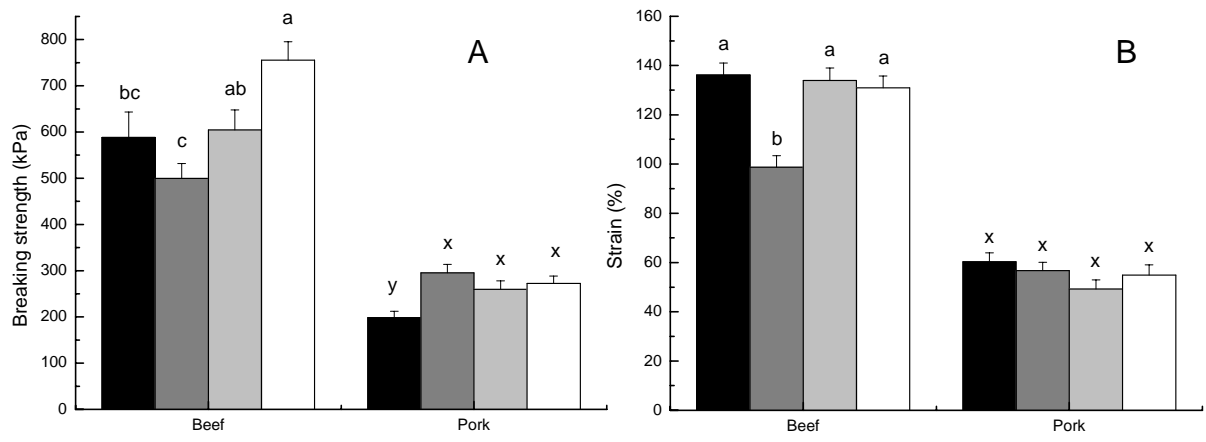


Figure 2

