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# PROTEOLYSIS AND PROTEIN OXIDATION IN DRY FERMENTED SAUSAGES

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AA | ascorbic acid |
| AAS | α-amino adipic semialdehyde |
| ABA | 4-aminobenzoic acid |
| BSA | bovine serum albumin |
| DHAA | dehydroascorbic acid |
| DNPH | 2,4-dinitrophenylhydrazine |
| DTNB | 5,5′-dithiobis(2-nitrobenzoic acid) |
| DTPA | diethylenetriaminepentaacetic acid |
| EGTA | ethylene glycol tetraacetic acid |
| GGS | γ-glutamic semialdehyde |
| MDA | malondialdehyde |
| MES | 2-(N-morpholino) ethanesulfonic acid |
| OPD | orthophenylenediamine |
| SA | sodium ascorbate |
| SDS | sodium dodecyl sulfate |
| SDS-PAGE | Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis |
| SN | sodium nitrite |
| TBARS | thiobarbituric acid reactive substances |
| TCA | trichloroacetic acid |
| TRIS | tris(hydroxymethyl)aminomethane |

# TABLE OF CONTENTS

# INTRODUCTION

## GENERAL BACKGROUND

Meat and meat products are an important part of the human diet providing essential nutrients such as proteins, vitamins and minerals. In ancient times, processing techniques have been developed that allowed storing meat at ambient temperature and increasing shelf life. In particular, salting and drying allows to reduce water activity protecting meat against spoilage (Zeuthen, 2014). Moreover, the discovery of meat fermentation introduced a further antimicrobial hurdle permitting the preservation of products with higher oxidative and microbial instability such as minced meat and fat (Leroy et al., 2013). Dry fermented sausages are the perfect result of these processing techniques, i.e. salting, drying and meat fermentation. Acidulation, caused by lactic acid production, and water activity reduction, due to salting and drying, drive the change from raw meat to a desirable fermented product (Ockerman and Basu, 2014).

The use of different ingredients, manufacturing processes and conditions gave rise to hundreds of dry fermented sausage types subdivided in regional specialities whose recipes have been transmitted through generations (Leroy et al., 2013). For this reason, dry fermented sausages are considered traditional products by consumers. Each dry fermented sausage type possesses its own flavour, texture, appearance and history (Leroy et al., 2013). Nowadays, all these features are still found in artisanal products which follow old and traditional methods and recipes, and often require a long ripening period or rely on microbes naturally present in the environment (Guerrero et al., 2009). On the other hand, industrial productions seek for fast, controlled and standardized

processing methods which require the use of selected starter cultures. Since the final sensory characteristics of the product highly depend on the ripening time and microbes used, industrial products often differ in quality from artisanal products (Leroy et al., 2013).

Dry fermented sausages are biological systems, in which a lot of physico-chemical changes occur. Therefore, the manufacturing of high quality and safe products with fast and standardized industrial processing methods needs profound knowledge and control of the process. However, the complexity of this biological system has not been fully elucidated yet. Therefore, further efforts are needed to clarify the mechanisms that regulate flavour formation and ensure safety.

**Sensory quality**

The final sensory characteristics of dry fermented sausages are determined by the ingredients and additives used, the processing conditions applied and bacteria present in the meat batter. During processing, numerous physico-chemical changes occur, of which the most important are fermentation, drying, proteolysis, lipolysis and oxidation phenomena. Indeed, flavour is given by the interaction of taste, which mainly derives from bacterial fermentation and tissue-generated proteolysis, and aroma, which is mainly determined by bacterial metabolism and lipid autoxidation (Leroy et al., 2006).

For these reasons, the great bulk of research in sensory quality of dry fermented sausages aimed at unravelling these physico-chemical changes. Several studies have focused on proteolysis (Molly et al., 1997; Hughes et al.,

2002, Hierro et al., 1999) and lipolysis (Ordóñez, et al., 1999; Hierro et al., 1997). Simultaneously, the use of exogenous proteolytic and lipolytic enzymes has been tested to speed up these processes (Fernández et al., 2000). The roles of several bacteria strains on fermentation and aroma compounds generation were evaluated as well (Leroy et al., 2006). Oxidation has also received attention: in particular lipid oxidation has been extensively studied (Zanardi et al., 2004 Ordóñez et al., 1999), whereas protein oxidation has gained interest only recently (Fuentes et al., 2014).

**Nutritional quality and safety**

In the history of dry fermented sausages, this product has often raised health concerns. Microbial hazards were a major issue in the past and, in particular, *Clostridium botulinum* made many victims (Erbguth, 2004). Nowadays, the regular use of nitrite and starter cultures has overcome this problem. Nevertheless, new concerns about dry fermented sausage consumption have been pointed out by meat scientists and nutritionists. Indeed, these products are rich in sodium chloride and fats, which, if consumed in high quantities, might contribute to the development of obesity and cardiovascular diseases (Leroy et al., 2013). Therefore, new dry fermented sausage formulations have been tested to reduce the sodium chloride (Zanardi et al., 2010) and fat content (García et al., 2002), and improve the fatty acid profile (Muguerza et al., 2004). The purpose of these approaches is to produce healthier products having similar physical and sensory properties as the traditional ones, though this is not always easy (Leroy et al., 2013).

In dry fermented sausages, research has also been carried out on biogenic amines, which are nitrogenous non-volatile low-molecular-weight substances of biological origin, and which have toxicological effects (Vidal-Carou et al., 2014).

Recently, the consumption of red meat and meat products has been associated with the incidence of colorectal cancer (CRC) (Demeyer et al., 2016). Although the mechanism that might induce CRC has not been revealed yet, different hypothesis have been proposed. Among the different hypothesis, scientists have focused on N-nitroso compounds (NOCs), which have potential carcinogenic activities (Demeyer et al., 2016). Processed meat products are an exogenous source of NOCs and might contribute to their endogenous formation due to the presence of nitrite (Demeyer et al., 2016). Indeed, nitrite is widely used in meat processing due to its role in colour formation, antioxidant properties and ability to prevent the growth of pathogenic bacteria (Leroy et al., 2013).

## RESEARCH OBJECTIVES AND THESIS OUTLINE

The overall aim of this PhD research was to study the physico-chemical changes occurring in the protein fraction during ripening of dry fermented sausages. More specifically, proteolysis and protein oxidation have been studied as a function of time and processing conditions. This PhD is an explorative study on the chemistry of protein oxidation and proteolysis in dry fermented sausages, and the results provide ground for further investigations on how these chemical changes may affect the quality of these meat products..

To achieve this objective, several laboratory experiments on dry fermented sausages and meat model systems were conducted, and the following research tasks were carried out:

1. Identifying small peptides derived from hydrolysis of three important sarcoplasmic proteins, i.e. myoglobin, creatine kinase and glyceraldehyde-3-phosphate dehydrogenase, and myosin heavy chain in a mildly acidified type dry fermented sausage.
2. Identifying actin-derived peptides during processing of dry fermented sausages at different pH values.
3. Studying the effect of pH on the activity of some proteolytic enzymes and release of free and peptide-bound α-NH2-N in a meat model system.
4. Studying the effects of pH and proteolysis on protein oxidation in a meat model system.
5. Studying the effect of protein oxidation on proteolysis in a meat model

system.

6. Studying the effects of the curing agents, sodium nitrite (SN) and sodium ascorbate (SA), on protein oxidation in dry fermented sausages.

The specific hypotheses that were formulated and their relation to the experimental chapters are the following:

| | Hypothesis | Chapter |
|---|---|---|
| 1 | Several unique peptides are released from proteolysis of myoglobin | 2 |
| 2 | Several unique peptides are released from proteolysis of creatine kinase | 2 |
| 3 | Several unique peptides are released from proteolysis of glyceraldehyde-3-phosphate dehydrogenase | 2 |
| 4 | Several unique peptides are released from proteolysis of myosin heavy chain | 2 |
| 5 | Several unique peptides are released from proteolysis of actin | 3 |
| 6 | More unique actin-derived peptides are released in strongly acidified sausages compared to mildly acidified sausages | 3 |
| 8 | Cathpesin B cleaves peptides bonds on actin releasing several peptides | 3 |
| 9 | Cathpesin D cleaves peptides bonds on actin releasing several peptides | 3 |
| 10 | Proteolysis, by releasing antioxidant peptides, limits protein oxidation | 4 |
| 11 | Protein oxidation negatively affects proteolysis | 4 |
| 12 | Sodium nitrite is an antioxidant against protein oxidation | 5 |
| 13 | Sodium ascorbate is an antioxidant against protein oxidation | 5 |

This PhD manuscript consists of six chapters.

**Chapter 1** provides an overview of the state of the art on the protein fraction of dry fermented sausages. More specifically, the main proteolytic enzymes, the occurrence of proteolysis and its effect on dry fermented sausages are discussed. In addition, the mechanism of protein oxidation in meat products is presented.

The results of the experimental work are described in four chapters divided in two parts. The first part deals with the identification of small peptides derived from proteolysis of sarcoplasmic and myofibrillar proteins. In **Chapter 2**, dry fermented sausages were manufactured in order to obtain a mildly acidified product, typical of Southern-European type sausages. The release of peptides upon proteolysis of three important sarcoplasmic proteins, i.e. myoglobin, creatine kinase and glyceraldehyde-3-phosphate dehydrogenase, and myosin was studied. Similarly, the release of peptides from the abundant myofibrillar protein actin was described in **Chapter 3**. Peptides were identified throughout the ripening of two types of dry fermented sausages, differing in the course of pH decline and ultimate pH values. The final pH of the sample was typical of Southern-European type (mildly acidified) and Northern-European type (strongly acidified) dry fermented sausages.

The second part deals with protein oxidation. In **Chapter 4**, meat model systems that mimick mildly acidified and strongly acidified dry fermented sausages were installed to study the effects of pH on proteolytic enzymes and protein oxidation. Moreover, the effect of protein oxidation on proteolysis was evaluated. The role of SA and SN on oxidation phenomena, with particular

attention to protein oxidation, is discussed in **Chapter 5**.

Finally, a general discussion and future perspectives are given in **Chapter 6**.

# CHAPTER 1

## STATE OF THE ART ON PROTEIN FRACTION OF DRY FERMENTED SAUSAGES

## 1.1 DRY FERMENTED SAUSAGES

### 1.1.1 Introduction

Dry fermented sausages are processed meat products manufactured and consumed worldwide. In Europe these products have a long tradition since their production goes back to the Roman Empire (Leroy et al., 2013). Indeed, the characteristic long shelf-life of these products allowed meat preservation in ancient times.

Lean meat, pork backfat and sodium chloride are the three basic ingredients. The use of different additives, casings and ripening conditions allowed the development of hundreds of types of dry fermented sausages (Leroy et al., 2013). Nevertheless, these products can be roughly divided in Northern-European and Southern-European types (Hui et al., 2004). These two types are characterised by different processing conditions resulting in different product features (Figure 1.1).

In Northern-European type dry fermented sausages both beef and pork are normally used. The shelf life and safety of these sausages are guaranteed by the high salt content, the use of nitrite, a fast pH drop and smoking (Holck et al., 2014). The ripening period is shorter compared to Southern-European type dry fermented sausages (Demeyer et al., 2000). In Southern-European type dry fermented sausages the raw material is usually pork, which is rarely mixed with beef (Hierro et al., 2014). Shelf life extension is mainly provided by a reduction of the water activity during the long drying phase. Nitrite is usually replaced with nitrate because of the longer ripening time (Sanz et al., 1997a). The amount of

sugar added and processing temperatures are normally lower than in Northern-European type sausages and, as a consequence, the pH drop is less pronounced (5.3-6.2 *vs.* <5.0) and slower (Demeyer et al., 2000). Weight loss is higher in Southern-European type sausages (~ 30 %) than in Northern-European type ones (~ 20 %) (Hui et al., 2004).

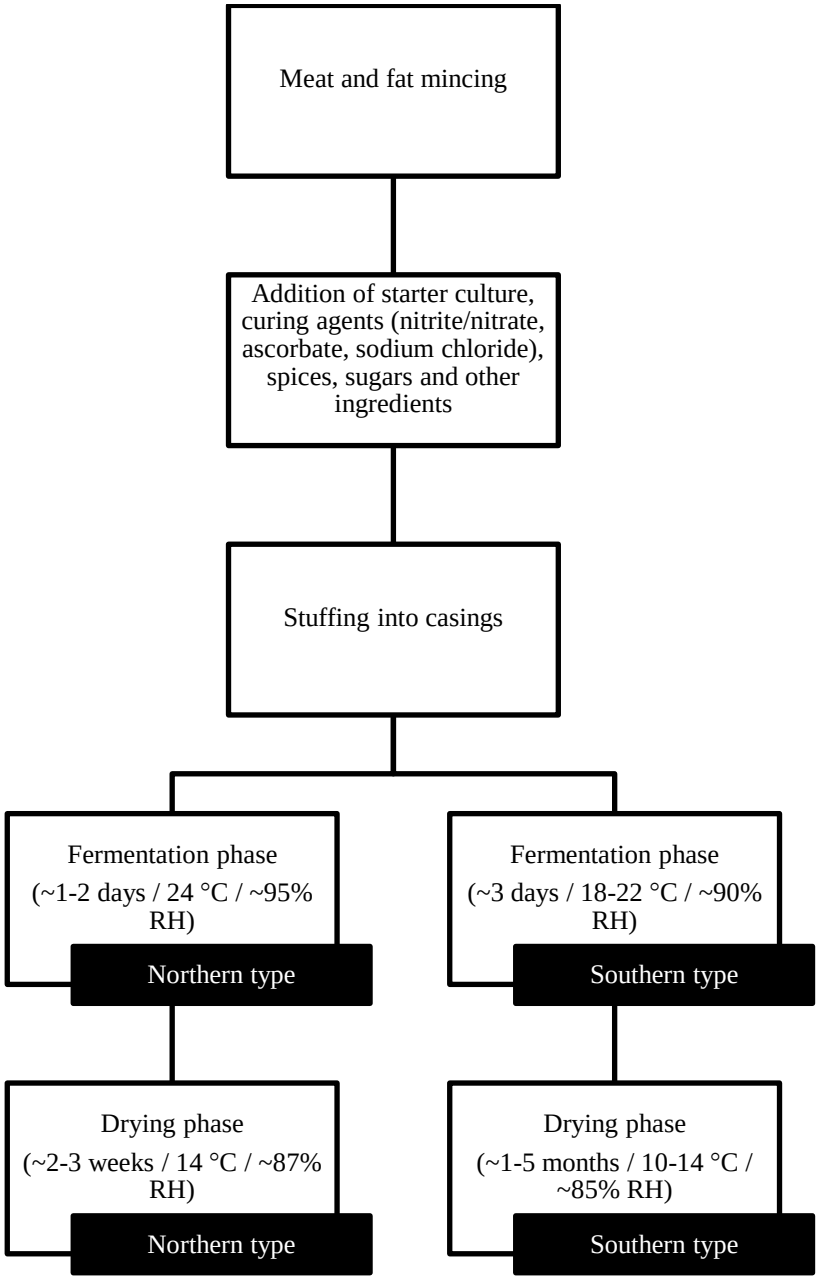



Figure 1.1 Manufacturing processes of Northern-European type and Southern-European type dry fermented sausages (Hierro et al., 2014; Holck et al., 2014).

### 1.1.2 Raw materials and additives

Lean meat is the major ingredient of dry fermented sausages comprising 60-80 % of the total mass (Ruiz and Pérez-Palacios, 2014). It is normally obtained from pork, although beef, ovine meat and turkey are used too. Pork backfat makes up 20-40 %, whereas sodium chloride is added between 2 % and 5 % (Ruiz and Pérez-Palacios, 2014). These three ingredients constitute together almost 100 % of the initial wet weight of dry fermented sausages.

Sodium nitrate/nitrite, SA, dextrose and starter cultures are common additives in dry fermented sausages (Roncalés, 2014). The first two are considered, together with sodium chloride, curing agents since they allow the development of the desired bacteria and colour (Roncalés, 2014). These compounds are not used in some local artisanal products; however, they are fundamental in industrial production to ensure safety. In particular, SN prevents the growth of undesired bacteria. Sodium nitrate/nitrite and SA are normally added at 150 mg/kg (the maximum dose allowed in the European Union) and 500 mg/kg, respectively (Honikel, 2008, Roncalés, 2014). The residual nitrite in cured products is between 10 and 20 mg/kg. The combination of ascorbate and nitrite exert an antioxidant lipid effect:

(1) $2NO^{2-}$ + Ascorbic acid (AA) → 2NO + $2OH^{-}$ + dehydroascorbic acid (DHAA)

(2) NO + LOO• → LOONO + LONO2

The reaction between ascorbate and nitrite forms nitric oxide (reaction 1), which is capable of scavanging lipid radicals (reaction 2) (Villaverde et al., 2014a). Nitrite can also yield nitrogen reactive species, which may initiate

nitration of proteins. However, the impact of protein nitration on the quality of meat products is poorly understood (Villaverde et al., 2014a)

Dextrose is added between 0.2 % and 0.7 % and serves as “food” for the added starter cultures. Although selected starter cultures and dextrose are always added in order to ensure standard quality and safety in industrial productions, also in this case, some artisanal productions rely on indigenous bacteria present in the environment (Leroy et al., 2014).

Spices and flavour enhancers, curing accelerators and water binders are other additives that may be present (Roncalés, 2014).

### 1.1.3 Preparation and ripening

The preparation of the dry fermented sausage batch comprises three steps:

- weighing the ingredients and additives
- chopping and mincing lean meat and fats
- mixing salt and the other additives present in the recipe with minced meat and fats

Weighing the ingredients and additives is an important step, which must be done accurately. Obviously, errors in this step compromises the desired quality of the final products.

Lean meat and fats are first coarsely chopped in a bowl chopper. For pork sausages, a mixture of shoulders, pork backfat and belly are normally used. Subsequently, the chopped meat is minced in a meat grinder. In the meat grinder, meat plates with holes ranging from 4 mm to 8 mm are normally used.

This allows to obtain a meat batch where fat particles are clearly visible and distinguishable from the lean meat matrix. The size of the plate holes depends on the desired size of the fat particles. In case the meat grinder is not available, the meat batch can be prepared in the bowl chopper. In this case, lean meat and fats are chopped till the desired dimension of fat particles is reached.

The last step of the batch preparation is the mixing of the minced meat and fats with salt and the other additives. When frozen lean meat is used, 20-30 % of the total lean meat should be defrosted to 0 °C before mincing to help the solubilization of salt.

Subsequently, the meat batch is stuffed into artificial or natural casings (Hierro et al., 2014). The latter provides the best flavour and appearance, whereas artificial casings are more resistant during processing and less expensive (Wu et al., 2014).

The fresh sausages are then ripened for a variable time that depends on the desired final water content. In general, sausages with higher diameter require longer ripening periods (Grau et al., 2014).

The first phase of ripening is called the fermentation phase, lasts between 2 and 4 days and is characterized by high temperature (20 – 24 °C) and high relative humidity (> 90 %) (Demeyer et al., 2014). During this phase, lactic acid bacteria convert sugars into lactic acid creating a more acidic environment that prevents the growth of undesired bacteria. The pH decreases during fermentation from about 5.7 to a value that depends on the amount of dextrose added. In Southern-European type sausages the amount of dextrose added is about 0.25 % and pH varies between 5.0 and 5.2 at the end of

fermentation. In the Northern-European types dextrose is added at about 0.7 % and pH drops to 4.7-4.8 (Demeyer et al., 2014).

Temperature and relative humidity are lowered at the end of fermentation and during the second phase, called the drying phase. This phase lasts until the desired weight loss is reached and varies between 21 days (Northern-European types) and 1-5 months (Southern-European types). During drying, pH slightly increases and remains below 5 in the Northern-European type sausages, whereas it may considerably increase and reach values above 6.0 in the Southern-European types (Demeyer et al., 2014). This high pH may favour the growth of undesired bacteria which is avoided by a longer drying period that reduces water activity and ensures safe products.

Smoking is sometimes applied and is mainly used in the Northern-European type sausages. This process gives both a drying effect and a desirable taste preventing rancidity and spoilage (Owen et al., 2001). Wood types commonly used for smoking are oak, hickory, cherry, etc. These are all hardwoods. Softwoods are not utilized because of their high content of resin acids which give an unpleasant flavour to the products (Randall et al., 1970). Pyrolysis of lignin produces phenolic compounds which give a characteristic flavour to the food (Owen et al., 2001).

### 1.1.4 Physico-chemical changes

During fermentation and drying processes, numerous physico-chemical changes occur leading to the characteristics of the final product. Sugars, proteins and lipids undergo enzymatic hydrolysis due to the action of meat

endogenous enzymes and bacteria which form new compounds of lower molecular weight affecting flavour and texture.

The initial microbial load of dry fermeted sausages is about $10^5$ CFU/g and comprises bacteria of different species, such as Lactobacilli, micrococci, enterbacteria, *Pseudomonas* spp., *Achromobacter* spp., *Flavobacterium* spp., *Bacillus* spp. (Ordóñez et al., 1999). As aforementioned, fermentation in many artisanal products is carried out by endogenous lactic acid bacteria (LAB), whereas in industrial production starter cultures are used. The most common LAB naturally found or added in dry fermented sausages are *Lactobacillus sakei*, *Lactobacillus curvatus* and *Lactobacillus plantarum*, *Pediococcus pentosaceus*, and *Pediococcus acidilacti* (Ordóñez et al., 1999). The lactobacilli are normally used in Europe to ensure a slow acidification, whereas pediococci are often used in USA where a rapid acidification is needed to cope with the high temperature applied (30-45°C) (Ordóñez et al., 1999). When LAB are added as starter culture the initial load wanted is about $10^6$ CFU/g, which increases to $10^8$ CFU/g during the fermentation phase. The LAB in dry fermented sausages are normally homofermentative, i.e. produces lactic acid from sugars (Ordóñez et al., 1999). This allows acidification of the product from the normal pH of meat (about 5.8) to 5.3 (typical for Southern-European type dry fermented sausages) or below (normal in Northern-European type dry fermented sausages). The pH drop favours the selection of the wanted microflora and the development of the desired texture (water retention is reduced and thus the drying process is favoured) (Ordóñez et al., 1999). The *Lactobacillus sakei* CTC 494 strain, used in the dry fermented sausages samples of this thesis, is a facultative

heterofermentative species. However, it mainly acts as a homofermentative species under the conditions present in dry fermented sausages. Indeed, this species was reported to be a potential starter culter for dry fermented sausages and was not found to produce undesirable byproducts such as carbon dioxide or acetic acid in relevant amounts (Leroy et al., 1999, 2001). Staphylococci are also used as starter culture in combination with LAB and generate volatile compounds such as amino acid catabolites, pyruvate metabolites, and methylketones influencing the aroma of dry fermented sausages (Stahnke et al. 2002).

Endogenous and microbial lipases hydrolyse triglycerides producing free fatty acids, monoglycerides and diglycerides (Ordóñez et al., 1999). Similarly, endogenous and microbial proteolytic enzymes hydrolyse the peptide bond of proteins releasing amino acids and small peptides (Flores and Olivares, 2014). Free fatty acids undergo further degradation generating hydroperoxides which are precursors of volatile compounds, such as alkanes, aldehydes, alcohols, esters, and carboxylic acids (Flores and Olivares, 2014). Some of these volatile compounds have low flavour detection thresholds and, hence, greatly affect odour. A high degree of lipid oxidation generates an excessive amount of compounds that are responsible of off-flavours. Although a certain degree of rancid taste is characteristic for dry fermented sausages, strategies to limit excessive lipid oxidation have been frequently studied.

The oxidative deamination and decarboxylation of free amino acids in the presence of a dicarbonyl compound generates aldehydes through the Strecker degradation reaction (Flores and Olivares, 2014). Volatile compounds

derived by this reaction, such as 2-methyl propanal, 2-methyl butanal, and 3-methyl butanal, are also characteristic of fermented products (Ordoñez, et al., 1999).

Microbes also influence lipid oxidation and free fatty acids degradation for their metabolism producing volatile and non-volatile compounds that influence the final flavour of dry fermented sausages (Leroy et al., 2013).

## 1.2 PROTEOLYSIS

### 1.2.1 Introduction

During ripening of dry cured meat products, meat endogenous proteolytic enzymes are still active and degrade proteins into small peptides and free amino acids. Yet, the addition of starter cultures with proteolytic activity contributes to the breakdown of the meat proteins.

In meat products, proteolysis starts with the hydrolysis of the major sarcoplasmic and myofibrillar proteins. This first step, which is carried out by endoproteases, generates smaller peptides. These peptides are further hydrolysed by exoproteases, releasing free amino acids, dipeptides and tripeptides.

Proteolysis takes place throughout the whole ripening period and influences the final quality of the product in terms of flavour and texture. Indeed, the low molecular weight compounds generated by proteolysis, such as free amino acids and dipeptides, directly affect flavour and serve as precursors of flavour compounds (Temussi, 2011; Ravyts et al., 2012).

### 1.2.2 Proteolytic enzymes

Several proteolytic enzymes are present in muscle tissue; some are located in the lysosomes, whereas others are in the cytosol. These enzymes are responsible for protein breakdown that occurs in meat products. The most important proteolytic enzymes in meat are summarised in Table 1.

Table 1. Muscle proteolytic enzymes (Toldrá and Reig, 2014).

| Enzymes | | Main Substrate | Main product |
|---|---|---|---|
| Endoproteases | Cathepsins | Myofibrillar proteins | Protein fragments |
| | Calpains | Myofibrillar proteins | Protein fragments |
| | Proteasome | Myofibrillar proteins | Protein fragments |
| | Caspase | Myofibrillar proteins | Protein fragments |
| Exoproteases | Tripeptidylpeptidases | Polypeptides | Tripeptides |
| | Dipeptidylpeptidases | Polypeptides | Dipeptides |
| | Aminopeptidases | Peptides (N-terminus) | Free amino acids |
| | Carboxypeptidases | Peptides (C-terminus) | Free amino acids |

#### 1.2.2.1 Endoproteases

Cathepsins and calpains are considered the most important endoproteases in meat products in terms of post-mortem activity (Toldrá and Reig, 2014).

Cathepsins are a family of proteolytic enzymes located in the lysosomes. The most studied cathepsins in meat science are cathepsins B, L

and D (Toldrá and Reig, 2014).

Cathepsins B and L are cysteine proteases. They are composed of two domains that form the active site cleft containing the two reactive site residues, a cysteine and a histidine. These enzymes are optimally active in a reducing and acidic environment (Toldrá and Reig, 2014). Cathepsin L and B have an optimum pH around 5.0 and between 5.5 and 6.5, respectively. Cathepsin D is an aspartyl protease synthetized as a pro-enzyme which undergoes proteolysis during translocation and within the lysosome to yield two active enzymes (15 kDa N-terminal fragment and a 31 kDa C-terminal) (Hughes et al., 2000). Cathepsin D has an optimum pH between 3.0 and 4.5 and does not require a reducing environment (Bechet et al., 2005).

Myofibrillar proteins are potential substrates for cathepsins. In particular, cathepsin D, which mainly cleaves between hydrophobic residues, degrades titin, myosin, actin, tropomyosin, troponins T and I, and myosin light chains (Zeece and Katoh, 1989). Cathepsin B degrades myosin, actin, troponin T, troponin I and tropomyosin (Schwartz and Bird, 1977) whereas cathepsin L degrades most myofibrillar proteins with the exception of troponin C and tropomyosin (Bechet et al., 2005). Nevertheless, the role of cathepsins on meat protein degradation has been debated since these enzymes must be released from the lysosomes to exert their proteolytic activity. To this regard, Ertbjerg et al. (1999a, 1999b) reported that a low pH favours the release of cathepsins from lysosomes; therefore the pH drop caused by fermentation might facilitate the release of these enzymes in dry fermented sausages. The importance of cathepsins in ripened meat products is also driven by the long stability of these

enzymes (Toldrà et al., 1993). Cathepsin D was found to be still active after 5-10 months of processing in dry-cured ham. Similarly, cathepsins B and L showed a slight activity even after 15 month of process (Toldrà et al., 1993). Sodium chloride reduces the activity of the proteolytic enzymes and in particular cathepsin D is highly affected (Toldrà et al., 1993). On the other end, the curing agents, SN and ascorbate do not significantly influence the activity of these enzymes. The reduction in water activity during the drying phase has an inhibitory effect on the activity of cathepsins (Toldà et al., 1992).

Calpains are calcium-dependent, non-lysosomal proteases, ubiquitous in mammalian cells. The two well-characterized calpain isoforms differ in the amount of calcium required for the activity (m-calpain requiring calcium concentrations in the millimolar range and μ-calpain in the micromolar range). μ-Calpain and m-calpain are heterodimers with a common subunit of 28 kDa. The large subunit (80 kDa) is composed of 4 subdomains; the catalytic site is located in the second subdomain which contains a cysteine and a histidine residue in a position common to all cysteine proteases (Huff-Lonergan et al., 2010). Calpains are the main enzymes responsible for the post mortem tenderization of meat through proteolysis of myofibrillar proteins (Koohmaraie, 1992). In particular, μ-calpain is able to degrade troponin-T, filamin, desmin, nebulin and titin (Huff-Lonergan et al., 2010). Nevertheless, it is generally believed that calpains do not play a relevant role in the proteolysis of cured meat products since these enzymes are highly unstable (Ordóñez et al., 1999) and are inactivated by sodium chloride (Sárraga et al., 1989).

Proteasome is a protease complex whose function is found in diverse

cellular pathways by degradation of proteins. Although proteasome retains some activity after 7 days post-mortem at pH 5.5 (Lamare et al., 2002), its role in meat tenderization is still not clear (Geesink et al., 2008, Kemp et al., 2010).

The caspase system is a family of cysteine proteases involved in apoptosis. Although only few studies investigated the activity of caspases in meat, it seems that the muscle early post-mortem conditions induce the process of cell death activating the caspase system. The role of these enzymes on myofibrillar proteolysis has not been clarified yet, although it has been suggested that the degradation of calpastatin, a calpain inhibitor, carried out by caspases 1, 3 and 7 increases the activity of calpains (Geesink et al., 2008, Kemp et al., 2010).

#### 1.2.2.2 Exoproteases

Exoproteases release amino acids and small peptides composed of few residues, such as dipeptides and tripeptides, from the N- or C-terminus of peptides and proteins. This process is of great importance in ripened products because low molecular compounds, such as free amino acids and small peptides, affect flavour.

Tripeptidylpeptidases (TPP) release tripeptides from the amino termini of peptides and proteins. TPP I, located in the lysosomes, has an acidic pH optimum; TPP II, located in the cytosol, has a neutral pH optimum (Toldrá and Reig, 2014).

There are four dipeptidylpeptidases (DPP) studied in meat science. DPP I and DDP II are located in the lysosomes (Toldrá and Reig, 2014). The former is

a cysteine protease whereas the latter is a serin protease and they both have an optimum pH at 5.5 (Sentandreu and Toldrà, 2000, 2001a). DPP III is a metallopeptidase located in the cytosol with optimum activity at basic pH (Sentandreu and Toldrà , 1998). Since this enzyme has no activity at acidic pH, it is unlikely to be active in dry fermented sausages. DPP IV is a serin peptidase located in cell membranes and has optimum pH at 7.0-8.0. Unlike DPP III, DPP IV retains some activity at acidic pH (Sentandreu and Toldrà, 2001b). DPP I and DPP III release dipeptides from substrates with alanine or arginine in N-penultimate position, whereas the serine proteases, i.e. DPP II and DPP IV, mainly release dipeptides with proline in N-penultimate position. In dry cured ham, these peptides show long term stability, indeed DPP I, DPP III and DPP IV remain active throughout the whole process whereas DPP II during the first 240 days (Sentandreu and Toldrà, 2001c). Sodium chloride reduces the activity of DDP II, III, and IV.

Aminopeptidases release amino acids from the N-terminus and, in meat science, five aminopeptidases located in the cytosol have been purified and characterized (Toldrá and Reig, 2014). Aminopeptidase B or arginyl aminopeptidase has optimum activity at pH 6.5 and is inactivated at pH 5.0. This enzyme has been reported to release arginine, phenylalanine, proline and alanine (Flores et al., 1993). Alanyl aminopeptidase has maximum activity at pH 6.5 and hydrolyses aromatic, aliphatic, and basic amino acids showing broad substrate specificity (Flores et al., 1996). Methionyl aminopeptidase releases methionine, lysine, alanine and leucine and has optimum activity at pH 7.5 (Flores et al., 2000). The activity of this enzyme is not inhibited by sodium

chloride; therefore it likely plays a relevant role in amino acid release in cured meat products. Leucyl aminopeptidase and pyroglutamyl aminopeptidase have optimum pH at 9.5 and 8.5, respectively (Mantle et al., 1991). These enzymes likely play a minor role, if any, in dry fermented sausages due to the high pH required for their activity.

Carboxypeptidases release amino acids from the C-terminus, however the knowledge about these enzymes is still limited. Carboxypeptidases A and B are located in the lysosomes and have optimal activity at acidic pH. Carboxypeptidase A releases hydrophobic amino acids whereas carboxypeptidase B has a wider activity (Toldrá, 2006).

### 1.2.3 Proteolysis in dry fermented sausages

Considering the great importance of protein breakdown in flavour development, proteolysis has been deeply studied in meat products and diverse methods have been used: determination of protein and non-protein nitrogen (Defernando at al., 1991, Beriain et al., 2000) and free α-NH2-N (Verplaetse et al., 1992), electrophoresis techniques (Hughes et al., 2002) and measurement of the release of amino acids during ripening (Defernando at al., 1991).

Proteolysis starts already in the first days of fermentation and lasts throughout the whole ripening. Evidence for increases in non-protein nitrogen and free amino acid contents during the fermentation period have been reported (Beriain et al., 2000). The fermentation conditions characterized by low pH (4.7-5.2) and high temperatures (18-23 °C) are stimulatory for proteolytic enzymes and in particular for cathepsins (Verplaetse et al., 1989). The proteolysis of

sarcoplasmic and myofibrillar proteins has been studied by electrophoresis techniques, especially SDS-PAGE. The two most intense bands in the myofibrillar patterns correspond to the molecular masses of myosin heavy chain (180 kDa) and actin (40 kDa) (Spaziani et al., 2009). Myosin and actin band intensities significantly decrease during ripening and new bands with lower molecular weight appear (Hughes et al., 2002,). Similar findings were reported for sarcoplasmic proteins (Hughes et al., 2002, Spaziani et al., 2009). As mentioned above, sarcoplasmic and myofibrillar proteins are degraded by meat endogenous enzymes both during the fermentation and drying phases. On the other hand, the contribution of starter cultures is more limited and is detectable by SDS-PAGE only in the drying phase (Hughes et al., 2002, Spaziani et al., 2009, Casaburi et al., 2007). The extent of proteolysis depends on several physico-chemical parameters, such as pH, salt content and ripening conditions (Demeyer et al., 2000). In particular, pH values lower than 5.0 increase the activity of cathepsins and enhance proteolysis (Verplaetse et al., 1989, Demeyer et al., 2000).

The development of new proteomic tools, such as mass spectrometry-based techniques, allowed the identification of some small peptides generated during ripening. In the sarcoplasmic fraction, myoglobin, creatine kinase, glyceraldehyde-3-phosphate dehydrogenase and beta-enolase are the most hydrolysed and detected proteins (López et al., 2015a, Mora et al., 2015). In the myofibrillar fraction, small peptides derived from the proteolysis of troponin T, myosin regulatory light chain-2, alpha actin skeletal muscle and myosin heavy chain have been identified (López et al., 2015a, 2015b; Mora et al., 2015).

#### 1.2.3.1 The role of meat endogenous and bacterial enzymes

It is generally believed that meat endogenous endoproteases initially proteolyse intact proteins producing smaller peptides. Subsequently, the generated peptides are further degraded by both meat endogenous exoproteases and bacterial proteolytic enzymes releasing free amino acids and dipeptides (Molly et al., 1997).

The use of antibiotics and proteolytic enzyme inhibitors has contributed to reveal the relative importance of meat endogenous and microbial enzymes (Molly et al., 1997). Cathepsin D-like enzymes seem the most important proteases responsible for proteolysis of myofibrillar proteins, whereas cathepsin B, H and L play a relevant role only in the degradation of actin (Molly et al., 1997). These findings were confirmed by Hierro et al. (1999) reporting that myofibrillar proteins were proteolysed by meat endogenous enzymes and the added starter cultures played, if any, a marginal role. Some starter cultures, like the genus micrococcus, possess the ability to proteolyse myofibrillar proteins. Molly et al. (1997) reported that microbial enzymes degrade myosin and small peptides and release free amino acids. However, the conditions normally found in dry fermented sausages, i.e. acidic pH, relative low temperature and high salt concentrations, drastically reduce the activity of microbial proteolytic enzymes (Kenneally et al., 1999).

## 1.3 PROTEIN OXIDATION

### 1.3.1 Introduction

Oxidative stress is induced in biological systems when oxidants surpass the antioxidant defence system. This occurs during post-mortem conditions, in which the antioxidant defence system is gradually depleted. In this scenario, the major meat components lipids and proteins undergo oxidative modifications. Lipid oxidation has been deeply studied because it, together with microbial spoilage, clearly provokes meat deterioration (Zanardi et al., 2004). Indeed, rancid taste is the evident sensory result of lipid oxidation. On the other hand, protein oxidation has been overlooked because there is less evidence that it affects flavour, and its impact on technological properties of proteins is more difficult to assess (Lund et al., 2011; Estevez, 2011; Soladoye et al., 2015).

Reactive oxygen species, generated during meat post-mortem conditions, can remove hydrogen atoms from lipids and proteins. In the latter case, protein radicals are formed and, depending on where the hydrogen atom is removed, proteins undergo different modifications that include protein fragmentation, protein crosslinking and amino acid side chain modifications (Lund et al., 2011) (Figure 1.2).

This large number of oxidative modification products makes the assessment of protein oxidation difficult. Protein carbonyls are often used as a marker of protein oxidation in meat: either by quantification of total carbonyl groups through derivatization with 2,4-dinitrophenylhydrazine (DNPH) or determination of specific protein carbonyls, such as α-amino adipic

semialdehyde (AAS) and γ-glutamic semialdehyde (GGS), derived from oxidation of lysine, arginine and proline (Estevez, 2011). Other amino acids, like cysteine and tryptophan are also susceptible toward oxidation. Therefore, loss of sulfhydryl groups and tryptophan depletion has been employed to assess protein oxidation in meat products (Estevez, 2011).

The impact of protein oxidation on the quality of meat products has been only partly revealed so far (Lund et al., 2011). Oxidation modifies the structure of meat proteins and therefore it likely affects functional and technological properties. For these reasons, further research is required to reveal the actual impact of protein oxidation on the final quality of meat products.

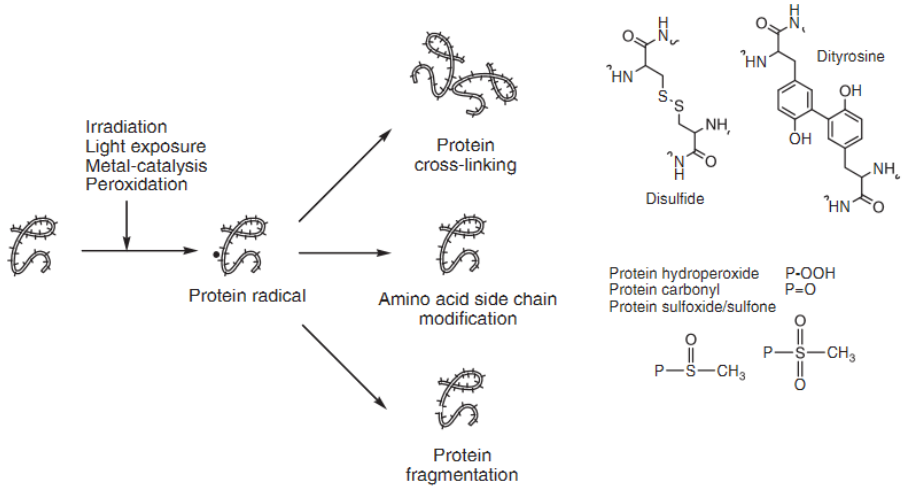



Figure 1.2. Most common chemical changes due to oxidation of proteins (Lund et al., 2011).

### 1.3.2 Mechanism of protein oxidation

Protein oxidation is a radical mediated chain reaction similar to lipid oxidation. However, in protein oxidation, a greater number of oxidation products can be formed depending on the target of oxidation, the oxidizing conditions and intensity (Davies, 2005).

Reactive oxygen species (ROS) are considered the initiators of protein oxidation. ROS abstract a hydrogen atom forming a carbon centred protein radical. In the presence of oxygen the carbon centred protein radical is converted into a peroxyl radical. The peroxyl radical is then converted into an alkyl peroxide by abstraction of a hydrogen atom from another molecule. Finally, further reactions with ROS or reduced forms of transition metals form alkoxyl radicals and its hydroxyl derivative (Lund et al., 2011; Estevez, 2011).

The hydrogen atom can be abstracted from either the protein backbone or the amino acid side chain. The former case leads to protein fragmentation. On the other hand, oxidation of amino acids introduces modifications on the side chain depending on the stricken amino acid. The most susceptible amino acids toward oxidation are cysteine, tyrosine, phenylalanine, tryptophan, histidine, proline, arginine, lysine and methionine (Lund et al., 2011). In particular, sulphur containing amino acids like methionine and cysteine are highly susceptible to oxidation, also at mild conditions, and their oxidation leads to formation of sulfone, sulfoxide and disulphide derivatives (Estevez, 2011). Tryptophan oxidation leads to formation of kynurenine or N-formylkynurenine. Histidine is oxidised into oxohistidine and imidazolone derivatives. Leucine and valine are oxidised into hydroxy derivatives (Stadtman & Levine, 2000). Finally, oxidation of

arginine, proline, lysine and threonine forms carbonyls (Estevez, 2011).

### 1.3.3 Markers of protein oxidation in meat products

#### 1.3.3.1 Formation of protein carbonyls

In meat science, protein oxidation is often assessed by determination of carbonyl groups. Carbonylation occurs through four different pathways: direct oxidation of amino acid side chains, non-enzymatic glycation in the presence of reducing sugars, oxidative cleavage of the peptide backbone and covalent binding to non-protein carbonyl compounds (Estevez, 2011). Among these pathways, the direct oxidation of amino acids is considered the most important. Threonine is oxidised into α-amino-3-keto butyric acid, lysine is oxidised into AAS, and arginine and proline into GGS (Estevez, 2011). This pathway is mediated by metal catalysed oxidation systems (Stadtman and Levine, 2003), in which transition metals reduce hydrogen peroxyde to form a hydroxyl radical through the Fenton reaction (Figure 1.3-1).

The second oxidation pathway is the non-enzymatic glycation (Figure 1.3-2): the Maillard reaction forms Schiff base and Amadori adducts to protein, which, in the presence of transition metal ions, undergo oxidation producing dicarbonyl compounds (Estevez, 2011).

The generation of alkoxyl radicals in the peptide backbone may provoke the peptide backbone cleavage by α-amidation (Figure 1.3-3). The peptide fragment produced from the N-terminal has an amide group at the C-terminal end, whereas the N-terminal amino acid residue of the fragment derived from

the C-terminal portion of the protein exists as an N-α-ketoacyl derivative (Berlett and Stadtman, 1997).

The last carbonylation pathway reported is due to the formation of protein adducts with lipid oxidation products, such as 4-hydroxy-2-nonenal (HNE) or malondialdehyde (MDA) (Figure 1.3-4) (Estevez, 2011).

In in vitro models, protein carbonylation has been induced through diverse ROS-generating systems. Transition metals, like iron and copper, in the presence of hydrogen peroxide form highly reactive hydroxyl radicals that can trigger protein carbonylation (Estevez, 2011). Ascorbic acid, which is often added as antioxidant in meat products, can feed the former reaction by reducing the oxidised form of the metal ion, favouring the generation of further oxygen radicals (Estevez, 2011). Similarly to transition metals, myoglobin can also react with hydrogen peroxide generating hypervalent myoglobin species capable to induce protein oxidation (Lund et al., 2011). Finally, oxidizing lipids, like peroxyl radical, can induce protein carbonylation (Estevez, 2011).

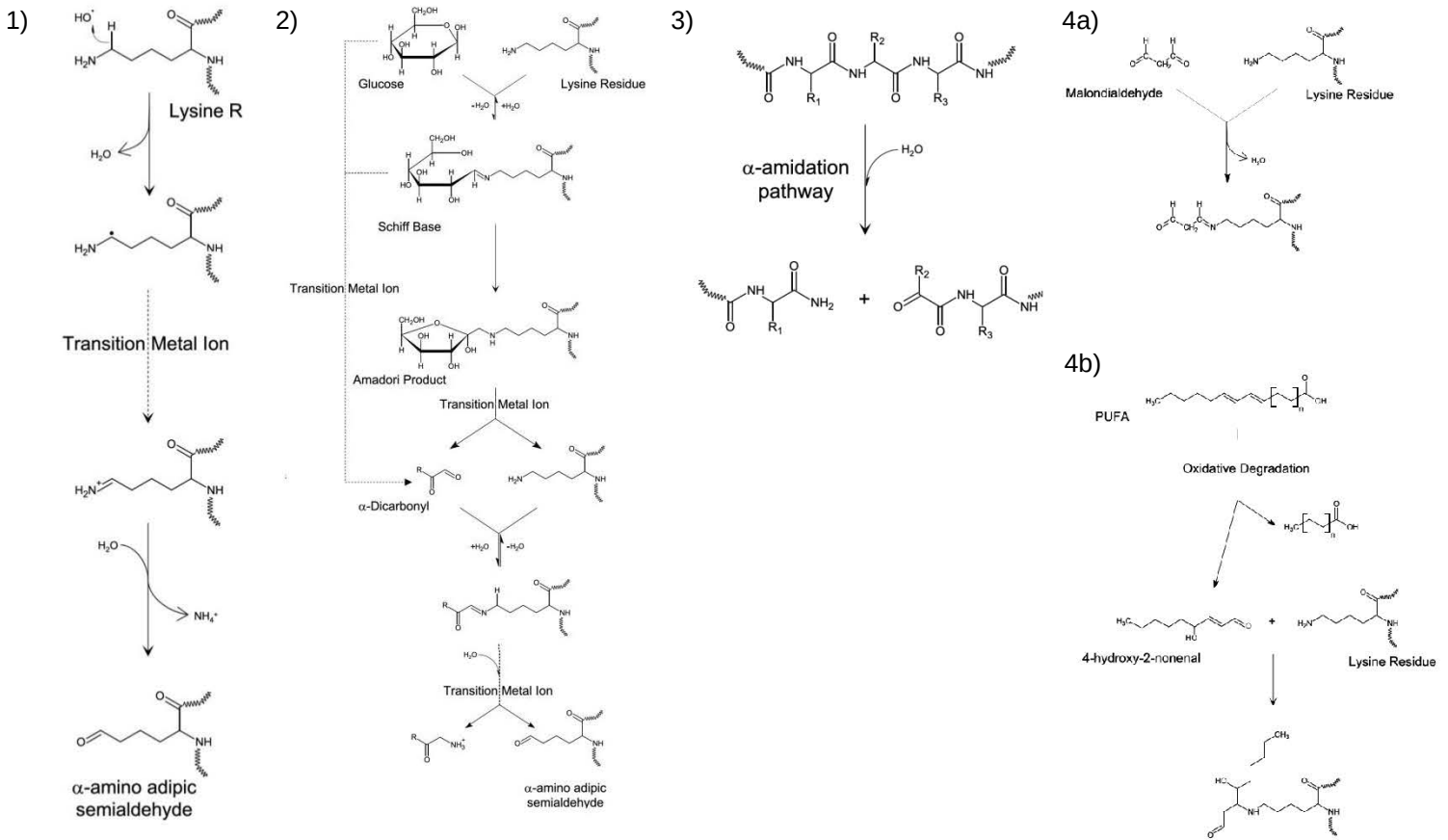



Fig 1.3. Protein carbonylations pathways: 1) Metal catalyzed oxidation of basic amino acid side chains; 2) Non-enzymatic glycation; 3) Peptide backbone cleavage by α-amidation; 4) Binding to non-protein carbonyl compounds such as 4-hydroxy-2-nonenal (HNE) (a) or malondialdehyde (MDA) (b) (Estevez, 2011)

For many years, the quantification of protein carbonyls has been done by derivatization with DNPH (Levine et al., 1994) in meat (Lund et al., 2004) and meat products, like dry fermented sausages (Berardo et la., 2015), liver pâtés (Vossen et al., 2012), meat patties (Estevez et al., 2005) and frankfurters (Vuorela et al., 2005). However, this method has been often criticized because of its low specificity and high variability among different laboratories; moreover it is impossible to understand how protein carbonyls were formed among the four aforementioned pathways (Estevez, 2011). Nevertheless, this method is often used thanks to its simplicity and low cost. Indeed, Soglia et al. (2016) recently developed an improved DNPH-based method to assess protein carbonylation by introducing an additional step to increase protein solubility and unfolding before labelling with DNPH.

Although the DNPH method provides an overview of the total carbonyls present, the need to understand the chemistry of protein carbonylation has required the identification and quantification of specific carbonyls, such as AAS and GGS, which are estimated to comprise 70 % of the total carbonyls in moderately oxidized meat products (Utrera et al., 2011). Estevez et al. (2009) identified and quantified AAS and GGS in food protein by derivatization with *p*-aminobenzoic acid (ABA) and detection through HPLC. This method provided new insights on the mechanism of protein carbonylation in isolated myofibrillar proteins (Utrera and Estévez, 2012) and meat products (Lorido et al., 2016; Lobo et al., 2016).

#### 1.3.3.2 Loss of thiol groups

Sulfhydryl groups of cysteine residues are highly susceptible to oxidation leading to formation of sulfenic acid, sulfinic acid and disulphide cross-links. Both metal and myoglobin oxidation systems are capable to induce oxidation of sulfhydryl groups in meat proteins (Martinaud et al., 1997). The determination of loss of thiol groups is done by derivatization with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). It has been reported that loss of thiol groups may occur during storage (Lund et al., 2008) and ripening of meat products (Berardo et al., 2015). Recently, Rysman et al. (2014) used 4,4′-dithiodipyridine (4-DPS) instead of DTNB for its higher sensitivity as thiol detection agent.

Meat proteins and in particular myosin have been found to form aggregates under oxidative conditions through the formation of disulphide cross-linking upon oxidation of sulfhydryl groups (Liu and Xiong, 2000). These aggregates have been observed in meat (Lund et al., 2007) and meat products (Jongberg et al., 2011) by gel electrophoresis techniques.

### 1.3.4 Impact on meat quality

The ultimate goal in the understanding of protein oxidation chemistry in meat science is to reveal its impact on product quality from technological, nutritional and sensory points of view.

In fresh meat, protein oxidation has been often associated with impaired tenderness and juiciness. Indeed, the formation of cross-links between myofibrillar proteins via disulphide bonds, Schiff bases and dityrosine increases meat toughness and reduces water holding capacity. Moreover, post-mortem

proteolysis, which plays a relevant role in meat tenderization, is affected by protein oxidation. Specifically, oxidation of proteolytic enzymes reduces their activity compromising proteolysis and tenderness (Chen et al., 2015). On the other hand, oxidation of proteins alters their susceptibility to proteolysis. It is generally believed that under mild oxidative conditions myofibrillar protein susceptibility to proteolysis is increased (Xue et al., 2012), conversely protein susceptibility is reduced under severe oxidative conditions due to formation of protein cross-links (Pacifici et al., 1993).

In some meat products where meat is minced, like in dry fermented sausages, the effect of protein oxidation on texture is associated with some technological aspects of proteins such as gelation and emulsifying properties. Also in this case, formation of cross-links between proteins deserves attention since it might favour the formation of the desired sliceable texture (Zhou et al., 2014, Jongberg et al., 2015).

From the nutritional point of view, loss of essential amino acids and altered digestibility are possible results of protein oxidation. Some essential amino acids, like lysine, histidine, arginine, threonine, phenylalanine and tryptophan, are susceptible toward oxidation and their loss can reduce the nutritional value of the product (Soladoye et al., 2015). Protein digestibility is reduced in case of intense oxidation due to the formation of protein aggregates. On the other hand, mild oxidation can increase protein digestibility (Soladoye et al., 2015).

Finally, although clear effects of protein oxidation on flavour have not been reported yet, protein carbonyls and Schiff bases might have an impact

(Lund et al., 2011). Villaverde et al. (2014b) speculated that intense proteolysis and protein carbonylation favour the formation of Strecker aldehydes which can influence flavour.

### 1.3.5 Protein oxidation in dry fermented sausages

Oxidative stability and strategies to improve it have been deeply investigated in dry fermented sausages in relation to lipid oxidation. Indeed, lipids undergo oxidative modifications that result in formation of off-flavours impairing the general quality of these products (Zanardi et al., 2004). On the other end, protein oxidation has been mainly related to reduced water-holding capacity and tenderness (Lund et al., 2011), therefore its impact on the quality of dry fermented sausages has been overlooked and only few studies have investigated the occurrence of protein oxidation in these products. Nevertheless, there are several research areas involving protein oxidation that might reveal new key information on the chemistry of these complex products. For instance, the understanding of how protein oxidation interferes in the mechanism of gelation, how lipid and protein oxidation interacts, how proteolysis is affected by protein oxidation and how the formation of oxidation products influences flavour might bring new manufacturing strategies in order to improve the final quality.

As mentioned before, dry fermented sausages are composed of approximately two thirds of lean meat in which one third of fat is dispersed. Therefore, the interaction between proteins and lipids in relation to oxidative stability is of great interest. Recent studies reported that a higher fat content has a pro-oxidant effect on thiol groups (Safa et al., 2015), but does not affect

protein carbonylation (Fuentes et al., 2014). The fatty acid profile and the presence of antioxidants in the fats used might also influence the oxidative stability of proteins in these products (Fuentes et al., 2014).

It is well-known that the curing agents SA and SN prevent lipid oxidation, conversely sodium chloride exerts pro-oxidant effects (Ruiz, 2007). On the other hand, studies on protein oxidation have reported contradictory results for these salts. Villaverde et al. (2014a, c) reported pro-oxidative effects of nitrite on tryptophan depletion and protein carbonylation. However, these pro-oxidant effects were prevented by the addition of SA which acted as antioxidant. Sodium chloride added in low amounts (0.55 %) entails lower oxidation of tryptophan but higher protein carbonylation compared to higher quantities (1.1 % and 2.2 %) (Lobo et al., 2016). Safa et al. (2015) reported no effects of salt content on loss of thiol groups when added in the range of 2.0 to 2.8 %.

# CHAPTER 2

## DEGRADATION OF THREE SARCOPLASMIC PROTEINS AND MYOSIN DURING RIPENING OF MILDLY ACIDIFIED DRY FERMENTED SAUSAGES

## 2.1 ABSTRACT

Peptides generated during ripening of a mildly acidified fermented sausages from three important sarcoplasmic proteins, i.e. myoglobin, creatine kinase and glyceraldehyde-3-phosphate dehydrogenase, and myosin heavy chain were identified through $LCMS^E$. These proteins are abundant in muscle cells and hence potential precursors of several peptides. Ten, thirty-nine, nine and twenty-eight peptides were reported from myoglobin, creatine kinase, glyceraldehyde-3-phosphate dehydrogenase and myosin, respectively.

Finally, the potential role of the proteolytic enzymes involved is discussed.

## 2.2 INTRODUCTION

Meat proteins are mainly divided in myofibrillar and sarcoplasmic proteins. Sarcoplasmic proteins are water soluble and make up 30-35 % of the total meat protein fraction (Toldrá and Reig, 2014). Sarcoplasmic proteins comprise hundreds of enzymes, generally of low molecular weight. Some of the these proteins are considered particularly important for meat quality and, such as myoglobin, creatine kinase and glyceraldehyde-3-phosphate dehydrogenase have been studied extensively in meat science for different reasons. Myoglobin is a water soluble globular protein, consists of 154 amino acids and contains a non-protein molecule, the heme ring, which has a chelated iron. In live animals, myoglobin function binds and delivers oxygen to mitochondria (Suman and Joseph, 2013). In meat, this protein is of great importance since it is the main responsible of the characteristic red colour (Suman and Joseph, 2013). Creatine kinase is a protein involved in the energy metabolism and consists of 381 amino acids. In vivo, creatine kinase regenerates adenosine triphosphate catalysing the transphosphorylation reaction between phosphocreatine and adenosine diphosphate (Daroit and Brandelli, 2008). This protein is involved in the muscle to meat conversion since it influences the post-mortem acidification (Daroit and Brandelli, 2008). Glyceraldehyde-3-phosphate dehydrogenase is a cell enzyme involved in glycolysis and consists of 333 amino acids (Sirover, 1999). It is a very abundant protein since it may constitute 10-20% of total cellular proteins (Sirover, 1999).

Myofibrillar proteins comprise ~55 % of the total muscle proteins among

which myosin is the most abundant one. Myosin is a high molecular weight protein which contains two heavy chains composed of 1939 amino acids. This protein, together with actin, is responsible for muscle contraction. This protein undergoes post-mortem degradation and some of myosin heavy chain fragments have been correlated to meat tenderization (Suman, 2012).

In dry fermented sausages, the activity of proteases, of both bacterial and meat origin, hydrolyses proteins generating small peptides and free amino acids. These low molecular weight proteins are important because they directly affect taste and serve as flavour precursors. Proteolysis has been studied for decades through electrophoretic techniques which allowed to understand the hydrolysis of intact proteins. However, little is known about the small peptides generated during ripening of meat products. Only recently, few studies identified some peptides generated from sarcoplasmic and myofibrillar proteins from dry cured ham and dry fermented sausages (López et al., 2015a; Mora et al., 2015).

Therefore, the aim of this study was to identify the small peptides generated by the hydrolysis of three important sarcoplasmic proteins, i.e. myoglobin, creatine kinase and glyceraldehyde-3-phosphate dehydrogenase, and the myofibrillar protein myosin heavy chain. These selected proteins are abundant in meat and were thus chosen for this study after a pre-screening through $LCMS^E$ analysis in which several peptides were identified in our samples.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Dry fermented sausage preparation

Dry fermented sausages were prepared by mixing lean pork (70.5 %), pork backfat (27.0 %), sodium chloride (2.5 %), SN (0.015 %, m/m), SA (0.05 %, m/m), dextrose (0.25 %, m/m) and a starter culture strain (*Lactobacillus sakei* CTC 494) originating from the culture collection of the Research Group of Industrial Microbiology and Food Biotechnology (Vrije Universiteit Brussel, Brussels, Belgium). The initial load of *L. sakei* CTC 494 was $10^6$ (CFU/g). The batter was stuffed into collagen casings of 50 mm diameter (Naturin, Weinheim, Germany) to make dry fermented sausages of about 200 g. Ripening lasted for 28 days in a climate chamber. During the first four days, fermentation was performed at a temperature of 24 °C and a relative humidity of 94 %. For the drying process, the temperature was dropped to 12 °C and relative humidity was set at 94 % for the first 14 days and at 80 % for the last 10 days. Samples were taken at days 0, 4 (end of fermentation), and 28 (end of ripening). The manufacturing processes and sampling were repeated in triplicate, resulting in three independent replicates.

### 2.3.2 pH and weight loss

In each manufacturing process, three randomly selected sausages per treatment were weighed and the pH was recorded after their preparation and during ripening. The pH was measured directly in the sausages [ISO 2917 (1999)] and the pH meter was calibrated in buffers of pH 4.0 and 7.0. Weight

loss was expressed as a percentage of the initial weight and the mean of the three records was calculated.

### 2.3.3 Peptide extraction

The peptide extraction was carried out as described by Mora et al. (2015). Briefly, peptides were extracted in 0.01 N HCl and proteins were precipitated by addition of EtOH. Finally, the peptide extract was dried in a rotary evaporator and the peptides were dissolved in 25 ml 0.01 N HCl.

### 2.3.4 Size-exclusion chromatography

Size-exclusion chromatography was carried out to select peptides between 500 and 4000 Da. Peptides were fractionated using an Akta Purifier (GE Healthcare Life Sciences, Uppsala, Sweden) on a Sephadex G25 Fine column (2.6 × 60 cm) and 0.01 N HCl was used as mobile phase at a flow rate of 1 mL/min. The fraction corresponding to elution volumes from 80 to 220 mL was collected and aliquots of 100 µL were lyophilised.

### 2.3.5 LCMS$^E$ analysis

Lyophilised peptides were suspended in 40 µL of 100 mM $NH_4HCO_2$ (pH 10). Samples were filtered (0.22 µm) and centrifuged. The peptide identification was done according to Devos et al. (2015) by liquid chromatography coupled to mass spectrometry in a data-independent positive mode of acquisition (LCMS$^E$). Briefly, 2 µl sample was injected on a NanoAcquity UPLC® system (Waters Corporation, Milford, MA, USA) for peptide separation. Solvent A1 and B1 were

composed of 20 mM ammonium formate in water and acetonitrile (pH 10), respectively. Solvent A2 and B2 were composed of 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile, respectively. The sample was loaded onto an Xbridge™ BEH130 C18 column (300 µm × 50 mm, 5 µm; Waters) at 50 % solvent B1 at 2 µL/min. Peptides were eluted and trapped on a Symmetry® C18 trapping column (180 µm × 20 mm, 5 µm; Waters Corporation) and finally separated on a HSS T3 C18 analytical column (75 µm × 250 mm, 1.8 µm; Waters Corporation) at 40°C at 250 nL/min by increasing the acetonitrile concentration from 5 to 50 % B2 over 60 min.

The outlet of the column was directly connected to a PicoTip Emitter (uncoated SilicaTip™ 10 ± 1 µm, New Objective, Woburn, MA, US) mounted on a Nanolockspray source of a SYNAPT™ G1 HDMS mass spectrometer (Waters Corporation). Accurate mass data were collected by alternating between low (5 V) and high (ramping from 15 to 35 V) energy scan functions (Geromanos et al., 2009). The selected m/z range was 125–2000 Da. The capillary voltage was set to 3.0 kV, the sampling cone voltage was 26 V, and the extraction cone voltage was 2.65 V. The source temperature was set at 80°C.

The $LCMS^E$ data were processed using the ProteinLynx Global SERVER™ v2.5 (PLGS, Waters Corporation) (Geromanos et al., 2009). The identification of actin peptides was done by using a Uniprot database containing 35416 protein entries from the organism Sus scrofa (downloaded from the Uniprot website, March 2016). The primary digest reagent was set as “none”. The precursor and fragment ion tolerance were determined automatically. The default protein identification criteria used included a detection of minimal three

fragment ions per peptide, and minimal three fragment ions per protein. A false positive rate of 4 % was allowed.

## 2.4 RESULTS AND DISCUSSION

During ripening of dry fermented sausages, the protein fraction undergoes intense degradation influencing the final characteristics of the product. It is generally believed that, in ripened meat products, muscle endogenous proteases start proteolysis breaking down intact proteins into small peptides (Molly et al., 1997). In particular, cathepsins play a relevant role; the pH drop caused by lactic acid bacteria during the fermentation phase favours the activity of these enzymes which have acidic pH optima (Verplaetse et al., 1989, Demeyer et al., 2000). In the present study, dry fermented sausages were ripened for twenty-eight days: four days of mild fermentation that dropped pH from 5.69 (± 0.07) to 5.23 (± 0.02), followed by twenty-four days of drying in which pH increased to 5.98 (± 0.07). Although the pH only decreased by approximately 0.5 pH units during the fermentation phase, this likely enabled proteolytic enzymes such as cathepsins D and B to display activity (Schwartz and Bird, 1977). These enzymes remain active for a long period during processing of ripened products (Toldrà et al., 1993) and it is reasonable to assume that they generated peptides till the end of the process. Only the activity of cathepsin D was probably reduced due to the pH increase during the drying phase (Bechet et al., 2005).

At the end of ripening, peptides derived from the proteolysis of three

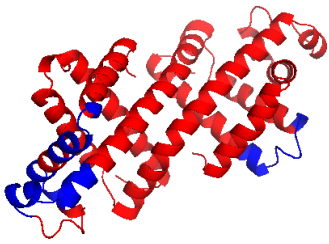
sarcoplasmic proteins, i.e. myoglobin, creatine kinase, glyceraldehyde-3-phosphate dehydrogenase, were identified by LCMS$^E$. A total of ten myoglobin-derived peptides were identified (Table 2.1). These peptides were released from four different regions of the myoglobin sequence (Figure 2.1). Previous studies reported myoglobin degradation in dry fermented sausages, in particular the intensity of the electrophoretic band of this protein was found to decrease during ripening (Hughes et al., 2002). Mora and Toldrá (2012) identified myoglobin-derived peptides in 8 month-old dry cured hams: comparing the results of the present study with the ones of these authors it is noticeable that the region between residues 47-65 is highly proteolysed although the ripening time is shorter in dry fermented sausages. The myoglobin-derived peptides n. 5 and 6 lying in the aforementioned region were also identified in the study of Mora and Toldrá (2012). Regions between residues 16-25 and 117-126 containing peptides n. 1-4 and 10 are also highly proteolysed. Indeed, Hughes et al. (2002) also identified myoglobin-derived peptides in these regions; interestingly the peptides identified between residues 117-126 by these authors were of bovine origin indicating that this region is susceptible to proteolysis in both porcine and bovine myoglobin.

**Table 2.1** Peptides of myoglobin identified by LCMS$^E$ dry fermented sausages

| N. | $P_1$[a] | Peptide sequence | $P_1$'[b] | P. s. s.[c] | z[d] | m/z[e] | Freq.[f] |
|---|---|---|---|---|---|---|---|
| 1 | V | WGKVEADVAGHGQE | V | 14 | 2 | 741.8 | 3 |
| 2 | V | WGKVEADVAGHG | Q | 14 | 2 | 613.3 | 1 |
| 3 | W | GKVEADVAGHGQE | V | 15 | 2 | 648.8 | 3 |
| 4 | K | VEADVAGHGQE | V | 17 | 2 | 556.2 | 1 |
| 5 | L | FKGHPETL | E | 33 | 2 | 464.7 | 1 |
| 6 | L | KSEDEMKASEDLKKHG | N | 50 | 4 | 458.7 | 2 |
| 7 | L | KSEDEMKASEDLK | K | 50 | 3 | 503.9 | 2 |
| 8 | K | SEDEMKASEDLK | K | 51 | 2 | 691.3 | 3 |
| 9 | K | SEDEMKASEDL | K | 51 | 2 | 627.2 | 3 |
| 10 | Q | SKHPGDFGADAQG | A | 117 | 2 | 643.7 | 2 |

[a] Amino acid residue preceding the peptide sequence (N-terminus).
[b] Amino acid residue following the peptide sequence (C-terminus).
[c] Peptide sequence start in myoglobin.
[d] Charge (+).
[e] Mass to charge ratio.
[f] Frequency of occurnace in the three replicates

**Figure 2.1** Myoglobin sequence and 3D structure. The majority of the identified peptides were lying in the underlined regions.



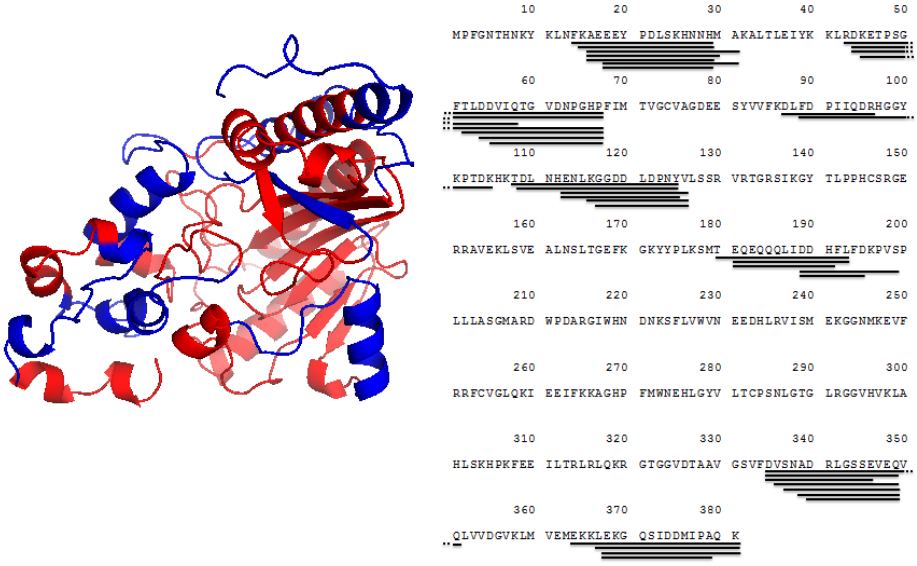
Creatine kinase is an abundant sarcoplasmic protein and its degradation in dry fermented sausages has been reported (López et al., 2015a). However, some technological conditions, like low fermentation and ripening temperature, that are prevalent in high pH dry fermented sausages, might reduce the activity of important proteolytic enzymes and limit the degradation of this protein (López et al., 2015a). In the present study, a total of thirty-nine creatine kinase-derived peptides were identified (Table 2.2). These peptides were released from seven regions which are highlighted in Figure 2.2. Mora et al. (2009) identified creatine kinase-derived peptides in dry cured ham subjected to a ripening process of 9 months. These authors reported several peptides hydrolysed from the seven regions highlighted in the present study, confirming the high susceptibility to proteolysis of these zones. Moreover, many of the cleavage sites reported by Mora et al. (2009) for creatine kinase are the same as found in the present study.

**Table 2.2** Peptides of creatine kinase identified by LCMS[E] dry fermented sausages

| N. | $P_1$[a] | Peptide sequence | $P_{1'}$[b] | P. s. s.[c] | z[d] | m/z[e] | Freq.[f] |
|---|---|---|---|---|---|---|---|
| 1 | N | FKAEEEYPDLSKHNNH | M | 13 | 3 | 653.3 | 3 |
| 2 | F | KAEEEYPDLSKHNNH | M | 14 | 3 | 604.2 | 1 |
| 3 | K | AEEEYPDLSKHNNHMA | K | 15 | 3 | 628.9 | 1 |
| 4 | K | AEEEYPDLSKHNNHM | A | 15 | 3 | 605.2 | 3 |
| 5 | K | AEEEYPDLSKHNNH | M | 15 | 3 | 561.5 | 3 |
| 6 | A | EEYPDLSKHNNHMA | K | 17 | 3 | 562.2 | 1 |
| 7 | A | EEYPDLSKHNNH | M | 17 | 2 | 741.8 | 2 |
| 8 | L | RDKETPSGFTLDDVIQTGVDNPGHP | F | 42 | 4 | 674.5 | 3 |
| 9 | R | DKETPSGFTLDDVIQTGVDNPGHP | F | 43 | 3 | 847.0 | 3 |
| 10 | R | DKETPSGFTLDDVIQ | T | 43 | 2 | 832.9 | 2 |
| 11 | D | KETPSGFTLDDVIQTGVDNPGHP | F | 44 | 3 | 808.7 | 2 |
| 12 | F | TLDDVIQTGVDNPGHP | F | 51 | 2 | 839.4 | 3 |
| 13 | L | DDVIQTGVDNPGHP | F | 53 | 2 | 732.3 | 3 |
| 14 | D | DVIQTGVDNPGHP | F | 54 | 2 | 674.8 | 3 |
| 15 | K | DLFDPIIQDR | H | 86 | 2 | 616.3 | 1 |
| 16 | L | FDPIIQDRHGGYKPTDK | H | 88 | 3 | 663.0 | 1 |
| 17 | K | TDLNHENLKGGDDLDPNY | V | 107 | 3 | 677.3 | 1 |
| 18 | T | DLNHENLKGGDDLDPNY | V | 108 | 3 | 643.6 | 1 |
| 19 | H | ENLKGGDDLDPNYV | L | 112 | 2 | 774.8 | 2 |
| 20 | H | ENLKGGDDLDPNY | V | 112 | 2 | 725.3 | 2 |
| 21 | L | KGGDDLDPNYV | L | 115 | 2 | 596.7 | 2 |
| 22 | K | GGDDLDPNYV | L | 116 | 1 | 1064.4 | 1 |
| 23 | N | TEQEQQQLIDDH | F | 179 | 2 | 742.3 | 2 |
| 24 | T | EQEQQQLIDDHFL | F | 180 | 2 | 821.8 | 3 |
| 25 | T | EQEQQQLIDDH | F | 180 | 2 | 691.8 | 3 |
| 26 | I | DDHFLFDKPVS | P | 188 | 2 | 660.3 | 2 |
| 27 | I | DDHFLFD | K | 188 | 1 | 908.3 | 3 |
| 28 | V | GSVFDVSNADRL | G | 330 | 2 | 640.3 | 1 |
| 29 | F | DVSNADRLGSSEVEQVQ | L | 334 | 2 | 916.9 | 2 |
| 30 | F | DVSNADRLGSSEVEQ | V | 334 | 2 | 803.3 | 3 |
| 31 | F | DVSNADRLGSSE | V | 334 | 2 | 625.2 | 3 |
| 32 | D | VSNADRLGSSEVEQ | V | 335 | 2 | 745.8 | 1 |
| 33 | V | SNADRLGSSEVEQ | V | 336 | 2 | 696.3 | 2 |
| 34 | N | ADRLGSSEVEQ | V | 338 | 2 | 595.7 | 1 |
| 35 | A | DRLGSSEVEQ | V | 339 | 2 | 560.2 | 3 |
| 36 | M | EKKLEKGQSIDDMIPAQK | | 363 | 3 | 686.6 | 1 |
| 37 | K | LEKGQSIDDMIPAQK | | 366 | 3 | 558.2 | 1 |
| 38 | L | EKGQSIDDMIPAQK | | 367 | 2 | 780.3 | 3 |
| 39 | L | EKGQSIDDMIPA | Q | 367 | 2 | 652.3 | 2 |

[a] Amino acid residue preceding the peptide sequence (N-terminus).
[b] Amino acid residue following the peptide sequence (C-terminus).
[c] Peptide sequence start in creatine kinase.
[d] Charge (+).
[e] Mass to charge ratio.
[f] Frequency of occurnace in the three replicates

**Figure 2.2** Creatine kinase sequence and 3D structure. The majority of the identified peptides were lying in the underlined regions.



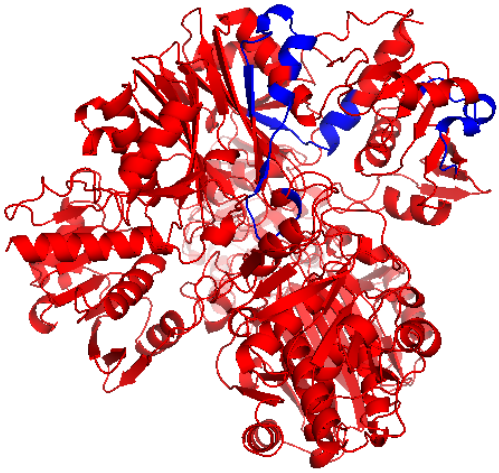

A total of nine peptides from glyceraldehyde-3-phosphate dehydrogenase have been identified (Table 2.3) and were generated from 4 different regions in the protein sequence (Figure 2.3). Glyceraldehyde-3-phosphate dehydrogenase is a sarcoplasmic protein whose fragments are often identified in ripened meat products (Mora et al., 2015). In particular, Mora et al., (2015) identified numerous peptides in the region between residues 193-224 in dry fermented sausages ripened for 43 days; in the present study 6 peptides were identified in the same region.

**Table 2.3** Peptides of glyceraldehyde-3-phosphate dehydrogenase identified by LCMS[E] dry fermented sausages

| N. | $P_1$[a] | Peptide sequence | $P_1'$[b] | P. s. s.[c] | z[d] | m/z[e] | Freq.[f] |
|---|---|---|---|---|---|---|---|
| 1 | Q | ERDPANIKWGDAGATY | V | 76 | 2 | 882.4 | 2 |
| 2 | Q | ERDPANIKWGDAG | A | 76 | 2 | 714.8 | 2 |
| 3 | R | DGRGAAQNIIPASTGAA | K | 195 | 2 | 785.4 | 2 |
| 4 | K | AVGKVIPELNGKLTG | M | 213 | 2 | 748.4 | 1 |
| 5 | K | AVGKVIPELN | G | 213 | 2 | 520.3 | 3 |
| 6 | K | AVGKVIPEL | N | 213 | 2 | 463.2 | 3 |
| 7 | A | VGKVIPEL | N | 214 | 2 | 427.7 | 2 |
| 8 | V | GKVIPEL | N | 215 | 1 | 755.4 | 3 |
| 9 | I | SWYDNEFGYSNR | V | 309 | 2 | 769.3 | 3 |

[a] Amino acid residue preceding the peptide sequence (N-terminus).
[b] Amino acid residue following the peptide sequence (C-terminus).
[c] Peptide sequence start in glyceraldehyde-3-phosphate dehydrogenase.
[d] Charge (+).
[e] Mass to charge ratio.
[f] Frequency of occurnace in the three replicates

**Figure 2.3** Glyceraldehyde-3-phosphate dehydrogenase sequence and 3D structure. The majority of the identified peptides were lying in the underlined regions.



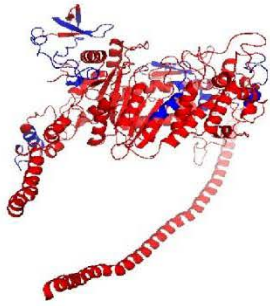
```
        10         20         30         40         50
MVKVGVNGFG RIGRLVTRAA FNSGKVDIVA INDPFIDLHY MVYMFQYDST

        60         70         80         90        100
HGKFHGTVKA ENGKLVINGK AITIFQERDP ANIKWGDAGA TYVVESTGVF

       110        120        130        140        150
TTMEKAGAHL KGGAKRVIIS APSADAPMFV MGVNHEKYDN SLKIVSNASC

       160        170        180        190        200
TTNCLAPLAK VIHDHFGIVE GLMTTVHAIT ATQKTVDGPS GKLWRDGRGA

       210        220        230        240        250
AQNIIPASTG AAKAVGKVIP ELNGKLTGMA FRVPTPNVSV VDLTCRLEKP

       260        270        280        290        300
AKYDDIKKVV KQASEGPLKG ILGYTEDQVV SCDFNSDTHS STFDAGAGIA

       310        320        330
LNDHFVKLIS WYDNEFGYSN RVVDLMVHMA SKE
```

As far as myosin heavy chain is concerned, twenty-eight peptides were identified (Table 2.4). These peptides were released from 10 regions (Figure 2.4). This protein is subjected to intense proteolysis in dry fermented sausages, being often completely degraded during ripening (Hughes et al., 2002). The peptides in the first region were also reported by López et al. (2015a) during ripening of dry fermented sausages.

**Table 2.4** Peptides of myosin identified by LCMS$^{E}$ dry fermented sausages

| N. | $P_1$[a] | Peptide sequence | $P_{1'}$[b] | P. s. s.[c] | z[d] | m/z[e] | Freq.[f] |
|---|---|---|---|---|---|---|---|
| 1 | A | FGEAAPYLRKSEKE | R | 9 | 3 | 542,2 | 3 |
| 2 | F | GEAAPYLRKSEKE | R | 10 | 3 | 493,2 | 2 |
| 3 | G | EAAPYLRKSEKERIEAQNKPFD | A | 11 | 4 | 655,5 | 3 |
| 4 | K | SEKERIEAQNKPFD | A | 19 | 3 | 564,2 | 2 |
| 5 | S | EKERIEAQNKPFD | A | 20 | 3 | 535,2 | 1 |
| 6 | K | ERIEAQNKPFD | A | 22 | 2 | 673,8 | 1 |
| 7 | K | ERIEAQNKPFDA | K | 22 | 2 | 709,3 | 1 |
| 8 | F | VAEPKESFVKGTVQSREG | G | 39 | 3 | 650,0 | 2 |
| 9 | V | AEPKESFVKGTVQSREG | G | 40 | 3 | 616,9 | 2 |
| 10 | A | EPKESFVKGTVQSREG | G | 41 | 3 | 593,3 | 1 |
| 11 | L | TVKEDQVFPMNPPKFD | K | 70 | 3 | 631,3 | 1 |
| 12 | L | TVKEDQVFPMNPPKFDKIED | M | 70 | 3 | 793,0 | 2 |
| 13 | T | VKEDQVFPMNPP | K | 71 | 2 | 700,8 | 2 |
| 14 | T | VKEDQVFPMNPPKFDKIED | M | 71 | 3 | 759,3 | 2 |
| 15 | V | KEDQVFPMNPPKFD | K | 72 | 2 | 846,4 | 2 |
| 16 | K | EDQVFPMNPPKFD | K | 73 | 2 | 782,3 | 2 |
| 17 | E | DQVFPMNPPKFD | K | 74 | 2 | 717,8 | 2 |
| 18 | E | DQVFPMNPPKFDKIEDMAM | M | 74 | 2 | 1127,0 | 2 |
| 19 | I | ISANPLLEAFGNA | K | 223 | 2 | 658,8 | 1 |
| 20 | Q | REEQAEPDGTEVADK | A | 371 | 2 | 837,3 | 2 |
| 21 | Q | REEQAEPDGTEVADKA | A | 371 | 2 | 872,8 | 2 |
| 22 | I | FDFNSLEQ | L | 470 | 2 | 500,1 | 1 |
| 23 | H | YAGTVDYNITGWLDKNK | D | 584 | 3 | 653,3 | 2 |
| 24 | S | AIPEGQFIDSKKASEKL | L | 732 | 3 | 621,0 | 3 |
| 25 | S | AIPEGQFIDSKKASEKLL | G | 732 | 3 | 658,7 | 2 |
| 26 | E | STMDIENDKQQ | L | 1068 | 2 | 654,7 | 1 |
| 27 | L | SRELEEISERLEEAGGAT | S | 1143 | 3 | 659,3 | 1 |
| 28 | S | ERLEEAGGATSAQIEM | N | 1151 | 2 | 846,3 | 1 |

[a] Amino acid residue preceding the peptide sequence (N-terminus).
[b] Amino acid residue following the peptide sequence (C-terminus).
[c] Peptide sequence start in myosin.
[d] Charge (+).
[e] Mass to charge ratio.
[f] Frequency of occurnace in the three replicates



```
        10         20         30         40         50
MSSDQEMAIF GEAAPYLRKS EKERIEAQNK PFDAKTSVFV AEPKESFVKG
        60         70         80         90        100
TVQSREGGKV TVKTEAGATL TVKEDQVFPM NPPKFDKIED MAMMTHLHEP
       110        120        130        140        150
AVLYNLKERY AAWMIYTYSG LFCVTVNPYK WLPVYNAEVV TAYRGKKRQE
       160        170        180        190        200
APPHIFSISD NAYQFMLTDR ENQSILITGE SGAGKTVNTK RVIQYFATIA
       210        220        230        240        250
VTGEKKKEEP TSGKMQGTLE DQIISANPLL EAFGNAKTVR NDNSSRFGKF
       260        270        280        290        300
IRIHFGTTGK LASADIETYL LEKSRVTFQL KAERSYHIFY QIMSNKKPEL
       310        320        330        340        350
IEMLLITTNP YDYAFVSQGE ITVPSIDDQE ELMATDSAIE ILGFTSDERV
       360        370        380        390        400
SIYKLTGAVM HYGNLKFKQK QREEQAEPDG TEVADKAAYL QGLNSADLLK
       410        420        430        440        450
ALCYPRVKVG NEFVTKGQTV QQVYNAVGAL AKAVYDKMFL WMVTRINQQL
       460        470        480        490        500
DTKQPRQYFI GVLDIAGFEI FDFNSLEQLC INFTNEKLQQ FFNHHMFVLE
       510        520        530        540        550
QEEYKKEGIE WEFIDFGMDL AACIELIEKP MGIFSILEEE CMFPKATDTS
       560        570        580        590        600
FKNKLYEQHL GKSNNFQKPK PAKGKVEAHF SLIHYAGTVD YNITGWLDKN
       610        620        630        640        650
KDPLNETVVG LYQKSSVKTL AFLFTGAAGA DAEAGGGKKG GKKKGSSFQT
       660        670        680        690        700
VSALFRENLN KLMTNLRSTH PHFVRCIIPN ETKTPGAMEH ELVLHQLRCN
       710        720        730        740        750
GVLEGIRICR KGFPSRILYA DFKQRYKVLN ASAIPEGQFI DSKKASEKLL
       760        770        780        790        800
GSIDIDHTQY KFGHTKVFFK AGLLGLLEEM RDEKLAQLIT RTQARCRGFL
       810        820        830        840        850
ARVEYQKMVE RRESIFCIQY NIRAFMNVKH WPWMKLYFKI KPLLKSAETE
       860        870        880        890        900
KEMANMKEEF EKTKESLAKA EAKRKELEEK MVALMQEKND LQLQVQAEAD
       910        920        930        940        950
SLADAEERCD QLIKTKIQLE AKIKEVTERA EDEEEINAEL TAKKRKLEDE
       960        970        980        990       1000
CSELKKDIDD LELTLAKVEK EKHATENKVK NLTEEMAGLD ETIAKLTKEK
      1010       1020       1030       1040       1050
KALQEAHQQT LDDLQAEEDK VNTLTKAKTK LEQQVDDLEG SLEQEKKLRM
      1060       1070       1080       1090       1100
DLERAKRKLE GDLKLAQEST MDIENDKQQL DEKLKKKEFE MSNLQSKIED
      1110       1120       1130       1140       1150
EQALAMQLQK KIKELQARIE ELEEEIEAER ASRAKAEKQR SDLSRELEEI
      1160       1170       1180       1190       1200
SERLEEAGGA TSAQIEMNKK REAEFQKMRR DLEEATLQHE ATAATLRKKH
```

**Figure 2.4** First part of the myosin sequence and 3D structure. The majority of the identified peptides were lying in the underlined regions.

In Tables 2.1 to 2.4, several peptides are truncated differing by a single amino acid. The release of single amino acids and the consequent shortening of peptides is done by aminopeptidases from the N-terminus and by carboxypeptidases from the C-terminus. In porcine skeletal muscle, several aminopeptidases have been purified and characterized (Flores et al., 1993; 1996; 2000) and their role was previously discussed in meat products (Mora et al., 2009; 2015). These enzymes have optimum activity at neutral pH, however the conditions present in the dry fermented sausages, especially in the final weeks of drying, allow activity of these enzymes (Flores et al., 1993; 1996; 2000). Carboxypeptidases A and B are considered the main responsible enzymes for the release of free amino acid from the C-terminus (Mora et al., 2015). These exopeptidases are located in the lysosomes and have acidic pH optima (Toldrá, 2006).

Besides meat endogenous proteases, bacterial enzymes hydrolyse proteins during ripening of dry fermented sausages. The contribution of these enzymes to proteolysis is very low under dry fermented sausage conditions (Kenneally et al., 1999). This is particularly true for myofibrillar proteins, whereas sarcoplasmic proteins are good substrates for some *Lactobacillus sakei* strains (Ammor and Mayo, 2007). Fadda et al. (2009) reported that some lactic acid bacteria are able to hydrolyse meat sarcoplasmic proteins and exert exopeptidase activity. Therefore, the *Lactobacillus sakei* strain used in the present study may have played a role in the generation of the peptides identified.

## 2.5 CONCLUSIONS

In this study, the small peptides generated by proteolysis of three important sarcoplasmic proteins, i.e. myoglobin, creatine kinase and glyceraldehyde-3-phosphate dehydrogenase, and myosin heavy chain have been identified through $LCMS^E$ in dry fermented sausages. These proteins were cleaved by endopeptidases in different points; the subsequent action of exopeptidases reduced the length of the peptides. These low molecular compounds are important flavour compounds and flavour precursors. Therefore, the identification of these peptides provides information for further studies to better understand their actual impact.

# CHAPTER 3

## ACTIN PROTEOLYSIS DURING RIPENING OF DRY FERMENTED SAUSAGES AT DIFFERENT PH VALUES

Redrafted after Berardo, A., Devreese, B., De Maere, H., Stavropoulou, D.A., Van Royen, G., Leroy, F., De Smet, S. (2016). Actin proteolysis during ripening of dry fermented sausages at different pH values. Food Chemistry, 221, 1322-1332.

## 3.1 ABSTRACT

In dry fermented sausages, myofibrillar proteins undergo intense proteolysis generating small peptides and free amino acids that play a role in flavour generation. This study aimed to identify small peptides arising from actin proteolysis, as influenced by the type of processing. Two acidification profiles were imposed, in order to mimic the pH normally obtained in Southern-European type and Northern-European type dry fermented sausages. The identification of peptides was done by liquid chromatography coupled to mass spectrometry in a data-independent positive mode of acquisition ($LCMS^E$). During manufacturing of the dry fermented sausages, actin was highly proteolysed, especially in nine regions of the sequence. After fermentation, 52 and 42 actin-derived peptides were identified at high and low pH, respectively, which further increased to 66 and 144 peptides, respectively, at the end of ripening. Looking at the cleavage sites, cathepsins B and D likely played an important role.

## 3.2 INTRODUCTION

During ripening of dry fermented sausages, the protein fraction undergoes proteolysis. The main actors of this process, i.e. proteolytic enzymes, have different pH optima (Hughes et al., 1999, 2000). Therefore, the prevailing acidification profile during processing is expected to affect proteolysis and thus flavour formation. Dry fermented sausages can be roughly divided in two groups with different acidity levels, namely Northern-European type and Southern-European type products (Ravyts et al., 2012). In the Northern-European type, the pH drops below 5.0 during fermentation and stays more or less at that level throughout ripening. On the contrary, in the Southern-European type, the pH drops only moderately during fermentation and increases during the drying phase, resulting in a final pH between 5.5 and 6.0 (Demeyer et al., 2000). As described in chapter 1, acidification is not the only difference between Northern-European type and Southern-European type dry fermented sausages. Nevertheless, only this difference is taken into account in this chapter; therefore a high pH sausage (mildly acidified) a low pH sausage (strongly acidified) were studied.

Since the pH drop during meat fermentation not only influences flavour by creating an acid taste but also through proteolysis, it is important to better understand the details of this mechanism. Therefore, the aim of this study was to identify the peptides originated from actin degradation, which is one of the most abundant proteins in meat, during ripening of dry fermented sausages at two different pH values.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Dry fermented sausage preparation

Dry fermented sausages were prepared by mixing lean pork (70.5 %), pork backfat (27.0 %), sodium chloride (2.5 %), SN (0.015 %, m/m), SA (0.05 %, m/m), and a starter culture strain (*Lactobacillus sakei* CTC 494) originating from the culture collection of the Research Group of Industrial Microbiology and Food Biotechnology (Vrije Universiteit Brussel, Brussels, Belgium). The initial load of *L. sakei* CTC 494 was $10^6$ (CFU/g). Two pH treatments were installed by adding either 0.25 %, m/m (high-pH treatment) or 0.7 %, m/m (low-pH treatment) of dextrose. The batter was stuffed into collagen casings of 50 mm diameter (Naturin, Weinheim, Germany) to make dry fermented sausages of about 200 g. Ripening lasted for 28 days in a climate chamber. During the first four days, fermentation was performed at a temperature of 24 °C and a relative humidity of 94 %. For the drying process, the temperature was dropped to 12 °C and relative humidity was set at 94 % for the first 14 days and at 80 % for the last 10 days. Samples were taken at days 0, 4 (end of fermentation), and 28 (end of ripening). The manufacturing processes and sampling were repeated in triplicate, resulting in three independent replicates.

### 3.3.2 pH and weight loss

In each manufacturing process, three randomly selected sausages per treatment were weighed and the pH was recorded after their preparation and during ripening. The pH was measured directly in the sausages [ISO 2917

(1999)] and the pH meter was calibrated in buffers of pH 4.0 and 7.0. Weight loss was expressed as a percentage of the initial weight and the mean of the three records was calculated.

### 3.3.3 Peptide extraction and size-exclusion chromatography

The peptide extraction and size-exclusion chromatography were carried out as described in Chapter 2.

### 3.3.4 LCMS$^E$ analysis

Lyophilised peptides were suspended in 40 µL of 100 mM $NH_4HCO_2$ (pH 10). Samples were filtered (0.22 µm) and centrifuged. The peptide identification was done according to the method described in Chapter 2.

The LCMS$^E$ data were processed using the ProteinLynx Global SERVER™ v2.5 (PLGS, Waters Corporation) (Geromanos et al., 2009). The identification of actin peptides was done by using a Uniprot database containing 753 protein entries from actin of the organism *Sus scrofa* (downloaded from the Uniprot website, March 2016). The primary digest reagent was set as “none”. The precursor and fragment ion tolerance were determined automatically. The default protein identification criteria used included a detection of minimal three fragment ions per peptide, and minimal three fragment ions per protein. Methionine oxidation was selected as a variable modification. A false positive rate of 4 % was allowed.

### 3.3.5 Identification of peptides cleaved by cathepsins B and D

To identify the actin-derived peptides that were likely generated by the action of cathepsins B and D during meat fermentation, the previously determined cleavage sites of these enzymes on bovine F-actin, were used (Hughes et al., 1999, 2000).

## 3.4 RESULTS

### 3.4.1 pH and weight loss

The initial pH in the raw meat was 5.69 and 5.68 for the high-pH and low-pH treatments respectively. During the fermentation phase, the pH decreased to 5.23 (± 0.02) and 4.89 (± 0.01) in the high-pH and low-pH treatments, respectively. In the high-pH treatment, the pH increased throughout the drying phase to a final pH of 5.98 (± 0.07) on day 28. On the contrary, in the low-pH treatment, the pH decreased to a minimum of 4.76 (± 0.01) on day 15 and subsequently increased to a final pH of 4.96 (± 0.01) at the end of ripening on day 28.

Weight loss showed similar trends in both treatments. Indeed, all samples lost about 2 % and 30 % of the initial weight at the end of fermentation and ripening, respectively.

### 3.4.2 Peptides identified from actin degradation

The extracted peptides from the raw meat and the sausages at the end of fermentation and ripening were subjected to identification by $LCMS^E$. In the raw meat, no peptides arising from actin were found. At the end of fermentation, however, 52 and 42 peptides were identified in the high-pH and low-pH sausages, respectively (Tables 3.1 and 3.2). At this stage, both sausage types showed 37 peptides in common (highlighted in bold in the Tables). At the end of ripening, the number of peptides identified increased to 66 in the high-pH sausages (Table 3.3). In the low-pH sausages, 144 peptides were identified, of which 47 peptides were also found in the high-pH variant (Table 3.4). The peptides identified covered more than 50 % of the entire actin sequence and were generated from all four actin subdomains, in which nine actin regions containing the majority of the identified peptides are highlighted in Figure 3.1. Most peptides were identified in the regions 1 and 9, which are near the N- and C-terminus and are located in the first subdomain together with the regions 3, 4 and 5 (region 1 is also partially lying in the second subdomain). Regions 2 and 7 were located in the second and fourth subdomain, respectively. Finally, in the third subdomain, peptides were mainly released from regions 6 and 8. On average, more peptides were identified at the end of ripening. However, in the regions 6 and 9, similar amounts of peptides were identified at both days.

**Table 3.1** Peptides of actin identified by LCMS[E] in high-pH dry fermented sausages after 4 days of ripening (fermentation phase)

| N. | $P_1$[a] | Peptide sequence | $P_1$,[b] | P. s. s.[c] | z[d] | m/z[e] | Info | Freq.[f] |
|---|---|---|---|---|---|---|---|---|
| 1 | E | DETTALVCDNGSGLVK | A | 4 | 2 | 811.3 | | 1 |
| 2 | C | DNGSGLVKAGFAGDDAPR | A | 12 | 3 | 582.9 | CD[f] (N-ter), CDB[h] (C-ter) | 1 |
| 3 | K | AGFAGDDAPR | A | 20 | 2 | 488.7 | | 1 |
| 4 | G | **FAGDDAPRAVFPSIVG** | R | 22 | 2 | 809.9 | CBD[h] (N-ter) | 3 |
| 5 | G | **FAGDDAPRAVFPS** | I | 22 | 2 | 675.3 | CBD[h] (N-ter) | 2 |
| 6 | D | SYVGDEAQSKRG | I | 53 | 2 | 648.8 | | 1 |
| 7 | Y | VGDEAQSKRG | I | 55 | 2 | 523.7 | | 1 |
| 8 | F | **YNELRVAPEEHPTL** | L | 92 | 3 | 556.6 | CBD[h] (N-ter) | 2 |
| 9 | F | **YNELRVAPEE** | H | 92 | 2 | 610.3 | CBD[h] (N-ter) | 3 |
| 10 | L | **RVAPEEHPTL** | L | 96 | 2 | 574.8 | | 3 |
| 11 | R | VAPEEHPTL | L | 97 | 2 | 496.7 | | 2 |
| 12 | A | **PEEHPTL** | L | 99 | 2 | 411.7 | | 3 |
| 13 | T | GIVLDSGDGVTHNVPIYEG | Y | 151 | 3 | 647.9 | | 1 |
| 14 | G | **IVLDSGDGVTHNVPIYEG** | Y | 152 | 2 | 942.9 | | 3 |
| 15 | G | IVLDSGDGVTHNVPIYE | G | 152 | 2 | 914.4 | | 2 |
| 16 | I | **VLDSGDGVTHNVPIYEG** | Y | 153 | 2 | 886.4 | CD[f] (N-ter) | 3 |
| 17 | I | **VLDSGDGVTHNVPIYE** | G | 153 | 2 | 857.9 | CD[f] (N-ter) | 2 |
| 18 | I | **VLDSGDGVTHNVPIY** | E | 153 | 2 | 793.3 | CD[f] (N-ter) | 2 |
| 19 | I | VLDSGDGVTHNVP | I | 153 | 2 | 655.3 | CD[f] (N-ter) | 2 |
| 20 | V | **LDSGDGVTHNVPIYEG** | Y | 154 | 2 | 836.8 | | 2 |
| 21 | L | **DSGDGVTHNVPIYEG** | Y | 155 | 2 | 780.3 | CD[f] (N-ter) | 3 |
| 22 | L | **DSGDGVTHNVPIYE** | G | 155 | 2 | 751.8 | CD[f] (N-ter) | 2 |
| 23 | L | **DSGDGVTHNVPIY** | E | 155 | 2 | 687.3 | CD[f] (N-ter) | 2 |
| 24 | D | **SGDGVTHNVPIYEG** | Y | 156 | 2 | 722.8 | | 2 |
| 25 | S | **DGVTHNVPIYEG** | Y | 158 | 2 | 650.8 | | 2 |
| 26 | L | EKSYELPDGQVITIGN | E | 238 | 2 | 881.9 | | 1 |
| 27 | S | **YELPDGQVITIGNERFR** | C | 241 | 3 | 669.6 | | 2 |
| 28 | S | **YELPDGQVITIGNERF** | R | 241 | 2 | 925.9 | | 2 |
| 29 | S | **YELPDGQVITIGNER** | F | 241 | 2 | 852.4 | | 3 |
| 30 | S | YELPDGQVIT | I | 241 | 1 | 1134.5 | CB[g] (C-ter) | 2 |
| 31 | Y | **ELPDGQVITIGNERF** | R | 242 | 2 | 844.4 | | 2 |
| 32 | Y | **ELPDGQVITIGNER** | F | 242 | 2 | 770.9 | | 3 |
| 33 | M | SGGTTMYPGIADRMQ | K | 301 | 2 | 792.8 | | 1 |
| 34 | M | SGGTTMYPGIADR | M | 301 | 2 | 663.3 | | 1 |
| 35 | S | TFQQMWITKQEY | D | 352 | 3 | 534.9 | | 2 |
| 36 | M | **WITKQEYDEAGPSIVH** | R | 357 | 3 | 624.9 | | 3 |
| 37 | M | **WITKQEYDEAGPS** | I | 357 | 2 | 762.3 | | 2 |
| 38 | W | **ITKQEYDEAGPSIVH** | R | 358 | 3 | 562.9 | CD[f] (N-ter) | 3 |
| 39 | W | **ITKQEYDEAGPS** | I | 358 | 2 | 669.3 | CD[f] (N-ter) | 3 |
| 40 | I | **TKQEYDEAGPSIVH** | R | 359 | 2 | 787.3 | | 2 |
| 41 | K | **QEYDEAGPSIVH** | R | 361 | 2 | 672.8 | | 2 |
| 42 | Q | **EYDEAGPSIVHRK** | C | 362 | 2 | 750.8 | | 2 |
| 43 | Q | EYDEAGPSIVHR | K | 362 | 2 | 686.8 | | 2 |
| 44 | Q | **EYDEAGPSIVH** | R | 362 | 2 | 608.7 | | 3 |
| 45 | E | **YDEAGPSIVHRK** | C | 363 | 3 | 457.9 | CB[g] (N-ter) | 2 |
| 46 | E | **YDEAGPSIVHR** | K | 363 | 2 | 622.3 | CB[g] (N-ter) | 2 |
| 47 | E | **YDEAGPSIVH** | R | 363 | 2 | 544.2 | CB[g] (N-ter) | 3 |
| 48 | Y | **DEAGPSIVHRK** | C | 364 | 2 | 604.8 | | 2 |
| 49 | Y | **DEAGPSIVHR** | K | 364 | 2 | 540.7 | | 2 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 50 | Y | **DEAGPSIVH** | R | 364 | 2 | 462.7 | 1 |
| 51 | E | **AGPSIVHR** | K | 366 | 2 | 418.7 | 1 |
| 52 | E | **AGPSIVH** | R | 366 | 1 | 680.3 | 2 |

[a] Amino acid residue preceding the peptide sequence (N-terminus).
[b] Amino acid residue following the peptide sequence (C-terminus).
[c] Peptide sequence start in actin.
[d] Charge (+).
[e] Mass to charge ratio.
[f] Cathepsin D cleavage site.
[g] Cathepsin B cleavage site.
[h] Cathepsin B and D common cleavage site.
[f] Frequency of occurnace in the three replicates
Peptides that were also found in low pH dry fermented sausages after 4 days of ripening are indicted in bold.

The identified peptides were mapped on to the 3D actin structure (Figure 3.2). Although after the fermentation phase the number of peptides identified in the two pH treatments was similar, some peptides (in red) were identified only at high pH (Figure 3.2A). On the contrary, at the end of ripening, the higher number of peptides identified in the low-pH compared to the high-pH treatment is reflected in the higher number of blue zones in the 3D structure (Figure 3.2B). However, the majority of peptides were identified in both treatments and are highlighted in yellow.

**Table 3.2** Peptides of actin identified by LCMS$^{E}$ in low-pH dry fermented sausages after 4 days of ripening (fermentation phase)

| N. | $P_1$[a] | Peptide sequence | $P_1'$[b] | P. s. s.[c] | z[d] | m/z[e] | Info | Freq.[f] |
|---|---|---|---|---|---|---|---|---|
| 1 | G | **FAGDDAPRAVFPSIVG** | R | 22 | 2 | 809.9 | CBD[h] (N-ter) | 1 |
| 2 | G | **FAGDDAPRAVFPS** | I | 22 | 2 | 675.3 | CBD[h] (N-ter) | 1 |
| 3 | H | GIITNWDDMEK | I | 75 | 2 | 661.3 | CB[g] (N-ter), CB[g] (C-ter) | 1 |
| 4 | F | **YNELRVAPEEHPTL** | L | 92 | 3 | 556.6 | CBD[h] (N-ter) | 3 |
| 5 | F | YNELRVAPEEHPT | L | 92 | 3 | 518.9 | CBD[h] (N-ter) | 2 |
| 6 | F | **YNELRVAPEE** | H | 92 | 2 | 610.3 | CBD[h] (N-ter) | 2 |
| 7 | L | **RVAPEEHPTL** | L | 96 | 2 | 574.8 | | 2 |
| 8 | A | **PEEHPTL** | L | 99 | 2 | 411.7 | | 1 |
| 9 | G | **IVLDSGDGVTHNVPIYEG** | Y | 152 | 2 | 942.9 | | 1 |
| 10 | I | **VLDSGDGVTHNVPIYEG** | Y | 153 | 2 | 886.4 | CD[f] (N-ter) | 3 |
| 11 | I | **VLDSGDGVTHNVPIYE** | G | 153 | 2 | 857.9 | CD[f] (N-ter) | 2 |
| 12 | I | **VLDSGDGVTHNVPIY** | E | 153 | 2 | 793.3 | CD[f] (N-ter) | 1 |
| 13 | V | **LDSGDGVTHNVPIYEG** | Y | 154 | 2 | 836.8 | | 3 |
| 14 | L | **DSGDGVTHNVPIYEG** | Y | 155 | 2 | 780.3 | CD[f] (N-ter) | 3 |
| 15 | L | **DSGDGVTHNVPIYE** | G | 155 | 2 | 751.8 | CD[f] (N-ter) | 2 |
| 16 | L | **DSGDGVTHNVPIY** | E | 155 | 2 | 687.3 | CD[f] (N-ter) | 2 |
| 17 | D | **SGDGVTHNVPIYEG** | Y | 156 | 2 | 722.8 | | 2 |
| 18 | G | **DGVTHNVPIYEG** | Y | 158 | 2 | 650.8 | | 3 |
| 19 | G | YALPHAIM | R | 170 | 2 | 458.2 | | 1 |
| 20 | S | **YELPDGQVITIGNERFR** | C | 241 | 3 | 669.6 | | 2 |
| 21 | S | **YELPDGQVITIGNERF** | R | 241 | 2 | 925.9 | | 2 |
| 22 | S | **YELPDGQVITIGNER** | F | 241 | 2 | 852.4 | | 2 |
| 23 | Y | **ELPDGQVITIGNERF** | R | 242 | 2 | 844.4 | | 2 |
| 24 | Y | **ELPDGQVITIGNER** | F | 242 | 2 | 770.9 | | 2 |
| 25 | M | WITKQEYDEAGPSIVHRK | C | 357 | 4 | 540.0 | | 2 |
| 26 | M | **WITKQEYDEAGPSIVH** | R | 357 | 3 | 624.9 | | 2 |
| 27 | M | **WITKQEYDEAGPS** | I | 357 | 2 | 762.3 | | 1 |
| 28 | W | **ITKQEYDEAGPSIVH** | R | 358 | 3 | 562.9 | CD[f] (N-ter) | 3 |
| 29 | W | **ITKQEYDEAGPS** | I | 358 | 2 | 669.3 | CD[f] (N-ter) | 1 |
| 30 | I | **TKQEYDEAGPSIVH** | R | 359 | 2 | 787.3 | | 2 |
| 31 | K | **QEYDEAGPSIVH** | R | 361 | 2 | 672.8 | | 3 |
| 32 | Q | **EYDEAGPSIVHRK** | C | 362 | 2 | 750.8 | | 1 |
| 33 | Q | **EYDEAGPSIVH** | R | 362 | 2 | 599.7 | | 3 |
| 34 | E | **YDEAGPSIVHRK** | C | 363 | 3 | 457.9 | CB[g] (N-ter) | 1 |
| 35 | E | **YDEAGPSIVHR** | K | 363 | 2 | 622.3 | CB[g] (N-ter) | 2 |
| 36 | E | **YDEAGPSIVH** | R | 363 | 2 | 544.2 | CB[g] (N-ter) | 3 |
| 37 | Y | **DEAGPSIVHRK** | C | 364 | 2 | 604.8 | | 3 |
| 38 | Y | **DEAGPSIVHR** | K | 364 | 2 | 540.7 | | 3 |
| 39 | Y | **DEAGPSIVH** | R | 364 | 2 | 462.7 | | 2 |
| 40 | D | EAGPSIVHRK | C | 365 | 2 | 547.3 | | 2 |
| 41 | E | **AGPSIVHR** | K | 366 | 2 | 418.7 | | 1 |
| 42 | E | **AGPSIVH** | R | 366 | 1 | 680.3 | | 1 |

[a] Acid residue preceding the peptide sequence (N-terminus). [b] Amino acid residue following the peptide sequence (C-terminus). [c] Peptide sequence start in actin. [d] Charge (+). [e] Mass to charge ratio. [f] Cathepsin D cleavage site. [g] Cathepsin B cleavage site. [h] Cathepsin B and D common cleavage site. [f] Frequency of occurnace in the three replicates Peptides that were also found in high pH dry fermented sausages after 4 days of ripening are indicted in bold.

**Table 3.3** Peptides of actin protein identified by LCMS[E] in high-pH dry fermented sausages after 28 days of ripening

| N. | $P_1$[a] | Peptide sequence | $P_1$',[b] | P. s. s.[c] | z[d] | m/z[e] | Info | Freq.[f] |
|---|---|---|---|---|---|---|---|---|
| 1 | C | DNGSGLVKAGFAGDD | A | 12 | 2 | 711.8 | CD[f] (N-ter) | 1 |
| 2 | K | AGFAGDDAPRAVFPSIVG | R | 20 | 2 | 873.9 | | 2 |
| 3 | A | GFAGDDAPRAVFPSIVG | R | 21 | 2 | 838.4 | | 2 |
| 4 | G | **FAGDDAPRAVFPSIVG** | R | 22 | 2 | 809.9 | CBD[h] (N-ter) | 3 |
| 5 | F | **AGDDAPRAVFPSIVGRP** | R | 23 | 3 | 575.6 | | 3 |
| 6 | F | **AGDDAPRAVFPSIVGR** | P | 23 | 3 | 543.2 | | 1 |
| 7 | F | **AGDDAPRAVFPSIVG** | R | 23 | 2 | 736.3 | | 2 |
| 8 | G | **MGQKDSYVGDEAQSKRG** | I | 48 | 3 | 619.2 | | 1 |
| 9 | G | **MGQKDSYVGDEAQ** | S | 48 | 2 | 714.3 | | 2 |
| 10 | M | **GQKDSYVGDEAQSKRG** | I | 49 | 3 | 575.6 | CB[g] (N-ter) | 1 |
| 11 | M | **GQKDSYVGDEAQSK** | R | 49 | 2 | 756.3 | CB[g] (N-ter) | 3 |
| 12 | M | GQKDSYVGDEAQS | K | 49 | 2 | 692.3 | CB[g] (N-ter) | 2 |
| 13 | M | GQKDSYVGDEAQ | S | 49 | 2 | 648.7 | CB[g] (N-ter) | 2 |
| 14 | Q | KDSYVGDEAQSKR | G | 51 | 3 | 494.9 | | 1 |
| 15 | H | **GIITNWDDMEK** | I | 75 | 2 | 661.3 | CB[g] (N-ter), CB[g] (C-ter) | 3 |
| 16 | G | **IITNWDDMEK** | I | 76 | 2 | 632.7 | CB[g] (N-ter), CB[g] (C-ter) | 3 |
| 17 | I | **ITNWDDMEK** | I | 77 | 2 | 576.2 | CB[g] (C-ter) | 1 |
| 18 | I | **TNWDDMEKIWHHT** | F | 78 | 3 | 571.5 | CD[f] (N-ter), CD[f] (C-ter) | 2 |
| 19 | W | **DDMEKIWHHT** | F | 81 | 2 | 656.2 | CD[f] (C-ter) | 2 |
| 20 | F | **YNELRVAPEEHPTL** | L | 92 | 3 | 556.6 | CBD[h] (N-ter) | 2 |
| 21 | F | **YNELRVAPEEHPT** | L | 92 | 3 | 518.9 | CBD[h] (N-ter), CB[g] (C-ter) | 2 |
| 22 | F | **YNELRVAPEE** | H | 92 | 2 | 610.3 | | 2 |
| 23 | Y | NELRVAPEEHPT | L | 93 | 2 | 696.3 | CB[g] (C-ter) | 2 |
| 24 | L | **RVAPEEHPTL** | L | 96 | 2 | 574.8 | | 1 |
| 25 | R | **VAPEEHPTLL** | T | 97 | 2 | 553.2 | CBD[h] (N-ter), CBD[h] (C-ter) | 2 |
| 26 | L | YASGRTTGIVLDSGDGVTHNVPIYEG | Y | 144 | 3 | 893.4 | CD[f] (N-ter) | 1 |
| 27 | T | GIVLDSGDGVTHNVPIYEG | Y | 151 | 2 | 971.4 | | 2 |
| 28 | G | **IVLDSGDGVTHNVPIYEG** | Y | 152 | 2 | 942.9 | | 3 |
| 29 | I | VLDSGDGVTHNVPIYEGYA | L | 153 | 2 | 1003.4 | CD[f] (N-ter) | 2 |
| 30 | I | **VLDSGDGVTHNVPIYEG** | Y | 153 | 2 | 886.4 | CD[f] (N-ter) | 3 |
| 31 | I | **VLDSGDGVTHNVPIYE** | G | 153 | 2 | 857.9 | CD[f] (N-ter) | 3 |
| 32 | I | **VLDSGDGVTHNVP** | I | 153 | 2 | 655.3 | CD[f] (N-ter) | 1 |
| 33 | V | LDSGDGVTHNVPIYEG | Y | 154 | 2 | 836.8 | | 3 |
| 34 | L | **DSGDGVTHNVPIYEG** | Y | 155 | 2 | 780.3 | CD[f] (N-ter) | 3 |
| 35 | L | **DSGDGVTHNVP** | I | 155 | 2 | 549.2 | CD[f] (N-ter) | 2 |
| 36 | D | **SGDGVTHNVPIYEG** | Y | 156 | 2 | 722.8 | | 3 |
| 37 | S | **GDGVTHNVPIYEG** | Y | 157 | 2 | 679.3 | | 1 |
| 38 | G | **DGVTHNVPIYEG** | Y | 158 | 2 | 650.8 | | 2 |
| 39 | S | SLEKSYELPDGQVITIGNER | F | 236 | 3 | 750.0 | | 1 |
| 40 | S | **SLEKSYELPDGQVIT** | I | 236 | 2 | 839.9 | CB[g] (C-ter) | 2 |
| 41 | L | EKSYELPDGQVITIGNER | F | 238 | 3 | 683.3 | | 3 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 42 | L | **EKSYELPDGQVITIGN** | E | 238 | 2 | 881.9 | | 2 |
| 43 | E | KSYELPDGQVITIGNER | F | 239 | 2 | 959.9 | | 2 |
| 44 | E | KSYELPDGQVITIGN | E | 239 | 2 | 817.4 | | 2 |
| 45 | K | **SYELPDGQVITIGNERF** | R | 240 | 2 | 969.4 | CD[f] (N-ter) | 2 |
| 46 | K | **SYELPDGQVITIGNER** | F | 240 | 2 | 895.9 | CD[f] (N-ter) | 3 |
| 47 | Y | **ELPDGQVITIGNERF** | R | 242 | 2 | 844.4 | | 2 |
| 48 | Y | **ELPDGQVITIGNER** | F | 242 | 2 | 770.9 | | 3 |
| 49 | I | GMESAGIHETTYNS | I | 269 | 2 | 748.8 | | 1 |
| 50 | R | KDLYANNVMSGGTTM | Y | 292 | 2 | 801.3 | | 1 |
| 51 | M | **SGGTTMYPGIADRMQ** | K | 301 | 2 | 792.8 | | 1 |
| 52 | M | **SGGTTMYPGIADR** | M | 301 | 2 | 663.3 | | 3 |
| 53 | M | **SGGTTMYPGIAD** | R | 301 | 1 | 1169.5 | | 2 |
| 54 | W | **ITKQEYDEAGPSIVH** | R | 358 | 3 | 562.9 | CD[f] (N-ter) | 3 |
| 55 | W | **ITKQEYDEAGPS** | I | 358 | 2 | 669.3 | CD[f] (N-ter) | 3 |
| 56 | I | **TKQEYDEAGPSIVHRK** | C | 359 | 3 | 619.9 | | 2 |
| 57 | I | **TKQEYDEAGPSIVHR** | K | 359 | 3 | 577.2 | | 1 |
| 58 | I | **TKQEYDEAGPSIVH** | R | 359 | 2 | 787.3 | | 3 |
| 59 | I | **TKQEYDEAGPS** | I | 359 | 2 | 612.7 | | 2 |
| 60 | K | QEYDEAGPSIVH | R | 361 | 2 | 672.8 | | 3 |
| 61 | Q | **EYDEAGPSIVHR** | K | 362 | 3 | 452.2 | | 2 |
| 62 | Q | **EYDEAGPSIVH** | R | 362 | 2 | 608.7 | | 3 |
| 63 | E | **YDEAGPSIVHR** | K | 363 | 2 | 622.3 | CB[g] (N-ter) | 3 |
| 64 | E | **YDEAGPSIVH** | R | 363 | 2 | 544.2 | CB[g] (N-ter) | 2 |
| 65 | Y | **DEAGPSIVHR** | K | 364 | 2 | 540.7 | | 2 |
| 66 | E | AGPSIVHR | K | 366 | 2 | 418.7 | | 1 |

[a] Amino acid residue preceding the peptide sequence (N-terminus).
[b] Amino acid residue following the peptide sequence (C-terminus).
[c] Peptide sequence start in actin.
[d] Charge (+).
[e] Mass to charge ratio.
[f] Cathepsin D cleavage site.
[g] Cathepsin B cleavage site.
[f] Frequency of occurnace in the three replicates
Peptides that were also found in low pH dry fermented sausages after 28 days of ripening are indicted in bold.

**Table 3.4** Peptides of actin protein identified by LCMS[E] in low-pH dry fermented sausages after 28 days of ripening

| N. | $P_1$[a] | Peptide sequence | $P_1$',[b] | P. s. s.[c] | z[d] | m/z[e] | Info | Freq.[f] |
|---|---|---|---|---|---|---|---|---|
| 1 | C | DNGSGLVKAGFAGDDAPRA | V | 12 | 3 | 606.6 | CD[f] (N-ter) | 1 |
| 2 | C | DNGSGLVKAGFAGDDAPR | A | 12 | 3 | 582.9 | CD[f] (N-ter), CBD[h] (C-ter) | 2 |
| 3 | D | NGSGLVKAGFAGDDAPRAVFPSIVG | R | 13 | 3 | 801.4 | | 2 |
| 4 | D | NGSGLVKAGFAGDDAPRA | V | 13 | 3 | 568.2 | | 2 |
| 5 | D | NGSGLVKAGFAGDDAPR | A | 13 | 3 | 544.6 | CBD[h] (C-ter) | 3 |
| 6 | N | GSGLVKAGFAGDDAPRA | V | 14 | 3 | 530.2 | | 2 |
| 7 | N | GSGLVKAGFAGDDAPR | A | 14 | 3 | 500.5 | CBD[h] (C-ter) | 2 |
| 8 | G | LVKAGFAGDDAPRA | V | 17 | 3 | 463.2 | | 2 |
| 9 | G | LVKAGFAGDDAPR | A | 17 | 2 | 658.8 | CBD[h] (C-ter) | 2 |
| 10 | L | VKAGFAGDDAPR | A | 18 | 2 | 602.3 | CBD[h] (C-ter) | 2 |
| 11 | V | KAGFAGDDAPRA | V | 19 | 2 | 588.2 | | 2 |
| 12 | K | AGFAGDDAPRAVFPS | I | 20 | 2 | 739.3 | | 2 |
| 13 | K | AGFAGDDAPRA | V | 20 | 2 | 524.2 | | 3 |
| 14 | K | AGFAGDDAPR | A | 20 | 2 | 488.7 | CBD[h] (C-ter) | 2 |
| 15 | A | GFAGDDAPRAVFPS | I | 21 | 2 | 703.8 | | 2 |
| 16 | A | GFAGDDAPRA | V | 21 | 2 | 488.7 | | 2 |
| 17 | G | FAGDDAPRAVFPSIVGRPRHQG | V | 22 | 4 | 588.3 | CBD[h] (N-ter) | 1 |
| 18 | G | FAGDDAPRAVFPSIVGRPRH | Q | 22 | 3 | 722.3 | CBD[h] (N-ter) | 2 |
| 19 | G | FAGDDAPRAVFPSIVGRP | R | 22 | 3 | 624.6 | CBD[h] (N-ter) | 3 |
| 20 | G | FAGDDAPRAVFPSIVGR | P | 22 | 3 | 592.3 | CBD[h] (N-ter) | 1 |
| 21 | G | **FAGDDAPRAVFPSIVG** | R | 22 | 2 | 809.9 | CBD[h] (N-ter) | 3 |
| 22 | G | FAGDDAPRAVFPS | I | 22 | 2 | 675.3 | CBD[h] (N-ter) | 2 |
| 23 | G | FAGDDAPRAVFP | S | 22 | 2 | 631.8 | CBD[h] (N-ter) | 1 |
| 24 | G | FAGDDAPRAVF | P | 22 | 2 | 583.2 | CBD[h] (N-ter) | 2 |
| 25 | F | **AGDDAPRAVFPSIVGRP** | R | 23 | 3 | 575.6 | | 2 |
| 26 | F | **AGDDAPRAVFPSIVGR** | P | 23 | 3 | 543.2 | | 2 |
| 27 | F | **AGDDAPRAVFPSIVG** | R | 23 | 2 | 736.3 | | 3 |
| 28 | A | GDDAPRA | V | 24 | 1 | 683.3 | CB[g] (N-ter) | 2 |
| 29 | D | DAPRAVFPS | I | 26 | 2 | 480.2 | | 1 |
| 30 | A | VFPSIVGRPRHQG | V | 31 | 3 | 483.9 | | 1 |
| 31 | F | PSIVGRPRHQG | V | 33 | 2 | 602.3 | | 2 |
| 32 | R | HQGVMVGMGQK | D | 41 | 2 | 586.2 | | 1 |
| 33 | G | VMVGMGQKDS | Y | 44 | 2 | 526.2 | | 2 |
| 34 | V | GMGQKDSYVGDEAQSKRG | I | 47 | 3 | 638.2 | | 1 |
| 35 | G | MGQKDSYVGDEAQSKRGILT | L | 48 | 3 | 728.3 | CB[g] (C-ter) | 2 |
| 36 | G | **MGQKDSYVGDEAQSKRG** | I | 48 | 3 | 619.2 | | 2 |
| 37 | G | MGQKDSYVGDEAQSKR | G | 48 | 3 | 600.2 | | 2 |
| 38 | G | MGQKDSYVGDEAQSK | R | 48 | 3 | 548.2 | | 2 |
| 39 | G | **MGQKDSYVGDEAQ** | S | 48 | 2 | 714.3 | | 2 |
| 40 | M | **GQKDSYVGDEAQSKRG** | I | 49 | 3 | 575.6 | CB[g] (N-ter) | 2 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 41 | M | GQKDSYVGDEAQSKR | G | 49 | 3 | 556.5 | CB[g] (N-ter) | 1 |
| 42 | M | **GQKDSYVGDEAQSK** | R | 49 | 2 | 756.3 | CB[g] (N-ter) | 3 |
| 43 | D | SYVGDEAQSKR | G | 53 | 2 | 620.3 | | 2 |
| 44 | S | YVGDEAQSKRGILT | L | 54 | 2 | 768.9 | CB[g] (C-ter) | 3 |
| 45 | S | YVGDEAQSKRG | I | 54 | 2 | 605.2 | | 2 |
| 46 | S | YVGDEAQSKR | G | 54 | 2 | 576.7 | | 2 |
| 47 | Y | VGDEAQSKRG | I | 55 | 2 | 523.7 | | 2 |
| 48 | V | GDEAQSKRGILT | L | 56 | 2 | 637.8 | CB[g] (C-ter) | 2 |
| 49 | L | TLKYPIE | H | 67 | 2 | 432.2 | | 2 |
| 50 | H | **GIITNWDDMEK** | I | 75 | 2 | 661.3 | CB[g] (N-ter), CB[g] (C-ter) | 3 |
| 51 | G | IITNWDDMEKIWHHTF | Y | 76 | 3 | 695.9 | CB[g] (N-ter), CBD[h] (C-ter) | 2 |
| 52 | G | IITNWDDMEKIWHHT | F | 76 | 3 | 646.9 | CB[g] (N-ter), CD[f] (C-ter) | 2 |
| 53 | G | **IITNWDDMEK** | I | 76 | 2 | 632.8 | CB[g] (N-ter), CB[g] (C-ter) | 3 |
| 54 | I | ITNWDDMEKIWHHTF | Y | 77 | 3 | 658.3 | CBD[h] (C-ter), CD[f] (C-ter) | 2 |
| 55 | I | ITNWDDMEKIWHH | T | 77 | 3 | 575.5 | | 2 |
| 56 | I | **ITNWDDMEK** | I | 77 | 2 | 576.2 | CB[g] (C-ter) | 2 |
| 57 | I | TNWDDMEKIWHHTF | Y | 78 | 3 | 620.6 | CD[f] (N-ter), CBD[h] (C-ter) | 2 |
| 58 | I | **TNWDDMEKIWHHT** | F | 78 | 3 | 571.5 | CD[f] (N-ter), CD[f] (C-ter) | 3 |
| 59 | I | TNWDDMEKIWHH | T | 78 | 3 | 537.9 | CD[f] (N-ter) | 2 |
| 60 | I | TNWDDMEK | I | 78 | 2 | 519.7 | CD[f] (N-ter), CB[g] (C-ter) | 3 |
| 61 | T | NWDDMEKIWHHT | F | 79 | 3 | 537.9 | CD[f] (C-ter) | 2 |
| 62 | N | WDDMEKIWHHTF | Y | 80 | 3 | 548.9 | CBD[h] (C-ter) | 2 |
| 63 | N | WDDMEKIWHH | T | 80 | 3 | 466.2 | | 2 |
| 64 | W | **DDMEKIWHHT** | F | 81 | 2 | 656.2 | CD[f] (C-ter) | 3 |
| 65 | F | **YNELRVAPEEHPTL** | L | 92 | 3 | 556.6 | CBD[h] (N-ter) | 2 |
| 66 | F | **YNELRVAPEEHPT** | L | 92 | 3 | 518.9 | CBD[h] (N-ter) | 2 |
| 67 | F | **YNELRVAPEE** | H | 92 | 2 | 610.3 | CBD[h] (N-ter) | 2 |
| 68 | E | LRVAPEEHPTL | L | 95 | 2 | 631.3 | | 2 |
| 69 | E | LRVAPEEH | P | 95 | 2 | 475.7 | CD[f] (C-ter) | 2 |
| 70 | L | **RVAPEEHPTL** | L | 96 | 2 | 574.8 | | 3 |
| 71 | L | RVAPEEHPT | L | 96 | 2 | 518.2 | CB[g] (C-ter) | 2 |
| 72 | R | **VAPEEHPTLL** | T | 97 | 2 | 553.2 | CBD[h] (N-ter), CBD[h] (C-ter) | 2 |
| 73 | R | VAPEEHPT | L | 97 | 2 | 440.2 | CBD[h] (N-ter), CB[g] (C-ter) | 2 |
| 74 | A | PEEHPTL | L | 99 | 2 | 411.7 | | 2 |
| 75 | L | LTEAPLNPKAN | R | 106 | 2 | 584.3 | | 1 |
| 76 | L | TEAPLNPKANREKM | T | 107 | 3 | 533.6 | CBD[h] (N-ter) | 2 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 77 | L | TEAPLNPKANREK | M | 107 | 3 | 489.9 | CBD[h] (N-ter), CD[f] (C-ter) | 2 |
| 78 | L | TEAPLNPKANRE | K | 107 | 2 | 670.3 | CBD[h] (N-ter) | 2 |
| 79 | L | TEAPLNPKAN | R | 107 | 2 | 527.7 | CBD[h] (N-ter) | 1 |
| 80 | G | **IVLDSGDGVTHNVPIYEG** | Y | 152 | 2 | 942.9 | | 2 |
| 81 | G | IVLDSGDGVTHNVPIYE | G | 152 | 2 | 914.4 | | 1 |
| 82 | I | **VLDSGDGVTHNVPIYEG** | Y | 153 | 2 | 886.4 | CD[f] (N-ter) | 3 |
| 83 | I | **VLDSGDGVTHNVPIYE** | G | 153 | 2 | 857.9 | CD[f] (N-ter) | 2 |
| 84 | I | VLDSGDGVTHNVPIY | E | 153 | 2 | 793.3 | CD[f] (N-ter) | 1 |
| 85 | I | **VLDSGDGVTHNVP** | I | 153 | 2 | 655.3 | CD[f] (N-ter) | 1 |
| 86 | L | **DSGDGVTHNVPIYEG** | Y | 155 | 2 | 780.3 | CD[f] (N-ter) | 3 |
| 87 | L | DSGDGVTHNVPIYE | G | 155 | 2 | 751.8 | CD[f] (N-ter) | 2 |
| 88 | L | DSGDGVTHNVPIY | E | 155 | 2 | 687.3 | CD[f] (N-ter) | 2 |
| 89 | L | **DSGDGVTHNVP** | I | 155 | 2 | 549.2 | CD[f] (N-ter) | 2 |
| 90 | D | **SGDGVTHNVPIYEG** | Y | 156 | 2 | 722.8 | | 2 |
| 91 | D | SGDGVTHNVP | I | 156 | 2 | 491.7 | | 2 |
| 92 | S | **GDGVTHNVPIYEG** | Y | 157 | 2 | 679.3 | | 2 |
| 93 | G | **DGVTHNVPIYEG** | Y | 158 | 2 | 650.8 | | 2 |
| 94 | Y | ALPHAIMRL | D | 171 | 2 | 511.2 | | 2 |
| 95 | T | AASSSSLEKSYELPDGQVITIGN | E | 231 | 2 | 1183.5 | | 1 |
| 96 | A | ASSSSLEKSYELPDGQVIT | I | 232 | 2 | 1005.9 | CB[g] (C-ter) | 1 |
| 97 | S | SLEKSYELPDGQVITIGNERF | R | 236 | 3 | 799.0 | | 3 |
| 98 | S | SLEKSYELPDGQVITIGN | E | 236 | 2 | 982.0 | | 2 |
| 99 | S | **SLEKSYELPDGQVIT** | I | 236 | 2 | 839.9 | CB[g] (C-ter) | 3 |
| 100 | S | SLEKSYELPDGQVI | T | 236 | 2 | 789.4 | | 2 |
| 101 | S | LEKSYELPDGQVITIGNER | F | 237 | 3 | 721.0 | | 2 |
| 102 | S | LEKSYELPDGQVIT | I | 237 | 2 | 796.4 | CB[g] (C-ter) | 3 |
| 103 | L | **EKSYELPDGQVITIGN** | E | 238 | 2 | 881.9 | | 2 |
| 104 | L | EKSYELPDGQVIT | I | 238 | 2 | 739.8 | | 2 |
| 105 | K | **SYELPDGQVITIGNERF** | R | 240 | 2 | 969.4 | CD[f] (N-ter) | 1 |
| 106 | K | **SYELPDGQVITIGNER** | F | 240 | 2 | 895.9 | CD[f] (N-ter) | 1 |
| 107 | S | YELPDGQVITIGNERFR | C | 241 | 3 | 669.6 | | 1 |
| 108 | S | YELPDGQVITIGNERF | R | 241 | 2 | 925.9 | | 2 |
| 109 | S | YELPDGQVITIGNER | F | 241 | 2 | 852.4 | | 2 |
| 110 | S | YELPDGQVIT | I | 241 | 1 | 1134.5 | | 2 |
| 111 | Y | **ELPDGQVITIGNERF** | R | 242 | 2 | 844.4 | | 2 |
| 112 | Y | **ELPDGQVITIGNER** | F | 242 | 2 | 770.9 | | 3 |
| 113 | Y | ELPDGQVI | T | 242 | 1 | 870.4 | | 2 |
| 114 | E | TLFQPSF | I | 261 | 1 | 839.3 | | 1 |
| 115 | Y | NSIMKCDIDIRK | D | 281 | 2 | 718.3 | | 1 |
| 116 | D | LYANNVMSGGTTMYPGIADRMQKE | I | 294 | 3 | 883.0 | | 1 |
| 117 | A | NNVMSGGTTMYPGIADRMQKE | I | 297 | 3 | 767.3 | | 2 |
| 118 | A | NNVMSGGTTMYPGIADR | M | 297 | 2 | 892.4 | | 3 |
| 119 | M | SGGTTMYPGIADRMQKE | I | 301 | 3 | 614.6 | | 3 |
| 120 | M | SGGTTMYPGIADRMQK | E | 301 | 3 | 571.6 | | 2 |
| 121 | M | **SGGTTMYPGIADRMQ** | K | 301 | 2 | 792.8 | | 2 |
| 122 | M | **SGGTTMYPGIADR** | M | 301 | 2 | 663.3 | | 2 |
| 123 | M | **SGGTTMYPGIAD** | R | 301 | 1 | 1169.5 | | 2 |
| 124 | K | IIAPPERKYS | V | 330 | 2 | 587.3 | CB[g] (N-ter) | 1 |
| 125 | M | WITKQEYDEAGPSIVHRK | C | 357 | 4 | 540.0 | | 1 |
| 126 | M | WITKQEYDEAGPSIVHR | K | 357 | 3 | 677.0 | | 1 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 127 | M | WITKQEYDEAGPS | I | 357 | 2 | 762.3 | | 2 |
| 128 | W | ITKQEYDEAGPSIVHRK | C | 358 | 4 | 493.5 | CDf (N-ter) | 3 |
| 129 | W | ITKQEYDEAGPSIVHR | K | 358 | 3 | 614. | CDf (N-ter) | 2 |
| 130 | W | **ITKQEYDEAGPSIVH** | R | 358 | 3 | 562.9 | CDf (N-ter) | 2 |
| 131 | W | **ITKQEYDEAGPS** | I | 358 | 2 | 669.3 | CDf (N-ter) | 3 |
| 132 | I | **TKQEYDEAGPSIVHRK** | C | 359 | 3 | 619.9 | | 2 |
| 133 | I | **TKQEYDEAGPSIVHR** | K | 359 | 3 | 577.2 | | 2 |
| 134 | I | **TKQEYDEAGPSIVH** | R | 359 | 2 | 787.3 | | 3 |
| 135 | I | **TKQEYDEAGPS** | I | 359 | 2 | 612.7 | | 1 |
| 136 | Q | EYDEAGPSIVHRK | C | 362 | 2 | 750.8 | | 2 |
| 137 | Q | **EYDEAGPSIVHR** | K | 362 | 2 | 686.8 | | 2 |
| 138 | Q | **EYDEAGPSIVH** | R | 362 | 2 | 608.7 | | 2 |
| 139 | E | YDEAGPSIVHRK | C | 363 | 2 | 686.3 | CB[g] (N-ter) | 2 |
| 140 | E | **YDEAGPSIVHR** | K | 363 | 2 | 622.3 | CB[g] (N-ter) | 3 |
| 141 | E | **YDEAGPSIVH** | R | 363 | 2 | 544.2 | CB[g] (N-ter) | 2 |
| 142 | Y | DEAGPSIVHRK | C | 364 | 2 | 604.8 | | 1 |
| 143 | Y | **DEAGPSIVHR** | K | 364 | 2 | 540.7 | | 1 |
| 144 | E | AGPSIVH | R | 366 | 1 | 680.3 | | 1 |

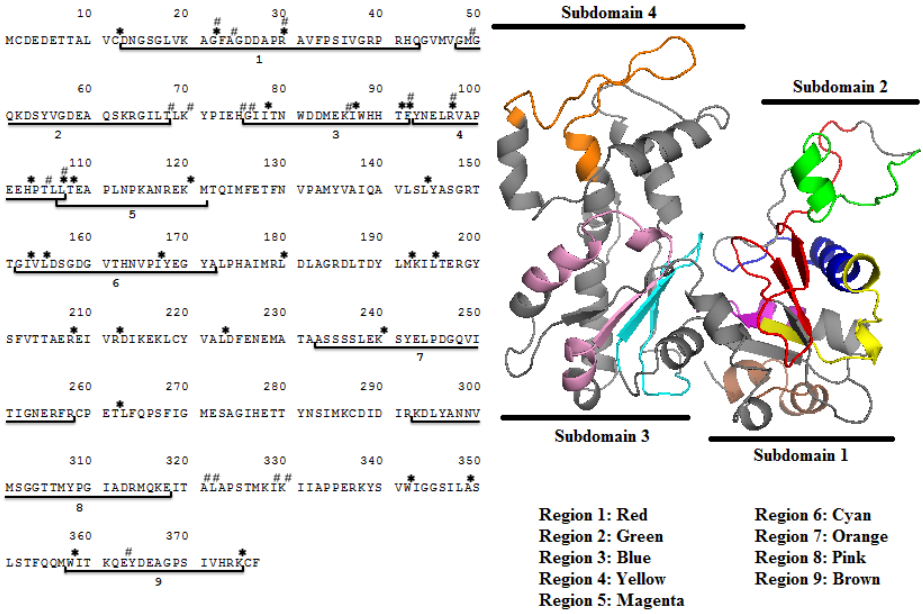
[a] Amino acid residue preceding the peptide sequence (N-terminus). [b] Amino acid residue following the peptide sequence (C-terminus). [c] Peptide sequence start in actin. [d] Charge (+). [e] Mass to charge ratio. [f] Cathepsin D cleavage site. [g] Cathepsin B cleavage site. [f] Frequency of occurnace in the three replicates. Peptides that were also found in high pH dry fermented sausages after 28 days of ripening are indicted in bold.

Based on the known specific cleavage sites of cathepsins B and D (Figure 3.1), an analysis of the proteolytic breakdown by the latter enzymes was done. At the end of fermentation, 19 and 17 peptides (on a total of 52 and 42) were cleaved off either from the N or C-terminus, at high and low pH, respectively (Tables 3.1 and 3.2). Throughout ripening, cleavage site analysis indicated that cathepsins B and D remained active leading to 29 and 67 identified peptides (on a total of 66 and 144) at high and low pH, respectively. The peptides $Thr_{108}$-$Lys_{120}$ and $Ile_{359}$-$Lys_{375}$ that were present in the low-pH samples were likely generated by cathepsin D. In addition, cathepsin D was probably responsible for the following cleavage sites: $Cys_{12}$-$Asp_{13}$, $Thr_{79}$-$Asn_{80}$, $Thr_{91}$-$Phe_{92}$, $Ile_{153}$-$Val_{154}$, $Leu_{155}$-$Asp_{156}$, $Lys_{240}$-$Ser_{241}$, $Trp_{358}$-$Ile_{359}$, $Thr_{91}$-$Phe_{92}$, $His_{103}$-$Pro_{104}$, and $Lys_{120}$-$Met_{121}$. According to the known cleavage sites for cathepsin B, this enzyme likely cleaved actin at the following sites: $Ala_{24}$-$Gly_{25}$, $Thr_{68}$-$Leu_{69}$, $Met_{49}$-$Gly_{50}$, $His_{75}$-$Gly_{76}$, $Gly_{76}$-$Ile_{77}$, $Lys_{86}$-$Ile_{87}$, $Thr_{105}$-$Leu_{106}$, $Thr_{251}$-$Ile_{252}$, $Lys_{330}$-$Ile_{331}$, and $Glu_{363}$-$Tyr_{364}$. Because of the occurrence of some common cleavage sites by both cathepsins, it was not possible to denote which of both enzymes mainly cleaved at the sites $Gly_{22}$-$Phe_{23}$, $Arg_{30}$-$Ala_{31}$, $Phe_{92}$-$Tyr_{93}$, $Arg_{97}$-$Val_{98}$, and $Leu_{107}$-$Thr_{108}$.

A lot of identified peptides were truncated differing only by a single amino acid. In particular for the peptides $Ile_{153}$-$Gly_{170}$, $Tyr_{242}$-$Arg_{258}$, and $Trp_{358}$-$His_{373}$, single amino acids were cleaved after already four days of fermentation and peptides were generated in both high-pH and low-pH sausages. In addition to the peptides reported above for the fermentation phase, the peptides $Met_{49}$-$Gly_{65}$ and $Ser_{237}$-$Arg_{256}$ (at high and low pH) as well as $Asp_{13}$-$Ala_{31}$ and $Phe_{23}$-

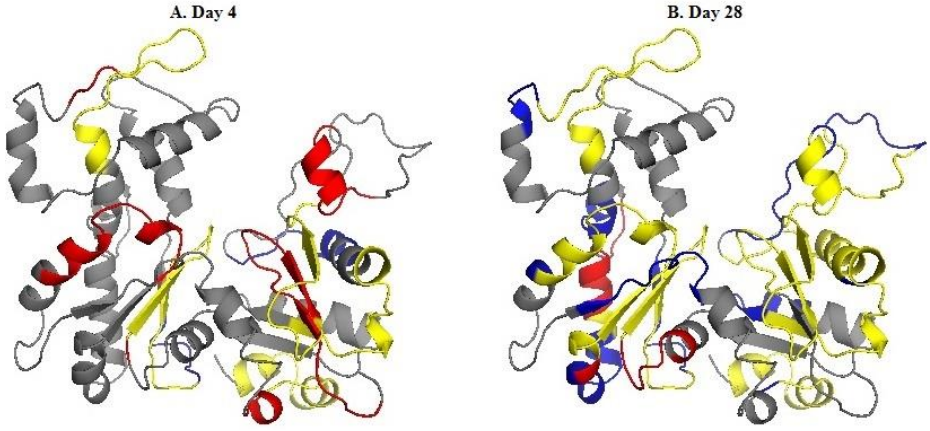
$Gly_{44}$ (at low pH only) were intensively hydrolysed at the end of the ripening phase. Similarly, several peptides were truncated differing by two or three amino acids, indicating the release of dipeptides and tripeptides. In the final products, the dipeptides Thr-Lys, Tyr-Ala, and Met-Gln were released in the high-pH sausages. The dipeptides and tripeptides Gln-Gly, Arg-His, Val-Phe, Met-Gln, Gly-Ser-Gly, Leu-Tyr-Arg, Ile-Val-Gly, Ile-Val-His, and Ile-Leu-Thr were released in the low-pH sausages.

**Figure 3.1** Actin sequence and 3D structure. The majority of the identified peptides were lying in the underlined regions.




* Cleavage sites of cathepsin D according to Hughes et al. (2000)
[#] Cleavage sites of cathepsin B according to Hughes et al. (1999)

**Figure 3.2** Peptides were mapped on the 3D structure. Peptides identified for high-pH and low-pH fermentations are shown in red and blue, respectively. Common peptides are shown in yellow.




## 3.5 DISCUSSION

Since actin is one of the most abundant proteins in muscle (Dominguez and Holmes, 2011), its degradation during ripening of dry fermented sausages is of interest for flavour formation. Breakdown of actin during ripening has been demonstrated previously, usually via electrophoresis techniques (Hughes et al., 2002). Yet, more detailed information on its degradation patterns requires state-of-the-art analysis via proteomic analysis. In the present paper, the small peptides generated from actin hydrolysis were identified by LCMS$^{E}$. The results showed that a high degree of proteolysis took place since the identified peptides covered more than 50 % of the actin sequence and peptides were identified from all four subdomains. In a recent study, Lopez et al. (2015) identified some peptides arising from actin in fermented sausage models. Several peptides

identified from that group originated from the regions 1, 3, and 7 that were also highlighted in the present study, confirming that those regions are susceptible to proteolysis.

It is evident that actin proteolysis started already during the fermentation phase: whereas no actin peptides were found in the raw meat, a more or less comparable peptide generation became already clear after four days of fermentation for both types of sausages. After 28 days of ripening, however, the number of peptides identified was more than twofold higher in the low-pH sausages. In contrast to the rather brief fermentation step, ripening lasted for a longer time and led to more pronounced pH differences (pH 4.9 *versus* pH 6.0 at the end of ripening). Demeyer et al. (2000) also reported lower actin degradation in sausages with higher pH and this might be due to the low optimum pH of cathepsin D like muscle enzymes, which play a major role in actin hydrolysis (Molly et al., 1997). In dry fermented sausages, proteolysis is generally divided in two steps: firstly, endopeptidases break down intact proteins generating small peptides; secondly, the generated peptides are further degraded by exopeptidases which release single amino acids, dipeptides, and tripeptides. The lysosomal cathepsins B and D are believed to be the main endopeptidases responsible for the first protein breakdown in dry fermented sausages (Molly et al., 1997). The identification of the cleavage sites of cathepsins B and D on bovine actin, which has the same sequence of pig actin, by Hughes et al. (1999, 2000) allowed to understand which peptides were likely generated by these enzymes. Indeed, some of these cleavage sites were also the starting point of several peptides identified at day 4 in the present study, supporting the finding

that cathepsins are already likely active during the first days of fermentation (Demeyer et al., 1992). The pH at the end of fermentation was in the activity range of cathepsin B and D in both treatments. Indeed, Schwartz and Bird (1977) reported that rabbit actin was degraded by cathepsins B and D at pH 5.0. These enzymes remain stable and active for several months during processing of dry cured meat products (Toldrà et al., 1993) and it is not surprising that the number of peptides likely generated by cathepsin B and D increased at the end of ripening in the present study. This was particularly evident in the low-pH treatment were the number of identified peptides greatly increased at the end of ripening. In the high-pH sausages, the contribution of these enzymes was more limited during the drying phase since their activity is very low at pH 6.0 (Schwartz and Bird, 1977). Although for some peptides the contribution of cathepsins B and D seems indisputable, this might be doubtful for other peptides. Indeed, in some cases the same peptide but with an additional residue after the cathepsin cleavage site was also identified. At this point, it is impossible to know whether the shorter peptide was the result of endopeptidase activity or if it was generated from the longer peptide by hydrolysis of the additional residue made by an exopeptidase.

The further degradation of peptides exerted by exopeptidases releases amino acids, dipeptides, and tripeptides from the N- and C-terminus. These enzymes, in dry fermented sausages, are either of muscle or microbial origin (Mora et al., 2015). In the present study, the activity of aminopeptidases and carboxypeptidases was evident since a lot of identified peptides were truncated differing by a single amino acid. There are five aminopeptidases known to be

active post-mortem: leucyl, arginyl, alanyl, pyroglutamyl, and methionyl aminopeptidases (Toldrá, 2006). Ile, Leu, Tyr, and Gly were probably released by alanyl aminopeptidase (Flores et al., 1996). Methionyl aminopeptidase mainly releases lysine, alanine and leucine (Flores et al., 2000) and arginyl aminopeptidase releases basic amino acids (Flores et al. 1993). These aminopeptidases have optimum activities at neutral pH and only retain some activity at pH 5.0 (Toldrá, 2006). Nevertheless, the action of exopeptidases generated more unique peptides at low pH. We suggest that some endopeptidases like cathepsins, which have optimum activity at very acidic pH, provided more substrates to exopeptidases at low pH. From the C-terminus, the activity of carboxypeptidases was also evident, although knowledge about these enzymes in meat products is still limited. There are two lysosomal carboxypeptidases (A and B) known to have optimal activity at acidic pH, with the former cleaving hydrophobic amino acids and the latter having a wider activity (Toldrá, 2006). Several peptides were also truncated differing by two or three amino acids, indicating that dipeptidyl, such as DPP I and DPP II (Sentandreu and Toldrá, 2000, 2001), and tripeptidyl peptidases were active during ripening.

In addition to the above, the contribution of microbial enzymes cannot be ignored. In the present study, all sausages were inoculated with *L. sakei*. This species, which has often been isolated from spontaneously fermented dry fermented sausages, is often used as starter culture for its high competitiveness and ability to produce antimicrobial compounds with strong antilisterial activity (Leroy and De Vuyst, 2005; Ravyts et al., 2012). Moreover, the background

microbiota that is usually constituted of coagulase-negative microbiota may also be relevant for proteolytic activity, as well as for the further conversion of amino acids in aroma compounds (Sánchez Mainar et al., 2017). It is generally believed that meat endogenous enzymes initiate proteolysis and the contribution of microbial enzymes is relevant only in a later stage of ripening (Hughes et al., 2002; Molly et al., 1997). The species *L. sakei* has been shown to possess some proteolytic activity (Candogan and Acton, 2004), which was in particular reported to release Leu and Ala from peptides (Sanz and Toldrá, 1997b).

Peptides, especially the ones with a low molecular mass < 5000 Da, are potential flavour compounds and flavour precursors. The results of the present study revealed that numerous peptides of low molecular weight are generated by actin degradation, especially at low pH conditions. Therefore, actin might be a key protein in determining the final taste characteristics in dry fermented sausages considering the high abundance of this protein.

## 3.6 CONCLUSIONS

In the present study, the generation of peptides from actin, which is one of the most abundant proteins in muscle, was studied during ripening of dry fermented sausages through $LCMS^E$. The understanding of proteolysis is of great importance since small peptides and amino acids influence the sensory characteristics of these products. The results showed that actin is already being hydrolysed during the fermentation phase, but proteolysis is most intense during the drying phase. In addition, a more acid pH profile, characteristic of Northern-

European type fermented sausages, generated more unique peptides. The relevance of the findings of the present study will need to be confirmed and contrasted with the analysis of proteolytic products of other major muscle proteins in future research.

# CHAPTER 4

## THE EFFECT OF PROTEIN OXIDATION ON PROTEOLYSIS IN A MEAT MODEL SYSTEM

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Redrafted after Berardo A., Claeys E., Vossen E., Leroy F., and De Smet S. (2015). Protein oxidation affects proteolysis in a meat model system. Meat Science 106, 78-84.

## 4.1 ABSTRACT

The effect of hydrogen peroxide-induced protein oxidation and pH (4.8 and 5.2) on meat proteolysis was investigated in a meat model system for dry fermented sausages. In oxidized samples, increased protein carbonyl contents and decreased thiol concentrations were found. The initial concentration of protein carbonyls was significantly lower in oxidized samples at pH 4.8 than in ones at pH 5.2, but after ten days comparable levels were reached. The inhibition of proteolysis by the addition of a protease inhibitor cocktail did not influence protein oxidation. Yet, proteolysis was negatively affected by low pH values as well as by oxidation, resulting in a reduced release of amino acids during ripening.

## 4.2 INTRODUCTION

The pH drop obtained during fermentation of dry fermented sausages provokes protein denaturation and enhances the activity of some important proteolytic enzymes (Astiasaran et al., 1990; Molly et al., 1997). Endogenous exopeptidases and endopeptidases are the main enzymes responsible for proteolysis in dry fermented sausages, while bacterial proteolytic enzymes seem to play a less pronounced role (Hierro et al., 1999, Toldrá et al. 2000). Several authors have indicated cathepsins as the most active endopeptidases involved in proteolysis in cured meat products (Demeyer et al., 1992; Molly et al., 1997; Toldrá et al., 1993; Verplaetse et al., 1992).

Besides the impact of acidification and proteolysis, fermented sausage production is affected by oxidation processes. Lipid oxidation might impair sensory quality since high levels of MDA correlate with rancid taste (Wood et al., 2008). The oxidative stability of dry fermented sausages is determined by the balance between pro-oxidant and antioxidant factors. Additives, like sodium chloride, exert pro-oxidant effects (Ruiz, 2007), whereas SA and nitrite might have either pro-oxidant or antioxidant activities. Myoglobin, abundantly present in meat, also exerts pro-oxidant effects (Carlsen and Skibsted, 2004). Moreover, some lactic acid bacteria used in fermented products produce hydrogen peroxide which is a strong oxidizer (O'Toole and Yuan, 2006). In contrast, meat-associated catalase-positive cocci, which are added as starter cultures or which are naturally present in the sausage batter, may neutralize peroxides (Ravyts et al., 2012). To a certain extent, protein breakdown taking place during the

ripening period may improve the oxidative stability since small peptides present higher antioxidant properties than intact proteins (Freitas et al., 2013).

Contradictory effects of protein oxidation on proteolysis were reported. On the one hand, the increased hydrophobicity due to oxidation favours the recognition and the subsequent degradation of oxidized proteins by proteases (Pacifici et al., 1993, Davies., 2001). This occurs in mild oxidative conditions, in which the proteolytic susceptibility of myosin heavy chain increases by the action of oxygen radicals (Xue et al., 2012). On the other hand, intense oxidative conditions generate cross-links between proteins so that the resulting aggregates are poor substrates for proteases (Pacifici et al., 1993). Moreover, the direct oxidation of proteolytic enzymes impairs their activity (Rowe et al., 2004), with cysteine proteases being highly susceptible (Lametsch et al., 2008).

To the best of our knowledge, the influence of oxidation on proteolysis in dry fermented sausages has not been studied before. Sausage preparation processes, like meat grinding and the consequent exposure to oxygen as well as the addition of sodium chloride might trigger protein oxidation and affect proteolysis. Yet, the understanding of how physico-chemical changes occurring in dry fermented sausages, including pH drop and proteolysis, interact with protein oxidation may enable strategies to control its negative effects. Therefore, the aim of this study was to investigate protein oxidation in a meat model system for dry fermented sausages and to assess its effect on proteolysis, and conversely the effects of pH and proteolysis on protein oxidation.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Sausage model preparation

The experimental set-up was a 2 × 2 × 2 full factorial design with two pH values installed, induction or not of oxidation, and the addition or not of a protease inhibitor cocktail. The experimental set-up was repeated twice at two different days and each time a batch of sausage models was prepared. Lean pork from shoulder muscles, which contained 4.5 % fat upon analysis by the ISO 1444-1973 method, was ground through a 3-mm plate and mixed with the curing agents sodium chloride (2.5 %, m/m), SN (0.015 %, m/m), and SA (0.05 %, m/m). The batch was subsequently divided in sub-batches for the different treatments. The batch was first divided in two equal parts and the pH was set at 5.20 ± 0.10 in the first batch and at 4.80 ± 0.10 in the second batch, in both cases by adding lactic acid. The pH remained in the ranges of 5.20 ± 0.10 and 4.80 ± 0.10 during ripening. The batches were further split in sub-batches for the oxidation treatment, control (C) versus oxidized (O), and for the addition or not of a protease inhibitor cocktail.

Oxidation was induced by adding hydrogen peroxide before the stuffing (12 µL/g meat of a 6 % hydrogen peroxide solution). Based on a preliminary test, the concentration of hydrogen peroxide was chosen to increase protein carbonylation to about 6 nmol/mg at pH 5.2. The cocktail of protease inhibitors was made by mixing pepstatin A (60 µM) and E-64 (1.4 mM). Pepstatin A was dissolved in ethanol and subsequently mixed with E-64 which had been previously dissolved in water (1:1, v/v). The protease inhibitor cocktail was

added at 0.02 mL per gram of meat. A solution containing ethanol and water (1:1, v/v) replaced the protease inhibitor cocktail in batches where it was not added.

The meat mixtures were stuffed into falcon tubes of 50 mL. Samples were taken after 0, 5 and 10 days of incubation at 22 °C. The length was chosen to allow sufficient proteolysis in order to mimic the first days of ripening.

### 4.3.2 Sarcoplasmic protein solubility

Sarcoplasmic protein solubility was measured in a low ionic strength solution (150 mM NaCl), as described previously (Claeys et al., 2002), and was expressed in mg soluble protein per gram of meat. Three grams of meat were homogenised in 30 mL of 150 mM NaCl and 0.01 mM iodo-acetic acid. The samples were centrifuged and filtered. The protein concentration of the supernatant, assumed to contain the soluble sarcoplasmic protein fraction, was determined using the biuret method.

### 4.3.3 Protein carbonyl content

The protein carbonyl content was determined by derivatization with DNPH as described by Levine et al. (1994) with some modifications. Three grams of meat with 30 mL of sodium phosphate buffer (20 mM, pH 6.5 containing 0.6 M NaCl) were homogenized and four aliquots of 0.2 mL were treated with 1 mL ice-cold trichloroacetic acid (TCA) (10 %) to precipitate the proteins. After centrifugation the supernatant was discarded and two aliquots were treated with 0.5 mL of 10 mM DNPH dissolved in 2.0 M HCl and two

aliquots were treated with 0.5 mL of 2.0 M HCl (blank). After 1 h of reaction, 0.5 mL of ice cold 20 % TCA was added. The samples were then centrifuged and supernatant discarded. Excess DNPH was removed by washing three times with 1 mL of ethanol:ethylacetate (1:1, v/v). The pellets were dissolved in 1 mL of 6.0 M guanidine hydrochloride in 20 mM phosphate buffer (pH 6.5). The carbonyl concentration (nmol/mg protein) was calculated from the absorbance at 280 nm and 370 nm of the samples using the following equation (Levine et al., 1994):

$$\frac{C_{hydrazone}}{C_{protein}} = \frac{A_{370}}{\varepsilon_{hydrazone,370} \times (A_{280} - A_{370} \times 0.43)} \times 10^6$$

Where $\varepsilon_{hydrazone,370}$ is 22000 $M^{-1}$ $cm^{-1}$ and the carbonyl concentrations obtained from the blanks were subtracted from the corresponding treated sample.

### 4.3.4 Thiol concentration

The thiol concentration was determined after derivatization by DTNB adopted from Jongberg et al. (2013). Two grams of frozen meat were homogenized in 50 mL of 5 % SDS in tris(hydroxymethyl)aminomethane (TRIS) buffer (pH 8.0) and incubated for 30 min in a water bath at 80 °C. The homogenate was centrifuged to eliminate insoluble particles. Two millilitres of 0.1 M TRIS buffer (pH 8) and 0.5 mL of 10 mM DTNB dissolved in TRIS buffer were added to 0.5 mL of supernatant. For each sample, a blank was included containing 0.5 mL of supernatant and 2.5 mL of TRIS buffer. A solution containing 0.5 mL of 5.0 % SDS in TRIS buffer, 0.5 mL of 10 mM DTNB, and 2.0

mL of TRIS buffer was used as reagent blank. All mixtures were protected against light and allowed to react for exactly 30 min. The absorbance was measured spectrophotometrically at 412 nm and the thiol concentration was calculated using the formula of Lambert-Beer ($\varepsilon_{412}$ = 14000 $M^{-1}$ $cm^{-1}$) and expressed in nmol thiols/mg protein. The protein concentration of the blank was determined spectrophotometrically at 280 nm using a bovine serum albumin (BSA) standard curve.

### 4.3.5 Electrophoresis

About 2.5 g of meat was homogenized in 50 mL of a 0.01 M imidazole buffer (pH 7.0), containing 2 % SDS, and kept at 95 °C for 10 min to dissolve proteins. After cooling to room temperature, protein solutions were centrifuged and filtered. After determination of protein concentration (Kjeldahl), solutions were diluted to obtain 3 mg of crude protein per mL and were divided in two equal aliquots. In one aliquot, 2 % of 2-mercaptoethanol was added to investigate proteolysis. In the aliquot without 2-mercaptoethanol, disulphide bonds remained intact, which was used to follow protein oxidation. Protein solutions were frozen and preserved at -18 °C until electrophoresis.

Gels were prepared according to Greaser et al. (1983). The separating gel contained 8.0 % of total (T) acrylamide plus N,N'-methylene-bis-acrylamide, with a cross-linking of 0.5 %, containing 0.1% of SDS at pH 8.8. The stacking gel contained 3.0 % of total (T) acrylamide plus N,N'-methylene-bis-acrylamide, with a cross-linking of 4.76 %, containing 0.1 % of SDS at pH 6.8.

Per well, 10 µL (30 µg protein) was loaded. The gels were mounted in a

vertical slab gel apparatus (Hoefer Scientific Instruments, SE 600 series), using constant power supply ECPS 2000/300 (Pharmacia, Sweden) for electrophoresis. Electrophoresis was performed at constant 20 mA and continued until the bromo-phenol blue front reached the bottom of the gel. The staining of the proteins was carried out by immersing the gels overnight in a solution containing 0.1 % of Coomassie brilliant blue R250 dissolved in a 20 % methanol and 2 % phosphoric acid (v/v) solution at room temperature.

After destaining in fresh fixing solution, the gels were scanned using a Bio-Rad computing densitometer model CDS-100, and the peak intensity was recorded.

### 4.3.6 Free and peptide-bound α-$NH_2$-N

Free and peptide-bound α-$NH_2$-N were determined according to the method described by Oddy (1974). Briefly, 1 g of frozen meat was homogenized in 20 mL of 0.6 M perchloric acid (PCA). The homogenate was centrifuged and filtered. For the total α-$NH_2$-N, 2 mL of PCA extract was mixed with 5 mL of HCl (8.4 M) and incubated at 100 °C for 24 h for hydrolysis. After hydrolysis, solutions were neutralised and diluted to 50 mL.

One mL of a buffer solution (25% propionic acid, 25% methyl cellosolve(2-methoxyethanol), pH 5.8) containing 0.5 % of ninhydrin (pH 5.8) and 100 µL of AA solution (50 mg AA was dissolved in 50 ml water), was added to either 0.2 mL of the PCA extract + 0.8 mL of water (for free α-$NH_2$-N) or 1 mL of the neutralized hydrolysed extract (for total α-$NH_2$-N). A solution containing 1 mL of distilled water, 1 mL of 0.5 % ninhydrin buffer solution (pH 5.8) and 100 µL

of 50 % AA solution was used as blank. All mixtures were left in boiling water for 20 min and, after cooling to room temperature, 5 mL of a 60 % ethanol solution was added. The absorbance was measured spectrophotometrically at 570 nm. Results were calculated as mg α-$NH_2$-N per 100 g of dry matter (ISO 1442-1973), by comparing with solutions of *L*-leucine of known concentration, used as a standard and treated the same way. The peptide-bound α-$NH_2$-N contents were calculated by subtracting the free α-$NH_2$-N from the total α-$NH_2$-N.

### 4.3.7 Cathepsin B+L and cathepsin D activities

Cathepsin B+L activity was determined fluorometrically (Fluoroscan Ascent FL, Thermo Scientific) with the common substrate N-CBZ-l-phenylalanyl-l-arginine-7-amido-4-methylcoumarin at 37°C as previously described (Uytterhaegen et al., 1992) with some modifications. Briefly, 3 g of meat was homogenised in 25 mL cold (2°C) 0.1 M citrate buffer pH 5.0, containing 0.2% Triton X100, and centrifuged (4000 g; 15 min.). Twenty-five µl of the filtered supernatant was mixed with 225 µl of 30 µM N-CBZ-l-phenylalanyl-l-arginine-7-amido-4-methylcoumarin in 0.1 M phosphate buffer (pH 6) containing 0.07 % Brij 35 and 1.29 mg/mL cysteine-HCl. The excitation wavelength was set at 335 nm and emission was recorded at 460 nm. Data were collected every minute for 20 minutes. The slope of the resulting line, describing the relationship between the time and the fluorescence readings, is a measure for the activity. Results are expressed as pmol 7-amino-4-methylcoumarin (NHMec) released per min and per g of meat.

Cathepsin D was extracted and its activity was assayed as previously

described by Claeys et al. (2001). Results are expressed as µg hemoglobin hydrolysed per min and per g of meat.

### 4.3.8 Statistical analysis

Data were analysed using the general linear model ANOVA procedure with the fixed effects of treatment (C and O), protease inhibitors (addition or not), and pH (4.8 and 5.2). Free and peptide-bound α-NH2-N data and cathepsin B+L and cathepsin D activities data were analysed using the general linear model ANOVA procedure with the fixed effects of treatment (C and O) and pH (4.8 and 5.2). In this case data from samples containing inhibitors were not considered. The 2-way interaction terms were only included in the model when significant ($P < 0.05$). The data at days 0, 5, and 10 were analysed separately. Tukey-adjusted post hoc tests were performed for all pairwise comparisons. *P*-values < 0.05 were considered significant. All the statistical analyses were carried out by SAS Enterprise guide 6.

## 4.4 RESULTS

### 4.4.1 Sarcoplasmic protein solubility

The solubility of sarcoplasmic proteins was assessed in a low ionic strength solution. The main effects of pH, oxidation treatment and use of inhibitors on protein solubility were all significant, whereas the interaction terms were not (Table 4.1). The sarcoplasmic protein solubility was almost 20 % lower in sausages at pH 4.8 than in ones at pH 5.2. The oxidation treatment resulted in an approximately 10 % lower protein solubility in comparison with the control treatment. In addition, samples treated with protease inhibitors displayed lower protein solubility. However, whereas the pH and oxidation treatments immediately exerted an effect on protein solubility, the addition of protease inhibitors revealed its effect only from day 5 of ripening on.

**Table 4.1** Effect of pH, induced oxidation and protease inhibitors on sarcoplasmic protein solubility (mg soluble protein/g meat) during ripening in a sausage model.

| | pH | | Oxidation treatment (O) | | Protease Inhibitors (PI) | | RMSE[1] | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 4.8 | 5.2 | Control | Oxida. | No | Yes | | pH | O | PI |
| Day 0 | 21.3 | 26.4 | 25.0 | 22.7 | 23.9 | 23.8 | 1.291 | <0.001 | 0.003 | 0.785 |
| Day 5 | 18.5 | 22.7 | 21.8 | 19.4 | 22.9 | 18.3 | 0.764 | <0.001 | <0.001 | <0.001 |
| Day 10 | 19.1 | 22.9 | 22.5 | 19.6 | 23.9 | 18.1 | 1.652 | <0.001 | 0.004 | <0.001 |

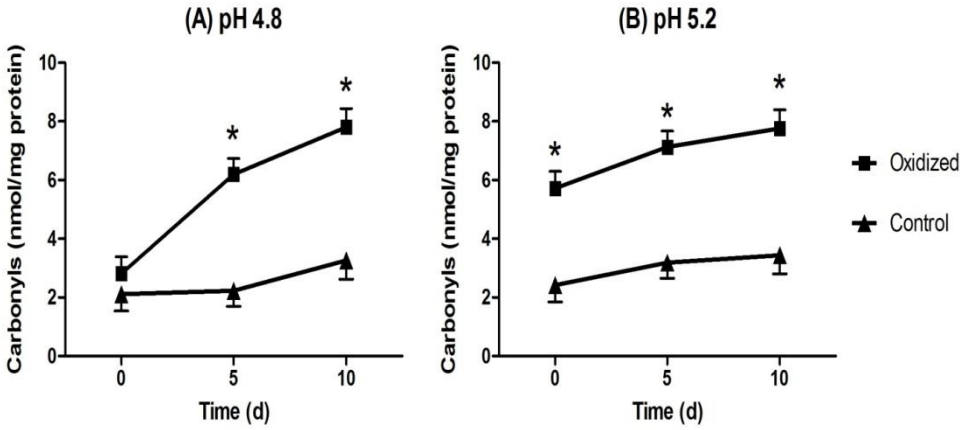
[1] Root mean square error

### 4.4.2 Protein oxidation

Protein oxidation was quantified by estimation of the protein carbonyl content (Figure 4.1) and thiol concentrations (Figure 4.2), as well as by protein electrophoresis (Figure 4.3). The pH and oxidation treatments significantly affected carbonyls and thiol concentrations, whereas the addition of the protease inhibitor cocktail did not significantly influence protein oxidation.

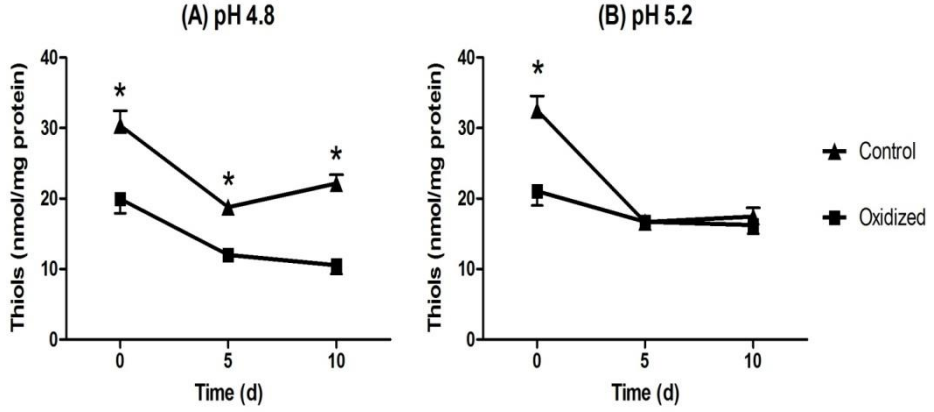
In the control treatment, carbonyl contents were restricted to values between 2.0 and 3.5 nm DNPH/mg protein, while the oxidation treatment always had significantly higher values, except for the sausages at day 0 and pH 4.8 (Figure 4.1), reflected in a significant pH × oxidation interaction term for that particular sampling moment. The control treatment displayed a slight but not significant increase in carbonyl content during ripening whereas the oxidation treatment resulted in a significant increase in carbonyl contents at day 5 of ripening compared to day 0.

**Figure 4.1** Effect of treatment on carbonyls in sausage models during ripening at pH 4.8 (A) and 5.2 (B).



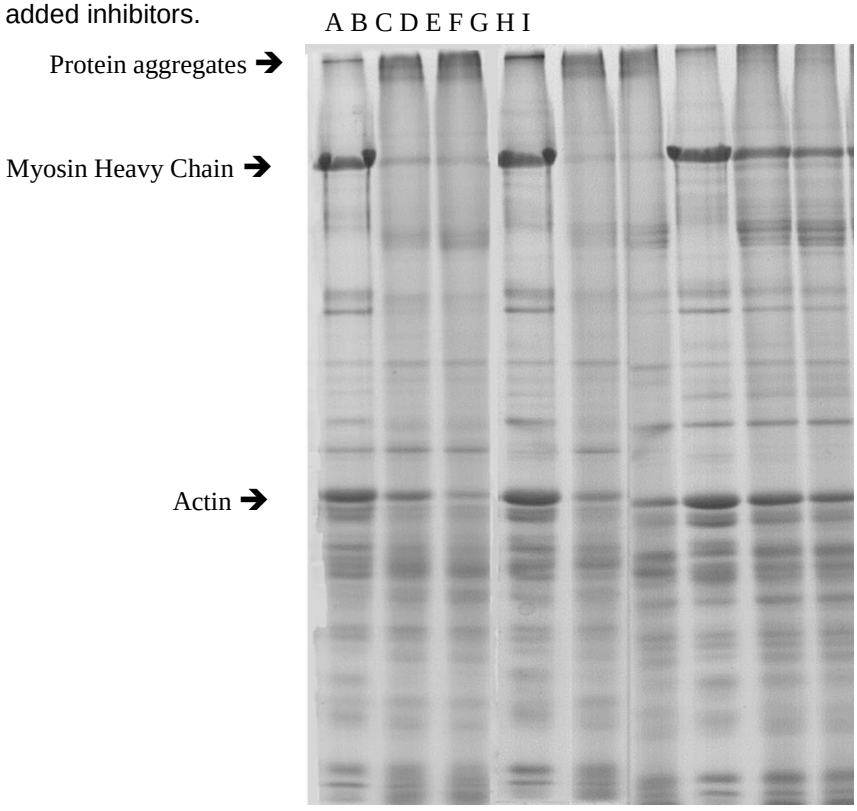

Error bars represent standard errors of the mean values.
* denotes significant difference between oxidized and control.

**Figure 4.2** Effect of treatment on thiol concentration in sausage models during ripening at pH 4.8 (A) and 5.2 (B).




Error bars represent standard errors of the mean values.
* denotes significant difference between oxidized and control.

**Figure 4.3** Electrophoresis patterns of protein solutions. A, B and C: control treatments at days 0, 5, 10, respectively (not treated with 2-mercaptoethanol). D, E and F: oxidation treatments at days 0, 5, and 10, respectively (not treated with 2-mercaptoethanol). G, H and I: oxidation treatments at days 0, 5, and 10, respectively (treated with 2-mercaptoethanol). All depicted samples originated from the experiment at pH 5.2 without added inhibitors.

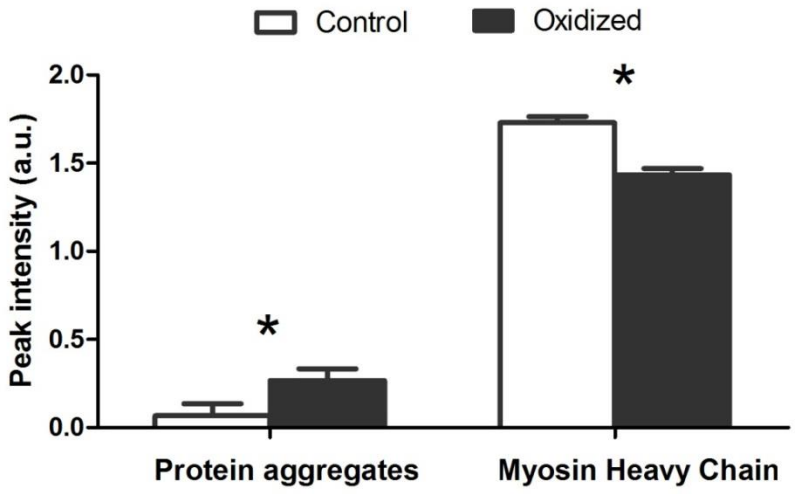



The initial thiol concentration (day 0) was significantly lower in the oxidation treatment than in the control treatment (Figure 4.2). Later on, after five days of ripening, all treatments displayed a significant loss of thiol groups. At pH 5.2, samples of the oxidation and control treatments converged to similar values,

both at days 5 and 10. For pH 4.8, however, samples from the oxidation treatment displayed considerably lower amounts of thiol groups after 5 and 10 days, than samples from the control treatment (significant pH × oxidation interaction term).

Finally, the electrophoresis patterns of proteins not treated with 2-mercaptoethanol displayed differences with respect to the oxidation treatment (Figure 4.3). The electrophoresis patterns were comparable for both pH values; therefore, only the ones obtained at pH 5.2 are shown. At day 0, oxidized samples (D band) showed a thicker band at the top of the gel (protein aggregates) in comparison with samples from the non-oxidized sausages. The peak intensities of protein aggregates and myosin heavy chain in control and oxidized samples at day 0 were measured and are shown in Figure 4.4. Protein aggregates were significantly higher in oxidized samples than in the control samples. On the contrary, the latter presented higher amount of myosin heavy chain. During ripening, proteins displayed a decreased intensity of all bands and the appearance of a broader band at the top of the gel. When oxidized proteins were treated with 2-mercaptoethanol (G, H, I bands), a disulphide bond breaker, the intensity of the upper band (aggregates) clearly decreased and the lower bands were recovered.

**Figure 4.4** Peak intensities of Protein aggregates and Myosin Heavy Chain in control and oxidized samples at day 0.




* denotes significant difference between oxidized and control.

**Table 4.2** Effect of induction of oxidation and pH on free α-$NH_2$-N (mg α-$NH_2$-N / 100 g dry matter) during ripening in a sausage model (samples in absence of inhibitors).

| | Oxidation treatment (O) | | pH | | RMSE[1] | *P*-value | |
|---|---|---|---|---|---|---|---|
| | Control | Oxidation | 4.8 | 5.2 | | O | pH |
| Day 0 | 269 | 269 | 250 | 288 | 38 | 0.989 | 0.266 |
| Day 5 | 390 | 333 | 337 | 386 | 24 | 0.027 | 0.033 |
| Day 10 | 520 | 381 | 403 | 497 | 51 | 0.009 | 0.048 |

[1] Root mean square error

### 4.4.3 Proteolysis

Sausage models prepared with the addition of inhibitors did not show any significant increase of free and peptide-bound α-$NH_2$-N during ripening (data not shown). On the contrary, free and peptide-bound α-$NH_2$-N significantly increased in samples in the absence of inhibitors during ripening, both in the control and oxidation treatments (Table 4.2). At day 0, both treatments had similar values for free α-$NH_2$-N, whereas at days 5 and 10 the oxidation treatment had significantly lower values compared to the control treatment. In contrast, peptide-bound α-$NH_2$-N did not show significant differences between the two treatments (data not shown). The samples at pH 4.8 had significantly lower values of free α-$NH_2$-N than the ones at pH 5.2, at all time points.

The electrophoresis patterns of proteins treated with 2-mercaptoethanol are reported in Figure 4.5. The electrophoresis patterns were comparable for both pH values; therefore, only the ones obtained at pH 5.2 are shown. In fermented sausage models with active endogenous proteases, the formation of new bands during ripening was found below the position of myosin heavy chain. When using protease inhibitors, no changes in the patterns were found during ripening.

Results of cathepsin D activity are reported in Figure 4.6. At day 0 control and oxidized samples at pH 5.2 presented, although not significantly, lower cathepsin D activities than the counterparts at pH 4.8. At day 5 and 10 of ripening significantly lower cathepsin D activity was recorded in oxidized samples at pH 5.2 compared to control samples at both pH values, illustrating the significant pH x treatment interaction term at these sampling days.

Cathepsin B+L activity is shown in Figure 4.7. The oxidized samples showed a strongly reduced cathepsin B+L activity at day 0 and almost no activity at day 5 and 10. At day 0 and 10, control samples at pH 4.8 reported a significantly lower cathepsin B+L activity than control samples at pH 5.2.

**Figure 4.5** Electrophoresis patterns of protein solutions treated with 2-mercaptoethanol. A, B, and C: samples treated with inhibitors at days 0, 5, and 10, respectively. D, E, and F: samples not treated with inhibitors at days 0, 5, and 10, respectively. All depicted samples originated from the experiment at pH 5.2 without oxidation treatment.

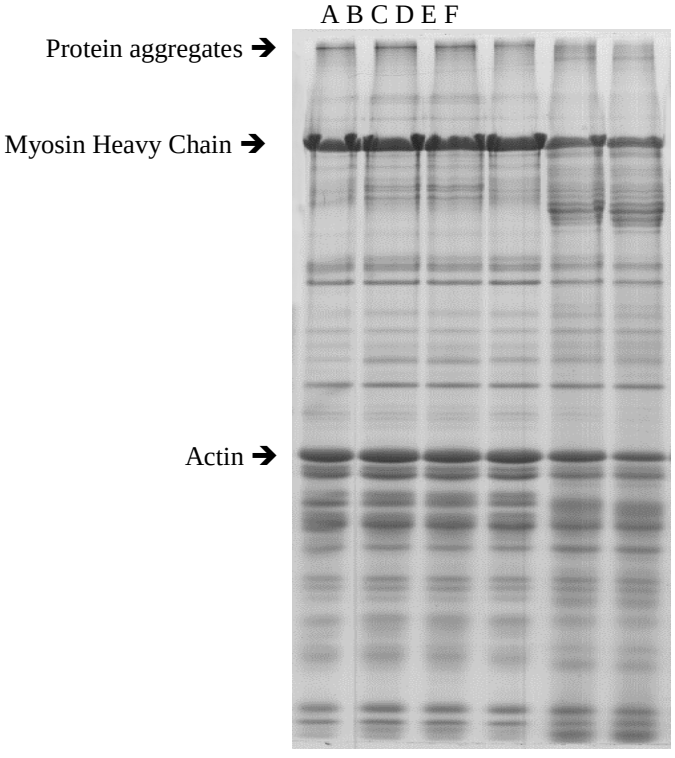
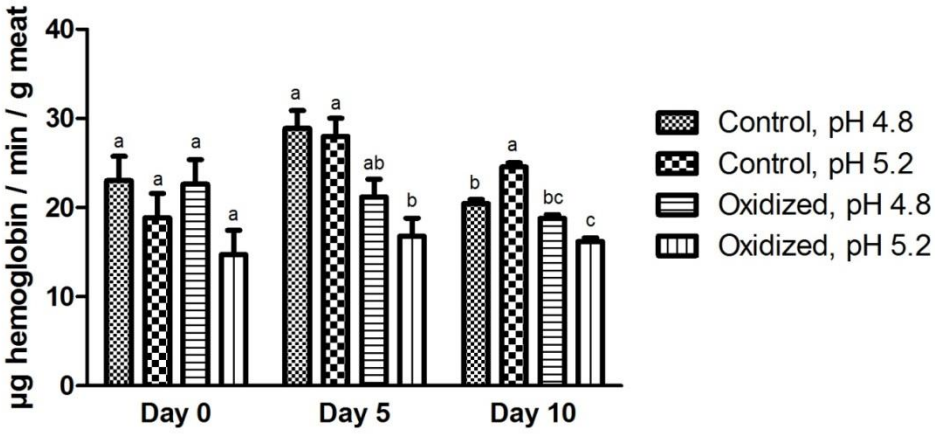
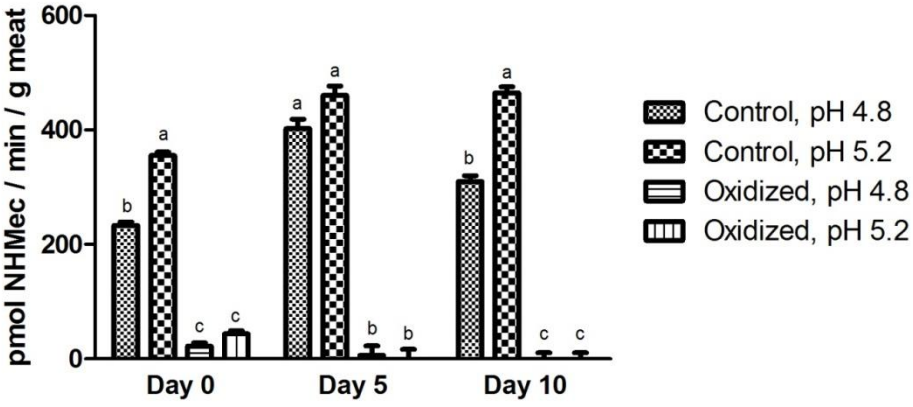


**Figure 4.6** Cathepsin D activity.




Error bars represent standard errors of the mean values.
Different letters (a, b, c) denote significant differences within sampling time.

**Figure 4.7** Cathepsin B+L activity.




Error bars represent standard errors of the mean values.
Different letters (a, b, c) denote significant differences within sampling time.

## 4.5 DISCUSSION

Proteolysis is an important process characteristic of meat fermentation, in particular during the first two weeks of ripening (Beriain et al., 2000; Casaburi et al., 2008). The electrophoresis patterns obtained in the present study underline this aspect, matching earlier observations in dry fermented pork sausages (Defernando and Fox, 1991). The occurrence of proteolysis was evident from the increase in both free and peptide-bound α-$NH_2$-N, reflecting the activity of amino peptidases and endopeptidases, respectively. Increases of free and peptide-bound α-$NH_2$-N were also found by Dierick et al. (1974) during the first nine days of dry fermented sausage ripening.

Little is known about the potential effects of proteolysis on protein oxidation in fermented meats. Small peptides may present antioxidant activity, which depends on their composition, structure and hydrophobicity (Sarmadi and Ismail, 2010). The antioxidant activity is exerted in different pathways: inactivation of reactive oxygen species (ROS), scavenging of free radicals, and chelating of transition metals (Broncano et al., 2011; Sarmadi and Ismail, 2010). The properties of peptides are in turn determined by protease specificity. In the present study, the addition of a protease inhibitor cocktail lowered protein solubility as a measure of proteolysis, but this did not result in differences in oxidative stability, even though proteolysis was successfully blocked at the level of both endopeptidases and exopeptidases. We therefore hypothesize that the active endogenous proteases either generated peptides with no antioxidant activity or that the antioxidant peptide concentration may not have been high

enough to exert detectable effects. On the other hand, radicals may have easier access to susceptible amino acids in meat peptides so that an effect of proteolysis on the susceptibility to oxidation cannot be excluded. However, this should result in an increased content of oxidation products, which was not observed. Additional experiments and analyses are required to independently check and quantify the antioxidant activity of muscle peptides and their susceptibility to oxidation in this type of product.

Vice versa, another objective of the present study was to elucidate if protein oxidation can affect proteolysis during meat fermentation. In meat, protein oxidation can be triggered by hydrogen peroxide, iron, or myoglobin. The reaction between iron and hydrogen peroxide forms highly reactive hydroxyl radicals. Yet, myoglobin can also react with hydrogen peroxide generating hypervalent myoglobin species (Lund et al., 2011). Metal-catalysed oxidation was reported to provoke increases of carbonyls, although formation of cross-links can also occur (Lund et al., 2011). Similarly, myoglobin-oxidizing systems generate protein carbonyls and cross-links (Lund et al., 2011; Estevez, 2011). Moreover, nitrite and hydrogen peroxide form peroxynitrite which is a strong oxidant. Consistently, the peroxide-induced samples in the present study indeed showed higher carbonyl contents and a loss of thiol groups, which was also accompanied by decreased protein solubility.

To the best of our knowledge, this is the first study indicating that the induction of protein oxidation reduces proteolysis in a model for fermented sausages. It has been suggested that a certain degree of oxidation may favour proteolysis, since increased protein hydrophobicity might facilitate the

degradation by proteases (Pacifici et al., 1993, Davies, 2001) and carbonylated proteins seem to be more susceptible to proteasome-driven proteolysis (Nyström, 2005). However, the present study indicates the opposite effect. Indeed, the induced oxidation decreased the activity of proteases. In particular, the activity of cathepsin B+L was completely stopped. These results show that these cysteine cathepsins are highly susceptible to oxidation. Similarly, Lametsch et al. (2008) reported that oxidation forms a disulphide bond within the μ-calpain active site, which inhibits the activity of that cysteine protease. On the contrary, the activity of cathepsin D was only slightly decreased suggesting a high resistance of this aspartic protease towards oxidation.

Moreover, protein oxidation generated protein aggregates through the formation of disulphide bonds, which were clearly visible in the upper part of the electrophoresis patterns of the oxidised samples. Similarly, Park et al. (2006) observed formation of protein aggregates on top of the electrophoresis patterns of oxidized myofibrillar proteins. In the same study, a decreased intensity of myosin heavy chain following oxidation was reported, which is confirmed in the present study. Lund et al. (2007) identified the high molecular weight on top of the gel as cross-linked myosin heavy chain. Consequently, protein aggregates, which have low proteolytic susceptibility, might also undergo limited proteolysis. Indeed, severe oxidation favours the formation of protein aggregates impeding the recognition of proteins by proteolytic enzymes (Pacifici et al., 1993). In particular, the formation of covalent bonds, *e.g.*, disulphide and dityrosine bonds, normally occurring during oxidation, induces proteins to cluster and precipitate (Chao et al., 1997; Davies and Delsignore, 1987; Meucci et al., 1991).

As a result of diminished proteolysis, the release of amino acids during ripening decreased in the oxidized samples. This may be of importance as amino acids can be converted by meat-associated catalase-positive cocci into aroma compounds (Ravyts et al., 2012), or even serve as alternative energy substrates for both the lactic acid bacteria and catalase-positive cocci (Janssens et al., 2014; Rimaux et al., 2010). Yet, amino acids can also be converted into aldehydes through the Strecker degradation. Villaverde et al. (2014b) hypothesized that protein carbonyls might also play a role in the formation of Strecker aldehydes.

As proteolysis is also affected by the overall fermentation and thus acidification process, the effect of pH also needs consideration. Therefore, two different pH levels were chemically imposed (pH 5.2 and 4.8) on the sausage batter, corresponding to typical pH values reached after fermentation in Southern-European type and Northern-European type fermented sausages, respectively. At pH 4.8, lower sarcoplasmic protein solubility was found, reflecting a higher protein denaturation. Indeed, a decrease of protein solubility usually occurs in dry fermented sausages during the fermentation step (Astiasaran et al., 1990; Klement et al., 1974). Therefore, it may be assumed that more residues were exposed to oxidation at low pH, leading to, among others, a higher protein carbonylation. Surprisingly, protein carbonylation in the oxidized samples was retarded during ripening at pH 4.8. Possibly, the lactic acid used to drop the pH might have played a role due to its potential antioxidant activity (Groussard et al., 2000). The higher amount of lactic acid needed for the samples at pH 4.8 might explain the lower carbonylation at day 0. Despite this

delayed protein oxidation, proteolysis was indifferently reduced in samples at pH 5.2 and pH 4.8, following induced oxidation. A pH of 5.2 also implied a higher release of amino acids during ripening. Verplaetse et al. (1992) reported similar results in sausages were the pH was dropped by using glucono-delta-lactone since the optimum activity of the major aminopeptidases in meat is at neutral pH (Toldrá et al., 1992). Moreover, the cathepsin B+L activity was higher at pH 5.2. Indeed, the optimum pH for cathepsins B and L are 5.5-6.5 and approximately 5, respectively (Geesink and Veiseth, 2009). Since cathepsin B can also act as an exopeptidase (Geesink and Veiseth, 2009), it might have increased the amount of free amino acids in those samples.

## 4.6 CONCLUSIONS

In summary, oxidation of proteins may reduce protein breakdown during dry fermented sausage ripening. Besides the negative effects of protein oxidation in terms of reduced oxidative stability, the lower amounts of free amino acids may negatively affect the sensorial qualities, as they are important for flavour development upon conversion by the meat microbiota and as a source of Strecker aldehydes. Further research is required to evaluate and quantify the antioxidant properties of the peptides generated by proteolysis and their susceptibility to oxidation, as well as the actual impact of the decreased proteolysis due to oxidation on quality deterioration.

# CHAPTER 5

## EFFECT OF SODIUM ASCORBATE AND SODIUM NITRITE ON PROTEIN AND LIPID OXIDATION IN DRY FERMENTED SAUSAGES

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Redrafted after Berardo A., De Maere H., Stavropoulou D. A., Rysman T., Leroy F., and De Smet S. (2016). Differential effects of sodium ascorbate and sodium nitrite on protein and lipid oxidation in dry fermented sausages. Meat Science, 121, 359-364.

## 5.1 ABSTRACT

The effects of SN and ascorbate on lipid and protein oxidation were studied during the ripening process of dry fermented sausages. Samples were taken at day 0, 2, 8, 14, 21 and 28 of ripening to assess lipid (MDA) and protein (carbonyls and sulfhydryl groups) oxidation. SA and nitrite were separately able to reduce the formation of MDA. Their combined addition resulted in higher amounts of carbonyl compounds compared to their separate addition or the treatment without any of both compounds. Moreover, SN limited the formation of GGS whereas SA showed a pro-oxidant effect. A loss of thiol groups was observed during ripening, which was not affected by the use of SA nor SN. In conclusion, SN and ascorbate affected protein and lipid oxidation in different manners. The possible pro-oxidant effect of their combined addition on carbonyl formation might influence the technological and sensory properties of these products.

## 5.2 INTRODUCTION

The stable character of dry fermented sausages is largely due to a combination of salting, bacterial acidification, drying and sometimes smoking. The salting process includes the addition of sodium chloride, nitrate and/or nitrite salts, and ascorbate salts. Nitrite and ascorbate salts are basic ingredients in fermented meat products. Nitrite can also be bacterially derived from nitrate (Sánchez Mainar and Leroy, 2015). In combination, these ingredients develop the desired red colour (Alley et al., 1992) and the cured flavour in fermented products (Toldrà et al., 2009). Moreover, nitrite exerts antimicrobial activity (Cassens, 1990).

The chemistry of nitrite and ascorbate in processed meat products is complex and not fully understood yet. Although nitrite is a natural electron acceptor and hence a potential oxidizing agent (Villaverde et al., 2014a), the ability of this compound to prevent lipid oxidation in meat products is well established (Balev et al., 2005; Zanardi et al., 2004). Ascorbate is also involved in redox reactions; this compound is an electron donor and its oxidized form dehydroascorbic acid is relatively unreactive and therefore ascorbate terminates the propagation of free radical reaction (Bendich et al., 1986). Nevertheless, ascorbate can act as pro-oxidant in the presence of metal ions. Indeed, its ability to reduce metal ions promotes the generation of reactive oxygen species through the Fenton reaction (Villaverde et al., 2014a). Hence, it has been shown that the use of ascorbate salts in processed meats inhibits lipid oxidation (Balev et al., 2005), but pro-oxidant effects have been reported as well (Haak et al.,

2009).

Proteins together with lipids are important constituents of meat products and undergo oxidation too. However, the effects of nitrite and ascorbate on protein oxidation have been much less investigated. Protein oxidation is potentially important for meat fermentation since it implies modifications at the protein level which can alter the structure and functionality of proteins, compromising their technological and sensory properties (Lund et al., 2011). Firstly, the reaction between lipid oxidation products and protein amines generates Schiff bases which may affect colour and flavour (Chelh et al., 2007). Secondly, oxidation of proteolytic enzymes may compromise their activity and indirectly influence the flavour (Berardo et al., 2015). Thirdly, the formation of crosslinks between proteins that are affected by protein oxidation may affect the texture of fermented sausages, in particular with respect to gelation (Zhou et al., 2014).

Considering the above-mentioned knowledge gap, the aim of the present study was to investigate the effects of SN and SA on the oxidation of both lipids and proteins during ripening in dry fermented sausages.

## 5.3. MATERIALS AND METHODS

### 5.3.1 Dry fermented sausage preparation

Dry fermented sausages were prepared mixing lean pork (70.5%), pork backfat (27.0%), sodium chloride (2.5%) and a starter culture containing a mixture of *Lactobacillus sakei* CTC 494, *Staphylococcus carnosus* 833 and

*Staphylococcus xylosus* 2S7-2 (Ravyts et al., 2010; Janssens et al., 2014). The initial load for the three starter cultures was $10^6$ (CFU/g). SA and SN were added according to a 2×2 factorial design with the following four treatments: 1) a control treatment without SA and SN (Control); 2) SA added at 500 mg/kg without SN (SA treatment); 3) SN added at 150 mg/kg without SA (SN treatment); 4) SA added at 500 mg/kg and SN at 150 mg/kg (SA+SN treatment). The batter was stuffed into collagen casings of 50 mm diameter (Naturin, Weinheim, Germany) to make dry fermented sausages of about 200 g. Ripening lasted for 28 days in a climate chamber (Kerres Anlagensysteme GmbH, Backnang, Germany). During the first two days, fermentation was performed at a temperature of 24 °C and a relative humidity of 94 %. For the drying process, the temperature was dropped to 12 °C and relative humidity was set to 82 % after the first two weeks. Samples were taken after 0 (day of production), 2 (end of fermentation), 8, 14, 21, and 28 (end of ripening) days. At each sampling day, one sausage per treatment was taken for analysis (except for pH and weight loss which were measured in three sausages per treatment throughout the ripening as described below). The manufacturing process was repeated once on a separate day, resulting in two independent replicate batches and samples.

### 5.3.2 pH and weight loss

In each manufacturing process, three randomly selected sausages per treatment were weighed and the pH was recorded after their preparation and during ripening. The pH was measured directly in the sausages [ISO 2917 (1999)] and the pH meter was calibrated in buffers of pH 4.0 and 7.0. Weight

loss was expressed as a percentage of the initial weight and the mean of the three records was calculated.

### 5.3.3 Residual AA

Residual AA was determined through high-performance liquid chromatography (HPLC) based on the reaction of DHAA with orthophenylenediamine (OPD) as described by Doolaege et al. (2012). Briefly, AA and DHAA were extracted using methanol/water (5/95; v/v) containing 0.1 M citric acid and 0.2 mM EDTA. DHAA is able to react with OPD but AA needs to be converted into DHAA first, using active carbon. By measuring total DHAA (i.e. present DHAA and DHAA formed from converted AA), and DHAA present in the samples, the AA concentration was calculated. Samples were analysed by reversed phase HPLC [150 × 4.6 mm Nucleosil 100 C18 column (3 µm) (Grace Davison Discovery Sciences, Lokeren, Belgium)] with fluorimetric detection (Agilent, Waldbronn, Germany) using excitation and emission wavelengths of 350 and 430 nm, respectively. The mobile phase was a mixture of methanol/water (5/95; v/v), containing 5 mM cetrimide and 50 mM $KH_2PO_4$ (pH 4.6). The elution was performed at a flow rate of 1.0 mL/min. Quantification was done by comparison of peak areas with those obtained from a standard solution of converted AA. Results were expressed in mg AA/kg sample.

### 5.3.4 Residual nitrite

Residual nitrite was determined according to ISO Standard 2918. Briefly, 4 g of sample was homogenized in 50 mL NaOH 0.02 M with 0.2 g of active

carbon and incubated for 2 hours in a shaking water bath at 80 °C. Then, 5 mL of $ZnSO_4$ was added and the homogenate was cooled to room temperature. The homogenate was then centrifuged at 1670 × *g* for 5 min. The supernatant was diluted to 100 mL with NaOH 0.02 M and filtered through a folded paper filter. Hundred µL of colour reagent A (0.2 g N-1-naftylethyleendiamine.2HCl in 150 mL 15% acetic acid) and 100 µL of colour reagent B (0.5 g sulfanilamide in 100 mL 15% acetic acid and 5 mL HCl 12 M) were mixed to 2.5 mL of supernatant or to 2.5 mL NaOH 0.02 M (blank). The absorbance was measured at 538 nm after 15 min. Residual nitrite in mg $NaNO_2$/kg of sample was calculated using a standard curve.

### 5.3.5 Lipid oxidation

Lipid oxidation was determined spectrophotometrically by measuring thiobarbituric acid-reactive substances (TBARS) as described by Doolaege et al. (2012). In brief, 5 g of meat was homogenised in 40.0 mL $HClO_4$ (0.6 M) and 1.0 mL butylated hydroxytoluene (BHT) solution. The homogenate was filtered and 5.0 mL was transferred in heat resistant glass test tubes together with 1 mL of TBA reagent. The resulting solutions were put in a boiling water bath for 35 min. They were subsequently cooled to room temperature and the absorbance was measured at 532 nm. Lipid oxidation was calculated using a standard curve and expressed as mg (MDA ) / kg sample.

### 5.3.6 Protein carbonyl content and thiol concentration of myofibrillar proteins

Protein carbonyl content and thiol concentration of myofibrillar proteins were assessed according to the methods described in Chapter 4.

### 5.3.7 Determination of GGS

Samples were prepared for ultra-performance liquid chromatography (UPLC) analysis of GGS according to Utrera et al. (2011) with some modifications. The vacuum-packed meat was thawed before 2.5 g of meat was homogenized in 30 mL of cold isolation buffer (10 mM sodium phosphate buffer, 0.1 M NaCl, 2 mM $MgCl_2$, and 1 mM ethylene glycol tetraacetic acid (EGTA), pH 6.5) using an Ultra Turrax (IKA, Staufen, Germany). Four aliquots of 0.2 mL were dispensed in 2 mL Eppendorf tubes. Proteins were precipitated with 1 mL of ice cold 20% TCA followed by centrifugation at 3000 x *g* for 30 min. The resulting pellets were treated again with 1.5 mL of ice cold 5% TCA followed by centrifugation at 5000 x *g* for 5 min. Pellets were treated with 0.5 mL of 250 2-(N-morpholino) ethanesulfonic acid (MES) buffer at pH 6.0 containing 1% SDS and 1 mM diethylenetriaminepentaacetic acid (DTPA). Two aliquots were treated with 0.5 mL of 50 mM ABA in 250 mM MES buffer (pH 6.0) and two aliquots were treated with 0.5 mL of 250 mM MES buffer (pH 6.0) as a blank. To create a reductive environment, an aliquot of 0.25 mL of 100 mM $NaCNBH_3$ in 250 mM MES buffer (pH 6.0) was added to all test tubes. The derivatization was completed by allowing the mixture to react for 90 min, while tubes were incubated at 37 °C and stirred regularly. The derivatization reaction was stopped

by adding 0.5 mL of ice cold 50% TCA followed by centrifugation (10000 x *g*, 10 min). Pellets were then washed twice with 1 mL of 10% TCA and 1 mL of ethanol:diethyl ether (1:1). Centrifugations at 8000 x *g* for 5 min were performed after each washing step. Following the final wash, the blank pellets were dissolved in 1.0 mL of 6 M guanidine hydrochloride in 20 mM phosphate buffer (pH 6.5) and placed on a rocking table for 30 min. After final centrifugation (3800 x *g*, 10 min) to remove insoluble material, the protein concentration was determined spectrophotometrically at 280 nm using an eight-point standard curve prepared from BSA. For the ABA-treated samples, protein hydrolysis was performed in 1.5 mL of 6 M HCl at 110 °C for 18 h. After that, hydrolysates were dried *in vacuo* at 45 °C using a SpeedVac (Thermo Fisher Scientific Inc., Waltham, MA, USA). Hydrolysates were finally reconstituted with 1 mL of Milli-Q water and filtered through a 0.22 µm Millex-HV Syringe filter (Millipore Corporation, Bedford, MA, USA). The GGS-ABA standard was prepared according to the procedure of Akagawa et al. (2006). Samples and standards were analysed using a Waters ACQUITY UPLC H-Class system with fluorescence detector (Waters Corporation, Milford, MA, USA). The UPLC system was equipped with an AccQ-Tag Ultra C 18 column (1.7 µm, 2.1 × 100 mm) (Waters Corporation). Eluent A and B were 5 mM sodium acetate buffer (pH 5.4) and acetonitrile, respectively, and a gradient was programmed by varying the eluent B from 0% to 8% in 3 min. The injection volume was 2.5 µL, the flow rate was kept constant at 0.5 mL/min, and the oven temperature was set at 30 °C. Excitation and emission wavelengths were set at 283 and 350 nm, respectively. The GGS-ABA peaks were identified by comparing the retention

time with that of the standard, and were manually integrated and plotted in an ABA standard curve ranging from 0.1 to 5 µM ($R^2$ > 0.999). Results were expressed as nmol GGS per mg protein.

### 5.3.8 Statistical analysis

All data were analysed using a repeated measures general linear model procedure with the fixed effects of addition or not of SA and SN, their interaction term, day of ripening, and the random effect of manufacturing batch. Tukey-adjusted *post hoc* tests were performed for all pairwise comparisons of means. P values < 0.05 were considered significant. All the statistical analyses were carried out by SAS Enterprise guide 6 (SAS Institute, Cary, NC, USA).

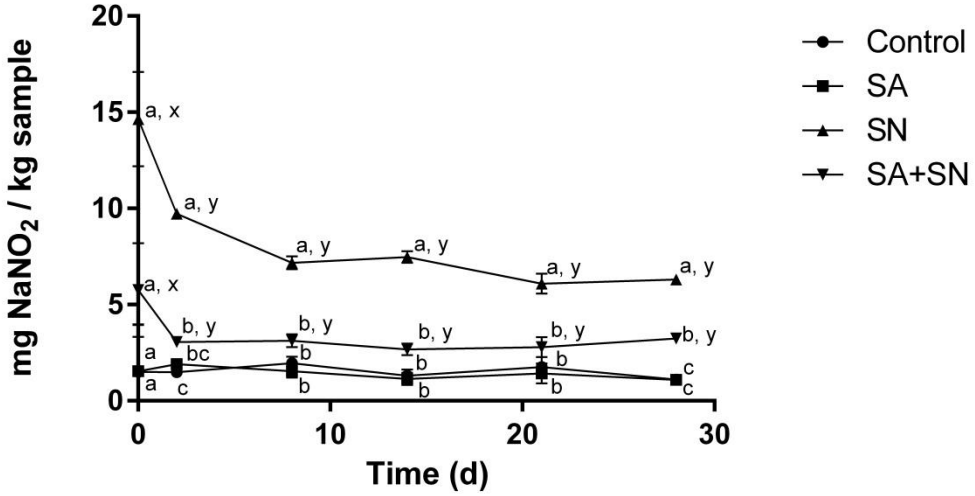
## 5.4 RESULTS AND DISCUSSION

The dry fermented sausages that served as study objects had an initial pH ranging from 5.6 to 5.8, which decreased to values ranging from 5.3 to 5.5 after two days of mild fermentation. During the drying phase, the pH gradually increased by 0.3 to 0.4 units and no significant differences were recorded among treatments. The weight loss of the final products was about 30% of the initial weight, without significant differences among treatments. The obtained weight loss and mild pH decrease were representative for Southern-European type dry fermented sausages (Demeyer et al., 2000).

### 5.4.1 Residual nitrite and ascorbate

Nitrite and ascorbate are essential additives in dry fermented sausages and they are involved in diverse reactions. In this study, the initial residual nitrite (day 0) was present in very low amounts compared to the ingoing dose (less than 10 % and 4 % in the SN and SA+SN treatments, respectively; Figure 5.1), confirming the high reactivity of this compound. Specifically, nitrite is partially oxidized to nitrate, partially lost as gas and another part reacts with lipids, proteins and metals (Cassens et al., 1979). Moreover, nitrite reacts with AA (Honikel, 2008), which may explain the lower amount found in the SA+SN treatment compared to the SN treatment, although the difference between these treatments was not significant. At the end of fermentation and during the drying phase, residual nitrite showed a significant difference between the SN and SA+SN treatments (less than 7 % and 3 % of the ingoing dose at the end of fermentation, respectively). In the treatments without added SN, only traces of nitrite were detected (less than 2 mg/kg).

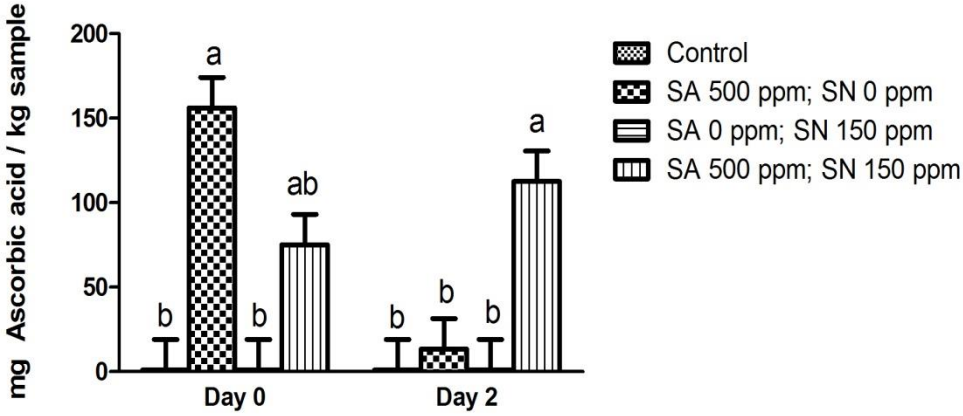
**Figure 5.1** Residual nitrite in dry fermented sausages during ripening. Error bars represent standard errors of the mean values. The a, b, c and x, y superscripts denote significant differences among means within sampling days and among means during ripening, respectively. Limit of quantification: 3 mg/kg (Iammarino et al., 2016).




Unsurprisingly, residual AA was not detected in sausages prepared without SA (Figure 5.2). In the SA treatment, the level of residual AA at day 0 was about 31 % of the added amount. The residual level of AA was twofold lower at day 0 in the SA+SN treatment than in the SA treatment (P = 0.10; 75 and 156 mg AA/kg sample, respectively). Its reaction with nitrite in the SA+SN treatment might have speeded up its oxidation to DHAA (Izumi et al., 1989). However, the contrary was observed at the end of fermentation, whereby the SA treatment tended to have 9-fold lower amounts of AA than the SA+SN treatment (P=0.09; 13 and 112 mg AA/kg sample, respectively). We speculate that the

anticipated higher oxidative stability of the SA+SN treatment due to nitrite addition, especially against lipid oxidation (see lipid oxidation results below), might have prevented further involvement of AA in oxidative reactions. The residual AA concentration from day 8 on was not consistent for the two replicates of the manufacturing process in the SA treatment since low amounts (values ranging between 10 and 50 mg AA/kg sample) were detected in the samples of the first batch, whereas no AA was detected in the samples of the second one. The SA+SN treatment showed values of residual AA ranging between 20 and 60 mg AA/kg sample from day 8 on in both replicates.

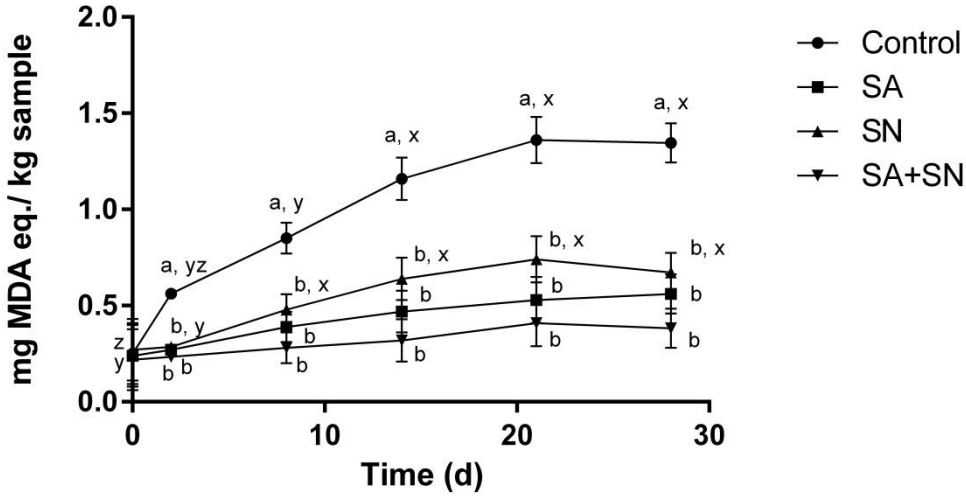
**Figure 5.2** Residual AA in dry fermented sausages during ripening. Error bars represent standard errors of the mean values. The a, b superscripts denote significant differences among means within sampling days.



### 5.4.2 Lipid oxidation

Oxidative reactions take place during ripening of dry fermented sausages and the oxidation of lipids generates aldehydes (Wójciak et al., 2012). In this study, the SA, SN and SA+SN treatments had significantly lower MDA equivalents compared to the control treatment throughout the ripening process (Figure 5.3). The combined addition of nitrite and ascorbate, although not significantly, resulted in a further slight decrease of MDA equivalents compared to the separate addition of nitrite or ascorbate. Ascorbic acid has the ability to scavenge reactive oxygen species and radicals (Bendich et al., 1986). Moreover, although AA is a hydrophilic compound, its antioxidant activity exerted in the water phase prevents the oxidation of the lipid component (Bendich et al., 1986). Balev et al. (2005) reported the antioxidant effects of AA against lipid oxidation in dry fermented sausages, but addition of AA lower than 500 mg/kg might convert AA into a pro-oxidant agent (Haak et al., 2009). The lipid antioxidant activity of nitrite is well known in meat products and often reported (Zanardi et al., 2004). Nitrite can limit the oxidation of lipids and the consequent formation of aldehydes in several ways. Indeed, nitrite blocks the pro-oxidant activity of iron by stabilizing heme iron and sequestering free iron (Bergamaschi and Pizza, 2011). Nitric oxide can be oxidized to $NO_2$ sequestering oxygen molecules (Honikel, 2008). The most important antioxidant pathway, however, seems to be due to the solubility of nitric oxide in fats where it reacts with lipid radicals and breaks the oxidative chain reaction (Skibsted, 2011).

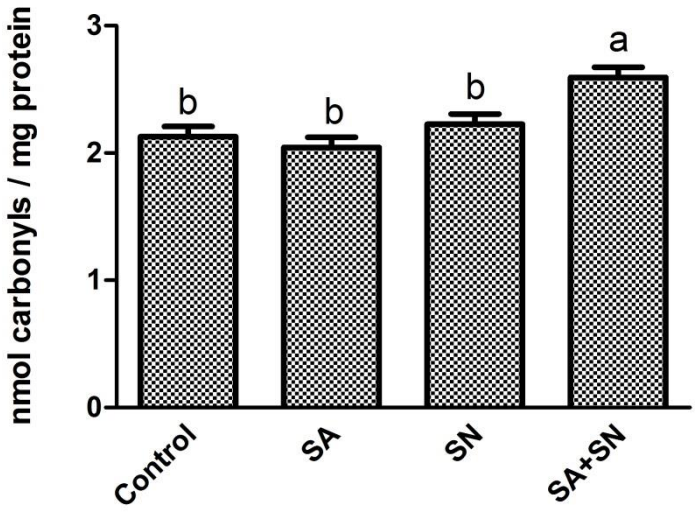
**Figure 5.3** Lipid oxidation evolution (TBARS) in dry fermented sausages during ripening. Error bars represent standard errors of the mean values. The a, b and x, y superscripts denote significant differences among means within sampling days and among means during ripening, respectively.




### 5.4.3 Protein oxidation

Little is known about the effects of AA and nitrite with respect to protein oxidation. Formation of carbonyl compounds is the most studied modification due to oxidation in meat products, usually based on the DNPH method (Estévez, 2011). In the present study, across all sampling points during the ripening period, the amount of protein carbonyls was significantly higher (approximately 20%) in the SA+SN treatment compared to the other treatments (interaction term $P < 0.01$; Figure 5.4).

**Figure 5.4** Nitrite and ascorbate effects on protein carbonyls (DNPH) in dry fermented sausages across days of ripening. Error bars represent standard errors of the mean values. Superscripts denote significant differences among treatments.



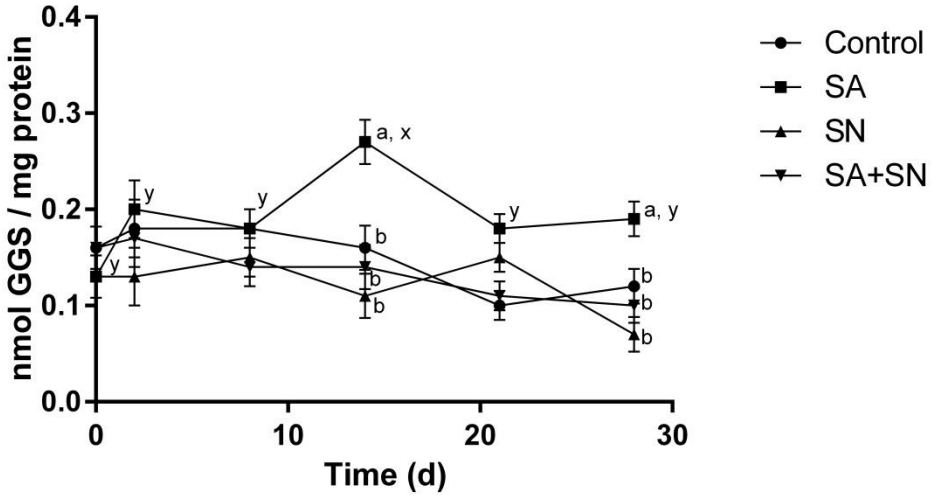

Protein carbonyls are mainly formed through three different pathways: metal-catalysed oxidation, non-enzymatic glycation and adduct formation with non-protein carbonyl compounds (Estévez, 2011). In metal-catalysed oxidation, carbonyls are formed in the side chain of arginine, lysine, proline and threonine (Stadtman and Levine, 2003). Metal ions, like $Fe^{2+}$, stimulate the generation of oxygen radicals from oxygen and $H_2O_2$ through the Fenton reaction in which the metal ion is then oxidized. In this scenario, AA can play an important pro-oxidant role reducing the oxidised metal ion, favouring the generation of further oxygen radicals by subsequent Fenton cycles (Villaverde et al., 2014a). However, in the present study, the only addition of ascorbate did not provoke an increase of

carbonyls, therefore it seems unlikely that the added SA increased protein carbonylation to a meaningful extent.

The second pathway of protein carbonylation is due to reducing sugars which can form carbonyls via glycation of lysine residues (Estévez, 2011). Ascorbic acid, a carbohydrate-like substance, might be involved in this pathway (Fan et al., 2009). Nitrite may oxidize AA to DHAA (Izumi et al., 1989), which is the first oxidation product of AA capable of glycating proteins (Ortwerth and Olesena, 1988). Therefore, the simultaneous addition of both compounds might have triggered the formation of carbonyls via glycation.

The third pathway of protein carbonylation is due to lipid oxidation products, like MDA and 4-hydroxynonenal, which can form adducts with proteins (Estévez, 2011). In this study, the control treatment did not show higher carbonyl compounds although it displayed higher MDA equivalents than the other treatments containing AA and/or nitrite. Therefore, it may be concluded that lipid oxidation products did not form protein adducts in detectable amounts, at least not under the investigated conditions. In addition, lipid peroxyl radicals, which are intermediates in the lipid oxidation reaction, did not seem to trigger protein carbonylation. In dry fermented sausages, lean pork and back fat are coarsely minced and meat and fat particles are clearly defined. As a consequence, interactions between lipids and proteins might be more limited in this type of products compared to more finely comminuted products.

**Figure 5.5** GGS evolution in dry fermented sausages during ripening. Error bars represent standard errors of the mean values. The a, b and x, y superscripts denote significant differences among means within sampling days and among means during ripening, respectively.



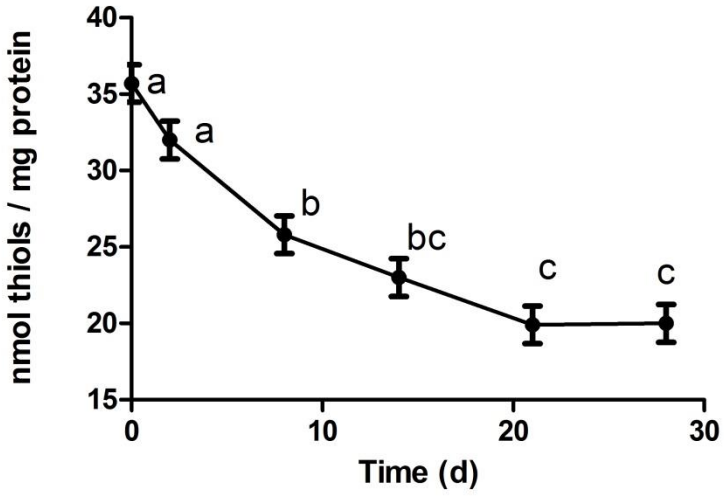

Among the carbonyls formed, GGS pointed towards an antioxidant effect of nitrite and a pro-oxidant effect of AA (Figure 5.5). The addition of AA generally increased GGS in the SA treatment becoming statistically significant at day 14 and 28 of ripening. Conversely, the addition of nitrite reduced the pro-oxidant effect of AA in the SA+SN treatment. GGS is an oxidative modification of arginine or proline and is formed during metal-catalyzed oxidation (Estévez, 2011). For this particular compound, the addition of AA in the SA treatment might have favoured the Fenton reaction promoting the generation of GGS, although the increase was insufficient to be detected with the DNPH method. This is possible as GGS may account for less than 20% of the total protein carbonyls in meat (Utrera et al., 2011). In the SA+SN treatment, the reaction

between AA and nitrite reduced the former to DHAA, which cannot perpetuate the Fenton reaction. Akagawa et al. (2005) reported an increase of GGS in BSA proteins in the presence of AA at pH 7.4. Recent research on protein oxidation in dry fermented sausage (Villaverde et al., 2014b, 2014c) reported pro-oxidant and antioxidant effects of nitrite and ascorbate, respectively, on AAS and AAS+GGS formation in dry fermented sausages. The contradictory results from these studies compared to the present one might be due to the different methods applied and the specific oxidative modification assessed to quantify protein carbonylation. Previous studies already revealed different results between the DNPH method and AAS and GGS methods (Armenteros et al., 2009; Fuentes et al., 2014).

In addition to carbonyl compounds, the loss of thiol groups in the myofibrillar fraction has been used as a marker of protein oxidation in meat products due to the high susceptibility of cysteine residues to oxidation (Lund et al., 2011). However, the loss of thiol groups was not affected by the use of SA nor SN. In this study, a significant loss of thiol groups occurred in all sausages during ripening (Figure 5.6; $P < 0.001$). Indeed, the final products had 40% less thiols than the fresh counterparts at day 0. These results suggest that cysteine oxidation occurred through a pathway independent of nitrite and ascorbate and that these two curing agents were not able to either prevent or promote it. The oxidation of thiols leads to formation of sulfenic acids, sulfinic acids, sulfonic acids and disulphide bonds causing aggregation between proteins (Zhang et al., 2013). These protein-protein interactions might be involved in the formation of a matrix which is necessary for the development of the desired sliceable sausage

texture (Zhou et al., 2014).

**Figure 5.6** Loss of thiol groups in the myofibrillar fraction of dry fermented sausages during ripening across the four treatments. Error bars represent standard errors of the mean values. Superscripts denote significant differences among means during ripening.




## 5.5 CONCLUSIONS

Nitrite and ascorbate are common additives in dry fermented sausages and are used, among other reasons, to control the oxidative stability. Their effect on lipid oxidation is well-established in contrast to protein oxidation. The results of the present study suggest that nitrite and ascorbate act differently against lipid and protein oxidation. Whereas ascorbate and nitrite reduce the formation of MDA, their simultaneous addition might increase the formation of carbonyl

compounds in proteins, although this does not affect the loss of thiol groups during ripening. This increased protein carbonylation might alter the structure and functionality of proteins compromising their technological and sensory properties. With regard to protein oxidation, the chemistry of nitrite and ascorbate revealed in this study provides grounds for further studies to better understand the reactions involved and to assess their actual impact on quality development within the product.

# CHAPTER 6

## GENERAL DISCUSSION AND FUTURE PROSPECTS

## 6.1 GENERAL DISCUSSION

Dry fermented sausages are meat products developed in ancient times to preserve meat at ambient temperature (Leroy et al., 2013). This product mainly consists of three basic ingredients: lean meat, fat and salt (sodium chloride). Therefore, the sensory characteristics are primarily given by these ingredients, their interaction and their development during the ripening time (Hui et al., 2004). Other ingredients and additives, such as spices, aromas, fibers etc., are sometimes added and contribute to typical flavour and texture attributes (Hui et al., 2004). However, in this thesis, we only used sausage models and samples of dry fermented sausage made of lean meat, fat and salt. The aim of this thesis was indeed to get a deeper view on the changes of the protein fraction during ripening. In particular, the generation of smaller peptides due to proteolysis and the occurrence of protein oxidation were studied.

The protein fraction of dry fermented sausages constitutes 20-30 % of the final product (Ockerman and Basu, 2014) and during ripening its intense degradation generates flavour compounds that affect taste and odour, as summarised in Chapter 1. Proteolytic degradation of a protein occurs in two steps, firstly the intact protein is cleaved into peptides by endoproteases and secondly peptides are shortened by exopapetidases that release amino acids, dipeptides and tripeptides (Molly et al., 1997). The composition of free amino acids in dry fermented sausages is well known and has been often studied

(Hierro et al., 1999). The further degradation of free amino acids into volatile compounds has been also investigated (Stahnke et al., 2002). In contrast, there is a lack of knowledge on the first step of proteolysis, i.e. the generation of peptides (< 5 KDa).

The use of liquid chromatography coupled to mass spectrometry enables the identification of small peptides derived from the degradation of several sarcoplasmic and myofibrillar proteins. In this thesis, we have identified a great number of peptides with a molecular weight between 500 Da and 3000 Da of the most abundant proteins (Chapters 2 and 3). Among the sarcoplasmic proteins, ten peptides were generated by proteolysis of myoglobin (H1 partially accepted), thirty-nine peptides were generated by proteolysis of creatine kinase (H2 accepted) and nine peptides were generated by proteolysis of glyceraldehyde-3-phosphate dehydrogenase (H3 partially accepted). From the myofibrillar proteins, twenty-eight peptides were generated by proteolysis of myosin (H4 accepted) and more than hundred peptides were generated by proteolysis of actin (H4 accepted). In the case of actin, peptides were identified in both mildly and strongly acidified dry fermented sausages, typical of Northern-European and Southern-European type of sausages respectively (Chapter 3). The identification was performed at the end of the fermentation (52 and 42 peptides were identified, in mildly and strongly acidified sausages, respectively) and at the end of ripening (66 and 144 peptides were identified, in mildly and strongly acidified sausages, respectively). The location of the peptides in the protein 3D structure and sequence was reported for both sarcoplasmic (Chapter 2) and myofibrillar proteins (Chapters 2 and 3). The results showed that peptides

were released from different protein regions thanks to the action of endopeptidases.

In the raw meat no peptides were found; on the other hand several peptides of actin were generated already after 3 days of ripening and more unique peptides were present at the end of ripening. The release of the peptides is probably mainly due to the activity of endogenous cathepsins (Molly et al., 1997), however little is known on their chemistry in dry fermented sausages. To better comprehend the role of these enzymes, it is necessary to reveal the peptides generated, which are the result of their activity. In addition, the cleavage sites of a proteolytic enzyme on a specific protein must be known. As far as actin is concerned, Hughes et al. (1999, 2000) identified the cleavage sites of cathepsins B and D on this protein, which allowed us to understand the actual role of these enzymes during processing of dry fermented sausages. A lot of peptides were cleaved at the peptide bonds corresponding to the cleavage sites of cathepsins B and D. These findings confirm and show the role of these enzymes that was so far only hypothesised and mainly based on their prolonged activity in this kind of product (H7 and H8 accepted).

As aforementioned, the peptides generated from the proteolysis of actin were identified both at the end of fermentation and ripening. We would have expected longer peptides at the end of fermentation, which would have been shortened by the action of exopeptidases during the drying phase. But this was not the case. The diverse proteolytic enzymes involved, of which several are active throughout the whole ripening period, and the activity of the starter culture, make proteolysis in dry fermented sausages a complex process that

cannot be simply explained as a linear evolution.

In the identification of the peptides, importance has been given to the characteristic pH conditions since these products can be roughly divided in two categories: mildly acidified products, typical of Southern-European type dry fermented sausages (pH 5.3-6.2, high final pH) and strongly acidified products, typical of Northern-European type dry fermented sausages (pH < 5, low final pH) (Hierro et al., 2014; Holck et al., 2014). The pH value in the final product directly influences taste; in the Northern-European type acidic taste is predominant compared to the Southern-European type (Holck et al., 2014). It is known that the pH evolution affects the activity of the proteolytic enzymes during ripening (Molly et al., 1997). It is reasonable to assume that pH values normally occurring in strongly-acidified dry fermented sausages favour the activity of some endopeptidases, such as cathepsins D, and hence protein breakdown into smaller peptides (Molly et al., 1997). Indeed, a higher number of actin-derived peptides was identified in dry fermented sausages with a lower pH value typical of Northern-European types (H6 accepted). On the other hand, the exopeptidases that are considered to be important in meat ripening have neutral pH optima (Toldrà et al., 2000). This implies a higher release of single amino acids in dry fermented sausages with higher pH, as reported in chapter 4. These findings demonstrate once more that the diverse proteolytic enzymes involved, having different pH optima, make proteolysis complex. For this reasons, it is import to reveal the products of proteolysis, i.e. the peptides. To this regard, the introduction of new proteomic tools in meat science, such as mass spectrometry-based proteomics, allows to get new insight in proteolysis.

However, this method gives good results in the identification of a target protein or peptide in a mixture, but it has still limitations in the identification of a complex mixture of peptides, such as the proteolysed pig proteome (Bantscheff et al., 2007; Vaudel et al., 2014). This limitation of the method used in this thesis implies that not all peptides present in the mixture are reported and quantification was not possible.

A deeper comprehension of the proteolysis in dry fermented sausages and peptides generated would provide a better understanding of the flavour formation. Indeed, peptides have taste properties, which depend on the conformational characteristics and the amino acid composition (Temussi, 2011). Charges on the side chains or on the terminals can give salty or sour taste, hydrophobic side chains can give bitter taste and umami taste was reported for some small peptides in meat products (Temussi, 2011). Nevertheless, there are only few studies that investigated the influence of peptides on the taste of meat products. Henriksen and Stahnke (1997) evaluated the effect of fractions of small peptides and free amino acids extracted from dry fermented sausages on taste. The results suggested that bitterness and sourness were correlated with hydrophobic amino acids and glutamic acid, respectively, and umami taste was provided by a mixture of different peptides and amino acids. Moreover, the amino acids released by the action of proteolytic enzymes during ripening, can be converted by meat-associated catalase-positive cocci into aroma compounds (Ravyts et al., 2012), or even serve as alternative energy substrates for both the lactic acid bacteria and catalase-positive cocci (Janssens et al., 2014; Rimaux et al., 2010). Peptides not only influence taste, but, depending on their sequence,

may show biological activity (Bhat et al., 2015). These peptides can have effects on health, such as reduction in blood pressure, antithrombotic properties, enhancement of mineral absorption and reduction of cholesterol blood levels, and exert preservative effects on foods, such as antimicrobial and antioxidative properties.

In dry fermented sausages, the proteome, in addition to proteolysis, undergoes protein oxidation. These two phenomena, hitting the same substrate, must somehow influence each other.

As previously reported, some peptides might have antioxidant properties, and therefore, if present in the pig proteome, might be released by proteolysis during ripening. This was investigated by inhibiting or not the action of proteolytic enzymes and checking the evolution of protein carbonyls and loss of sulphidryl groups. However, no differences were found between the two treatments, showing that the generation of peptides did not provoke an increased oxidative stability. Moreover, none of the peptides identified in chapters 2 and 3 were previously reported to have antioxidant activity. These findings allow us to speculate that proteolysis in dry fermented sausages does not generate peptides that may prevent or reduce protein oxidation and, in this sense, there seems to be no influence of proteolysis on the extent of protein oxidation (H9 rejected).

On the contrary, protein oxidation influences proteolysis. Indeed, the results reported in Chapter 4 shows that the activity of proteolytic enzymes is compromised by protein oxidation and the degree of inhibition depends on the

amino acids in the active site (H10 accepted). Cysteine residues are highly susceptible to oxidation and hence the activity of cysteine proteases, such as Cathepsin B and L, is quickly compromised in a oxidative environment (chapter 4). On the other hand, the aspartic proteolytic enzyme Cathepsin D is not easily oxidised and maintains its activity under oxidative conditions (chapter 4).

Besides oxidation of proteolytic enzymes, oxidation of proteins that serve as substrates for proteases also affects proteolysis. More specifically, oxidation of cysteine residues might result in formation of disulphide bonds between proteins causing protein aggregation (Lund et al., 2011). Protein aggregates are less susceptible to proteolysis compared to the original single proteins (Lund et al., 2011). In meat, myosin has been reported to form protein aggregates in samples under oxidative conditions (Park et al., 2006). This was confirmed in the experiment reported in Chapter 4, in which meat model systems were oxidised through direct addition of hydrogen peroxide. The oxidised samples formed protein aggregates which were visible at the top of the electrophoresis patterns, whereas the intensity of the myosin heavy chain band decreased. It can be concluded that intense oxidative conditions compromise proteolysis in dry fermented sausages by formation of aggregates and by reducing the activity of proteolytic enzymes. Therefore, the use of oxidised meat or applying conditions that favour protein oxidation can alter proteolysis and hence the final quality of dry fermented sausages.

It must be said that mild oxidative conditions may alter protein structure towards increased susceptibility to proteolysis of the oxidised proteins (Lund et al., 2011). In this research, oxidation was chemically induced by addition of

hydrogen peroxide and the amount added was chosen in order to increase protein carbonylation to 6 nmol/mg. To the best of our knowledge, the real content of hydrogen peroxide in dry fermented sausages is not known, although most lactobacilli used for fermentation are able to form hydrogen peroxide by oxidizing lactate (Ammor and Mayo, 2007). The target level of 6 nmol/mg protein carbonyls was chosen since levels of 7 nmol/mg were found in processed products (Estevez, 2011). In the experiments of this thesis, when oxidation was not induced (chapters 4 and 5), the level of protein carbonyls was between 2 and 2.5 nmol/mg, meaning that the ripening conditions applied induced moderate protein oxidation.

Essential additives, such as nitrite and ascorbate, are curing agents that develop the desired colour, exert antimicrobial activity and limit lipid oxidation (Honikel, 2008). Therefore, additives have a great influence on oxidation phenomena. The combined addition of 150 mg/kg of SN and 500 mg/kg of SA reduces the occurrence of lipid oxidation (Chapter 5). The separate use of nitrite or ascorbate also reduces lipid oxidation although less effectively. These curing agents can keep the level of MDA below the threshold of rancid taste detection (0.5 mg MDA/kg for a trained person) (Zdolec, 2006). Since these compounds influence oxidative processes, their role on protein oxidation should be clarified. Some research has been recently done, but clear results, due to the complex mechanism of protein oxidation and the high number of pathways and oxidation products, are not available yet (Villaverde et al. 2014a, 2014b, 2014c). In our experiment described in Chapter 5, the combined addition of nitrite and

ascorbate increased protein carbonylation (measured by the DNPH method) during ripening of dry fermented sausages. On the contrary, their sole use showed no differences compared to samples prepared without nitrite and ascorbate (H10 and H11 rejected). Based on these results, it can be speculated that protein carbonylation occurred through glycation: nitrite initially oxidized ascorbate into DHAA which is a glycation agent (Ortwerth and Olesena, 1988).

The DNPH method has been often used as a marker of protein oxidation in meat products. This method is robust but not specific for a particular protein carbonyl. The recent development of the GGS method (Estevez et al., 2009) is specific for the oxidation of two amino acids, i.e. arginine or proline, and was used in this thesis to get more information on the oxidised amino acids. The results showed a significant pro-oxidant effect of ascorbate at two time points. Nevertheless, the evolution in time of GGS in the four treatments did not show large differences. Thus, based on the results of this thesis, the amino acids arginine and proline did not undergo protein oxidation under the present experimental conditions and their monitoring is probably not necessary in dry fermented sausages.

As far as oxidation of thiol is concerned, the curing agents SA and SN seem not to exert any effect. However, during ripening of dry fermented sausages there is continuous loss of thiol groups due to oxidation (Chapter 5) and this may affect the structure of dry fermented sausages. In meat steaks, the inactivation of proteolytic enzymes due to oxidation may reduce meat tenderization and the formation of protein crosslinks may strengthen the myofibrillar structure increasing hardness (Lund et al., 2011). However, for dry

fermented sausages, to the best of our knowledge, effects of protein oxidation on meat texture have not been reported. Being a minced and a dried product, the hardness mainly depends on the water content. Nevertheless, the formation of protein crosslinks may play a role (Lund et al., 2011; Zhou et al., 2014). We speculate that the loss of thiol groups and the consequent formation of disulphide bonds, shown in chapter 5, might be beneficial for the meat matrix of these products contributing to the desired sliceable sausage texture (Zhou et al., 2014). Similarly, Estevez et al. (2005) found an increased hardness in frankfurters with higher protein carbonyls speculating that this was due to the loss of protein functionality and the formation of cross-links between proteins. The addition of exogenous proteolytic enzymes was correlated to softer sausage texture due to excessive proteolysis (Diaz et al., 1997). Similarly, the inhibition of endogenous proteolytic enzymes, as reported in chapter 4, might affect the texuture of dry fermented sausages.

## 6.2 FUTURE PROSPECTS

The peptide fragments released during ripening of dry fermented sausages and identified in this PhD thesis provide valuable information for further studies. These peptides can be used to identify potential bioactive peptides that may have beneficial health properties and exert antimicrobial and antioxidant activities (Bhat et al., 2015). Moreover, small peptides have taste properties covering the entire range of the five basic tastes: sweet, bitter, umami, sour and salty (Temussi, 2011). In this thesis we could not quantify the peptides reported, but understanding the taste properties of the most abundant peptides might reveal new important information on flavour development in dry fermented sausages. Therefore, the improvement of proteomic tools, such as mass spectrometry-based proteomics, which allows identification and accurate quantification of peptides in a complex mixture, may provide further insight on flavour formation in the future.

Protein oxidation is a complex mechanism that generates diverse oxidation products. The scarce interest on this type of protein modification has caused a lack of methodology. Indeed, results from different laboratories are often not comparable, as in the case of protein carbonyls analyses by the DHPH method (Estévez, 2011). New methods have been recently developed, but further efforts to this regard are still needed. In this thesis, we have used the GGS method to identify the oxidation product of arginine and proline during ripening. The results obtained did not show a clear evolution and arginine and proline seem not to be oxidised under the present experimental conditions,

although a pro-oxidant effect of ascorbate might be speculated. Nevertheless, this method provides information on specific protein carbonyls, in contrast to the DNPH method that measures total carbonyls from unspecific and different pathways. Future studies should be done using specific methods and markers, such as AAS and GGS, to better understand this complex chemistry.

In this thesis we did not focus on the effect of protein oxidation on sensory analysis. To the best of our knowledge, there are no clear evidences that the protein oxidation products can affect taste and odour in cured meats (Lund et al., 2011; Estevez, 2011; Zhang et al., 2013). Similarly, the effect of protein oxidation on texture on dry fermented sausages is not clear yet, although we speculated that loss of thiol groups affects gelation. The effect of protein oxidation on flavour and texture should be clarified in further studies. Besides sensory properties, nutritional and health aspects should also be taken into account. Indeed, oxidation of amino acids and impaired digestibility of oxidised proteins reduce nutritional quality (Soladoye et al., 2015). In addition, consumption of oxidised products increases oxidation markers in live animals and may thus similarly in humans contribute to the development of diseases conditions (Soladoye et al., 2015).

There are a lot of factors influencing protein oxidation in dry fermented sausages. As reported in this thesis, additives such as ascorbate and nitrite have an impact on oxidation phenomena. However, the controversial results found in literature make the understanding of their role rather complicated and further attention is needed (Villaverde et al., 2014a, 2014b, 2014c). Besides curing agents, other additives which were not included in the samples that

served as study objects of thesis, such as herbs, might also influence protein oxidation (Armenteros et al., 2016).

Protein nitration, which is connected to protein oxidation because reactive oxygen species may convert into RNS and initiate oxidative and nitrosative reactions, deserves also further attention (Honikel, 2008). Recently, Villaverde and others (2014a) reported the occurrence of protein nitration during ripening of fermented sausages. The effect of protein nitration on food quality, nutrition, and health is still unknown (Soladoye et al., 2015). Nevertheless, deeper insights on the redox chemistry of nitrite and ascorbate might reveal new interesting technological and scientific aspects.

Finally, the interaction between protein oxidation and lipid oxidation deserves also additional rsearch. More specifically, the role of oxidizing agents in raw meat should be clarified. Fuentes et al. (2014) speculated that the fatty acid profile and the presence of antioxidants in the fats affects protein oxidation in dry fermented sausages. Revealing the chemistry of the interaction between lipid and protein oxidation might provide strategies to limit oxidation in meat products.

# SUMMARY - SAMENVATTING

## SUMMARY

Dry fermented sausages are meat products, developed in ancient times to preserve meat. This product consists of lean meat, fat, spices and curing agents, which are minced, mixed and subsequently stuffed into a casing. Thereafter, this sausage undergoes a ripening period, in which physico-chemical changes occur and the characteristics of the product develop. During the initial fermentation period the pH of the product drops due to the action of lactic acid bacteria, whereafter the product loses water during the drying period, acquiring the name “dry fermented sausages”.

Other chemical changes occurring in the protein fraction, such as oxidation and hydrolysis, influence the sensory characteristics. The aim of this thesis was to deepen the knowledge on these changes, reveal the mechanism behind and test the effect of pH.

Chapter 1 provides background knowledge on dry fermented sausages. Strong attention was given to the protein fraction of this product, its oxidation and hydrolysis during the ripening time.

In Chapter 2 and 3, the generation of peptides (< 5000 Da) during the ripening period was studied. The identification of peptides, the understanding of which proteolytic enzymes are involved and the effect of pH might reveal new insights on the flavour formation. The hydrolysis of the sarcoplasmic proteins myoglobin, creatine kinase, glyceraldehyde-3-phosphate dehydrogenase and the myofibrillar proteins myosin heavy chain and actin was studied. The identification of these peptides was done through LCMS$^{E}$. These are abundant

proteins in muscle cells and, indeed, they released several peptides throughout the whole ripening period. Peptides are potential flavour compounds hence the evolution of these proteins is likely important to develop the final sensory characteristics of dry fermented sausages. The action of endopeptidases primarily generated peptides which chain was further shortened by the action of exopeptidases. In the case of actin, most of the identified peptides were released at the cleavage sites of cathepsins B and D. Moreover, a more acidic pH profile, characteristic of Northern-European type fermented sausages, likely favours the release of actin-peptides.

In Chapter 4 and 5, focus was given to protein oxidation. Firstly, the effects of the proteolysis and pH were investigated in a model for dry fermented sausages. Results showed that the concentration of protein carbonyls as marker for protein oxidation in dry fermented sausage models was similar after ten days of ripening in samples kept at both pH 4.8 and 5.2. Proteolysis did not influence protein oxidation. On the other hand, a higher level of oxidation negatively affected proteolysis by reducing the release of amino acids during ripening. Indeed, an oxidative environment leads to the inactivation of the cysteine proteases, such as cathepsins B and L and to formation of protein aggregates which have limited proteolytic susceptibility. Secondly, the role of the curing agents sodium nitrite and sodium ascorbate was investigated on oxidation in dry fermented sausages. Results suggested that nitrite and ascorbate act differently against lipid and protein oxidation. Sodium ascorbate and nitrite were separately able to reduce the formation of malondialdehyde as marker for lipid oxidation. Their combined addition, however, offered no further benefit and resulted in

higher amounts of protein carbonyl compounds compared to their separate addition or the treatment without any of both compounds. A loss of thiol groups was observed during ripening, which was not affected by the use of sodium ascorbate nor sodium nitrite.

## SAMENVATTING

Gefermenteerde droge worsten zijn vleesproducten waarvan de bereiding op een traditie van duizenden jaren teruggaat. Deze producten bevatten als ingrediënten mager vlees, vet, kruiden en pekelzout, die gemalen en gemengd worden en vervolgens in een darm gestopt worden. Nadien ondergaat de worst een rijpingsperiode, waarin fysicochemische veranderingen optreden en de typische eigenschappen van het product ontwikkelen. Gedurende de initiële fermentatieperiode treedt pH verlaging op door de werking van melkzuurbacteriën waarna het product water verliest tijdens de drogingsperiode, waarvan de naam gefermenteerde droge worsten afkomstig is.

Chemische veranderingen die optreden in de eiwitfractie zoals oxidatie en proteolyse beïnvloeden de sensorische eigenschappen van het eindproduct. Het doel van deze thesis was om meer kennis te verwerven in de veranderingen in de eiwitfractie van gefermenteerde droge worsten, de onderliggende mechanismen te ontrafelen en het effect van pH te testen.

In Hoofdstuk 1 wordt achtergrondinformatie gegeven over gefermenteerde droge worsten. Bijzondere aandacht wordt geschonken aan de eiwitfractie van dit type vleesproduct, en oxidatie en proteolyse tijdens de rijpingsperiode.

In de Hoofdstukken 2 en 3 wordt de vorming van kleine peptiden tijdens de rijpingsperiode bestudeerd. De identificatie van kleine peptiden, en een beter begrip van welke proteolytische enzymen hierbij betrokken zijn en de invloed van pH kan nieuwe inzichten verlenen in de aromavorming van dit type product.

De hydrolyse van de sarcoplasmatische eiwitten myoglobine, creatine kinase, glyceraldehyde-3-fosfaat dehydrogenase en de myofibrillaire eiwitten myosine zware keten en actin werden bestudeerd. De identificatie van de gevormde peptiden gebeurden d.m.v. LCMS$^{E}$. Dit zijn kwantitatief belangrijke eiwitten in spierweefsel, en inderdaad brachten zij verschillende peptiden voort tijdens de rijpingsperiode. Kleine peptiden zijn potentieel belangrijke aromacomponenten zodat de evolutie van deze eiwitten tijdens de rijpingsperiode wellicht belangrijk is voor de ontwikkeling van de uiteindelijke sensorische eigenschappen van gefermenteerde droge worsten. De activiteit van endopeptidasen genereert vooral peptiden die verder verkleind worden door de werking van exopeptidasen. In het geval van actine worden veel peptiden vrijgesteld op de splitsingsplaatsen van cathepsine B en D. Bovendien blijkt een zuurder pH profiel, kenmerkend voor Noord-Europees type gefermenteerde droge worsten, de vrijstelling van actine peptiden te bevorderen.

In Hoofdstukken 4 en 5 werd vooral aandacht geschonken aan oxidatie. Vooreerst werden de effecten van proteolyse en pH onderzocht in een model van gefermenteerde droge worsten. De resultaten toonden aan dat de concentratie van eiwitcarbonyls als merker voor eiwitoxidatie in de modelproducten gelijk was na tien dagen rijping in monsters met pH 4.8 of 5.2. Proteolyse had geen invloed op eiwitoxidatie. Daartegenover had een hoge graad van oxidatie een negatieve invloed op proteolyse door vermindering van de vrijstelling van aminozuren tijdens de rijping. Inderdaad leidt een oxidatieve omgeving tot de inactivatie van cysteine proteases, zoals cathepsine B en L en tot de vorming van eiwitaggregaten die een beperkte proteolytische gevoeligheid

hebben. Ten tweede werd de rol van de pekelzoutingrediënten natriumnitriet en ascorbinezuur onderzocht op oxidatie in gefermenteerde droge worsten. De resultaten suggereerden dat nitriet en ascorbaat verschillend inwerken op vet- en eiwitoxidatie. Nitriet en ascorbaat waren beiden in staat om de vorming van malondialdehyde als merker voor vetoxidatie te verminderen. Hun gecombineerde toevoeging daarentegen had geen voordeel en resulteerde in een hogere vorming van eiwitcarbonyls vergeleken met hun afzonderlijke toevoeging of de behandeling zonder één van beide additieven. Niettemin bleek eiwitoxidatie niet beïnvloed te worden door vetoxidatie in dit type producten. Natriumnitriet beperkte de vorming van GGS, terwijl natriumascorbaat een pro-oxidant effect vertoonde. Een verlies aan thiolgroepen werd waargenomen gedurende de rijping, die niet beïnvloed werd door het gebruik van natriumnitriet of natriumascorbaat.

In besluit, de eiwitfractie van gefermenteerde droge worsten ondergaat aanzienlijke veranderingen tijdens de rijpingsperiode. Proteolytische enzymen en radicalen veroorzaken respectievelijk proteolyse en oxidatie, en deze processen beïnvloeden de sensorische eigenschappen van de producten. Deze thesis levert nieuwe informatie over de fysicochemische veranderingen die optreden in gefermenteerde droge worsten. Een beter begrip van deze veranderingen kan leiden tot de implementatie van nieuwe technologische strategieën om de kwaliteit van deze vleesproducten te verbeteren.

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# CURRICULUM VITAE

Alberto Berardo was born on the 15th of November 1987 in Legnago (Italy). He graduated in B.Sc. Agricultural Science and Technology at Padua University (Italy) in 2010 and M.Sc. Agricultural Science and Technology at Padua University (Italy) in 2012. Since January 2013, he has been a PhD student in the study program of Doctor of Applied Biological Sciences, in the Laboratory for Animal Nutrition and Animal Product Quality, Faculty of Bioscience Engineering, Ghent University, Belgium. His work was financially supported by the Fund for Scientific Research – Flanders (FWO-Vlaanderen), through the Project Qualitative and Quantitative Analysis of Enzymatic and Oxidative Effects on the Protein Fraction of Fermented Meat Products (G.0327.12). This was a collaborative project between VUB, Research Group Industrial Microbiology and Food Biotechnology (IMDO), Prof. F. Leroy (project coordinator) and UGent, Laboratory for Animal Nutrition and Animal Product Quality (LANUPRO), S. De Smet.

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**Conference, workshop, seminars**

**2014**

2nd Symposium “Meet the Belgian Meat Researchers” of the Belgian Association for Meat Science and Technology, Melle, Belgium. *Oral presentation.*

Second International Symposium on Fermented Meat, Valencia, Spain. *Oral presentation.*

**2015**

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