

# Peptides and Ribonucleotides in Fresh Meat as a Function of Aging in Relation to Sensory Attributes of Pork

Meelis Tikk

*Faculty of Natural Resources and Agricultural Sciences*

*Department of Food Science*

*Uppsala*

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### Abstract

The objective of the present thesis was to obtain a more fundamental understanding of the development and importance of the flavour precursors, peptides and ribonucleotides on flavour development in pork. This will contribute to a better understanding of the influence of specific enzyme activities and production parameters on essential meat quality attributes.

The developed approach proved to be an appropriate tool to follow post-mortem changes in the peptide profile of pork during aging and hereby make it possible to identify specific peptides with potential importance for meat quality development and final meat quality. Changes in peptide patterns during aging of pork proceeded through continuous increase and decrease of the individual peptide fractions, which refer to gradual degradation of individual proteins/peptides throughout aging. Moreover, a decreased rate in the development in the peptide pattern was induced by glycogen-reducing finishing feeding and in animals with low slaughter weight. Pan-fried pork chops showed some tendency to more pronounced bitter notes and reduced juiciness, whereas whole roasts showed a positive relationship between meaty notes and a peptidic peak containing sequenced peptides. The effect of troponin T-derived sourness-suppressing peptide was confirmed on sour odour suppression in pork at 4 days of aging.

Finally, the obtained results clearly demonstrate that aging and cooking were important for the development of essential meat flavour precursors. In contrast, the difference in meat quality (pH) within the limits studied here had only negligible effect on the development of the investigated flavour precursors. The flavour enhancer inosine monophosphate was found to contribute to the sensory attributes brothy and meaty, whereas its degradation product hypoxanthine was related to the sensory attribute bitter. Finally, the change in sensory attributes from brothy/meaty to bitter taking place during the storage of pork was found to coincide with the continuous degradation of inosine monophosphate to hypoxanthine.

*Keywords:* pork, aging, meat quality, flavour, IMP, peptide profile, peptide identification, mass spectrometry

*Author's address:* Meelis Tikk, Department of Food Science, University of Aarhus  
P.O. Box 50, 8830 Tjele, Denmark  
E-mail: meelis.tikk@agrsci.dk



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## List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Tikk, M., Nebel, C., Dalsgaard, T.K., Rasmussen, J.T., Larsen, L.B. and Andersen, H.J. (2008) Specific peptide profiling during aging of pork. *Meat Science* (received for revision).
- II Tikk, M., Aaslyng, M.D., and Andersen, H.J. (2008) The significance of peptide profile on pork flavour (manuscript).
- III Tikk, M., Aaslyng, M.D., Karlsson, A.H., Tikk, K. and Andersen, H.J. (2008) The significance of sex, carcass weight and diet on peptide profiles and sensory quality in pork (manuscript).
- IV Tikk, M., Tikk, K., Tørngren, M.A., Meinert, L., Aaslyng, M.D., Karlsson, A.H. and Andersen, H.J. (2006) Development of Inosine Monophosphate and Its Degradation Products during Aging of Pork of Different Qualities in Relation to Basic Taste and Retronasal Flavor Perception of the Meat. *Journal of Agricultural and Food Chemistry* 54(20), 7769-7777.
- V Meinert, L., Tikk, K., Tikk, M., Brockhoff, P.B., Bredie, W.L.P., Bjerregaard, C., & Aaslyng, M.D. (2009) Flavour development in pork; influence of flavour precursor concentrations in longissimus dorsi from pigs with different raw meat qualities. *Meat Science* 81(1), 255-262.

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## Abbreviations

ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
HPCE	high-performance capillary electrophoresis
HPLC	high-performance liquid chromatography
IMP	inosine 5'-monophosphate
LC	liquid chromatography
LD	<i>M. longissimus dorsi</i>
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometry
PC	principal component
PCA	perchloric acid
PCA	principal component analysis
PLS1	partial least squares regression
PLS2	partial least squares regression
SPE	solid phase extraction
TOF	time-of-flight
UV	ultraviolet

## Introduction

Biochemical processes proceed during handling, processing and/or storage of raw material of animal origin. This directly affects the quality of the product, e.g. fish, cheese, fresh meat, dry-cured meat products, etc. (Spurvey *et al.*, 1998; Toldra *et al.*, 1997; Cliffe *et al.*, 1993). Moreover, the degree of hydrolytic breakdown of ribonucleotides, protein structures and proteins is highly dependent on environmental and physical factors (Flores *et al.*, 2000; Feidt *et al.*, 1998).

Proteolytic activity during the conversion of muscle to meat and the subsequent aging of meat is of importance for several meat quality parameters. These include flavour development, tenderness, formation of bioactive peptides and water-holding capacity of meat (Bauchart *et al.*, 2006; Gill *et al.*, 1996; Koohmaraie, 1994; Nishimura *et al.*, 1988). Consequently, an understanding of the proteolytic activity is essential in relation to control of the production of high quality of meat.

Even though presence and formation of peptides in meat are known to have both direct effects (flavour, bioactivity) and indirect effects on essential meat quality parameters (tenderness, water holding capacity), the identification of specific peptides and the origin of these in relation to fresh meat quality have only been investigated in a limited number of studies (Kitamura *et al.*, 2005; Okumura *et al.*, 2003; Lametsch *et al.*, 2002).

The degradation of adenosine 5'-triphosphate (ATP) to inosine 5'-monophosphate (IMP), hypoxanthine and ribose during aging and cooking of meat is considered to take part in the flavour development of meat (Cambero *et al.*, 2000; Imafidon & Spanier, 1994). IMP is known to be one of the chemical compounds that possess the fifth basic taste, "umami", and, similarly to glutamates, display an effect as flavour enhancers (Teleky-Vamosy & Szepes-Krell, 1989). The degradation of IMP contributes to formation of the bitter compound hypoxanthine, and ribose a reducing

sugar that potentially takes part in Maillard reactions (Cerny & Davidek, 2003; Farmer *et al.*, 1989; Salter *et al.*, 1989).

As the hydrolytic processes mentioned above are essential for the development and formation of the final meat flavour, there is a considerable need for and interest in understanding and controlling these hydrolytic processes in animal origin foods in much more detail in order to be able to produce and control the flavour quality in meat. In the following a state of the art of the knowledge of these proteolytic processes is given.

## Muscle proteins

### Function of muscle proteins

Muscles are contractile “organs”, creating force and movement. These functions are performed through muscle tissue, the second biggest components of which are proteins. Proteins in muscle are classified as myofibrillar proteins, sarcoplasmatic proteins, connective tissue and organelles. Insoluble myofibrillar proteins, together with the connective tissue, create the physical structure for the muscle, whereas the soluble sarcoplasmatic proteins function in various biochemical processes (Lawrie, 1998).

The main structural proteins in muscle are myosin, actin, titin, nebulin, tropomyosins, troponins (subtypes C, I, and T), desmin and others. Actin and myosin participate in a contractile function, creating the actomyosin complex with subsequent allosteric changes in myosin when a muscle is contracted, and disengaging the complex when a muscle is relaxed. Troponin acts as regulatory complex in muscle contraction, and titin, nebulin and desmin create an additional filamentous cytoskeleton network. An I-band in the muscle covers the section from A-band to A-band in consecutive sarcomers, whereas a z-disk creates borders between sarcomeres. In case of desirable contraction,  $\text{Ca}^{2+}$  ions, released from the sarcotubular system, bind to troponin C, causing an allosteric change in this protein and allowing troponin T to unblock the binding site on the molecule of actin. For muscle relaxation, ATP is used to break actin-myosin binding,  $\text{Ca}^{2+}$  is pumped back to the sarcotubular system, and troponin T blocks the binding site on the molecule of the actin. Sarcoplasmatic proteins carry regulatory and transport functions in the muscle. These include enzymatic activity in glycolytic pathways and protein degradation, and e.g. oxygen transport by myoglobin (Lawrie, 1998).

## Post-mortem degradation of muscle proteins

Upon slaughter of an animal, the nervous impulses stop, the energy and oxygen supply to muscles are cut off, which leaves all ongoing biochemical processes biologically unregulated. Uncontrolled  $\text{Ca}^{2+}$  release from the sarcotubular system induces the engagement of actin-myosin crossbridges. Simultaneously with the formation of the crossbridges, they are also disengaged in a manner similar to relaxation in the living muscle until ATP depletion. To produce additional energy, the glycolytic pathway becomes anaerobic which generates lactic acid and hereby a drop in meat pH. The high rate of pH decline in combination with the slow rate of temperature decrease at cooling decides the extent of protein denaturation which can lead to pale, soft, exudative (PSE) meat (Wismer-Pedersen & Briskey, 1961) and thereby also affect post-mortem protein degradation.

The most obvious effect is seen on the contracted myofibrillar proteins, resulting in toughening of the meat. However, along the aging, subsequent biochemical changes in meat induce a tenderizing effect, which is attributed to proteolytic enzymes that break down myofibrillar protein structures (Morrison *et al.*, 1998; Koohmaraie *et al.*, 1996). Fragments from e.g. troponin T, myosin light chain, desmin and actin occurring during aging have been reported among myofibrillar proteins (Hwang *et al.*, 2005; Lametsch *et al.*, 2002; Taylor *et al.*, 1995a). For troponin T-specific substrates, cleavage sites in amino acid sequence/protein structure during aging of pork has been proposed by Kitamura *et al.* (2005); however, the actin-myosin complex has not been found to be degraded (Hopkins & Thompson, 2002).

Similarly to myofibrillar proteins, degradation of sarcoplasmatic proteins has been found in a few studies, and devoted as markers for aging (Sayd *et al.*, 2006; Okumura *et al.*, 2003); however, as it is not part of post-mortem tenderization process, the interest in relation to meat quality has been moderate. Moreover, gel electrophoresis techniques applied in these studies often separate protein fragments in a relatively high molecular weight range (ranging from 3 to 200 kDa) (Hollung *et al.*, 2007; Claeys *et al.*, 2004; Lametsch & Bendixen, 2001) in comparison to a few chromatographical methods, allowing a decline to dipeptide level (Sforza *et al.*, 2006; Hansen-Møller *et al.*, 1997; Roudot-Algaron *et al.*, 1994). Differences in the molecular weight range allow discrimination of different aspects in post-mortem degradation of proteins. The higher molecular weight range shows the whole proteins or the primary degradation products, whereas the lower molecular weight range shows smaller peptides, which have potentially gone through more than one step of degradation (Kristensen *et al.*, 2003; Lamare

*et al.*, 2002; Feidt *et al.*, 1998), therefore making it more interesting in understanding the flavour development.

## Proteolysis

Enzymes are proteinous compounds in living tissues and carry the function of a catalyst. The energy to break a chemical bond in the presence of a catalyst is usually significantly lower than under normal conditions (Branden & Tooze, 1991; Mathews & Holde, 1990). The role of proteolytic enzymes in living muscle tissue is to catalyze the cleavages in damaged protein structures that need to be exchanged or repaired during the cell cycle, apoptosis etc. (Turk *et al.*, 2001; Molinari & Carafoli, 1997; Sorimachi *et al.*, 1997). The activity of proteolytic enzymes taking part in the degradation of structural proteins decides the rate of protein turnover, therefore it has been studied extensively in relation to muscle development and to growth rate using various feeding strategies (Therkildsen *et al.*, 2004; 2002a; Sazili *et al.*, 2004; Rosenvold *et al.*, 2001). Enzymatic activity is regulated by specific ions, inhibitors or by physical isolation from the substrate. Malfunction in enzymatic activity is known to play a role in ischemia, cancer and Alzheimers disease (Kuester *et al.*, 2008; Raynaud & Marcilhac, 2006; Troy *et al.*, 2004). The main muscle proteases are calpains, cathepsins and a proteasome complex. Additionally, proteases can be classified according to the specific location of cleavage in relation to protein structures and amino acid sequence as endo- or exopeptidases, cleaving either interdomain boundaries or amino acids and small peptides, respectively (Toldra & Flores, 2000; Sentandreu & Toldra, 2000). Calpains are classified as  $\mu$ - and m-calpains, based on the  $\text{Ca}^{2+}$  levels of  $\sim 10\text{--}50\ \mu\text{M}$  in  $\mu$ -calpains and  $\sim 200\text{--}500\ \mu\text{M}$  in m-calpains in vitro (Hosfield *et al.*, 1999), which is higher than the physiological concentration,  $<1\ \mu\text{M}$  in vitro (Goll *et al.*, 1992b). Furthermore, activation of calpains has been suggested to be influenced by endogeneous activators (Melloni *et al.*, 1998) and by the presence of  $\text{Ca}^{2+}$  that converts the enzyme to active protease by autolysis of calpains through the removal of a subunit (Cong *et al.*, 1989), or by the  $\text{Ca}^{2+}$ -induced conformational change, reorienting the protease domains to form a functional active site (Hosfield *et al.*, 1999). The inhibition by calpain inhibitors calpastatins is avoided by  $\text{Ca}^{2+}$ -dependent translocation to the cell membrane, which in combination with the localization in the cytoskeletal matrix also affects the substrate specificity (Molinari & Carafoli, 1997; Mellgren, 1987). Lysosomal cathepsins are known to take part in muscle protein turn-over (Barrett & Kirschke, 1981) and to be related to increased

muscle growth (Kristensen *et al.*, 2004). The broad range of various types of cathepsins allows them to possess endopeptidase, peptidyl dipeptidase and aminopeptidase activities. Cathepsins are concentrated into lysosomes, as they are physically separated from substrates (Goll *et al.*, 1983; Barrett & Kirschke, 1981; Venugopal & Bailey, 1978). Moreover, their activity is regulated by the inhibitors cystatins. The preference of cathepsins for lower pH is suggested to be an inhibiting factor, thereby controlling the proteolytic activity, and also to be a promoting factor in protein degradation by substrate denaturation (Pillay *et al.*, 2002).

Upon slaughter of the animal, the protein synthesis stops; however, the activity of proteolytic enzymes continues as long as the prerequisites for enzymatic activity are present. Factors of importance are suitable pH, temperature, substrate availability and presence of specific ions or inhibitors (Ertbjerg *et al.*, 1999; Rosell *et al.*, 1996; Dransfield, 1994; Koohmaraie, 1992). Moreover, as also mentioned for the living muscle, the enzyme activity is dependent on the presence and the amount of specific inhibitors that affect the proteolytic activity. The degradation of proteins in meat is mainly influenced by the calpain system, the proteasome complex and the lysosomal cathepsins (Feidt *et al.*, 1998; Koohmaraie, 1994; Koohmaraie *et al.*, 1991). The calpain system is mainly active during the early post-mortem processes (pH > 6) (Spanier *et al.*, 1990), whereas the proteasome complex is active during both the early post-mortem processes (the ATP-dependent activity) (Peters *et al.*, 1994) and at low pH (the cathepsin like activity) (Dutaud *et al.*, 2006; Lamare *et al.*, 2002), while the cathepsins are mainly active at low pH, i.e. after disintegration of the lysosomes (Spanier *et al.*, 1990; Goll *et al.*, 1983). The proteolytic activity within the living muscle is mainly determined by the rate of protein turn-over (Koohmaraie *et al.*, 2002). Consequently, several studies have focused on factors of importance for the rate of muscle protein turn-over in relation to the subsequent quality of derived meat in the search for potential tools to control the meat quality (Therkildsen *et al.*, 2004; 2002b).

The specificity of calpains to cleave proteins in relation to primary amino acid sequence in vitro studies is not specified in detail; however, the effect of calpains has been investigated on the separated myofibrillar proteins. Calpains in living muscle are associated with myofibrillar structures (Goll *et al.*, 1992b) and are suggested to be located near the z-disk (Kumamoto *et al.*, 1980). The substrate accessibility plays an important role, and proteins are cleaved at interdomain boundaries. Therefore, the calpain system has been suggested to be of utmost importance for tenderness development in meat (Hopkins & Thompson, 2002; Koohmaraie, 1994; 1992), as it influences the

degradation of structural proteins (Goll *et al.*, 1998; Huang & Forsberg, 1998; Koohmaraie, 1996; Koohmaraie, *et al.*, 1996; Taylor *et al.*, 1995a). Moreover, it has recently been suggested to also have a potential effect on water holding capacity in meat by degrading the cytoskeleton during aging (Zhang *et al.*, 2006; Melody *et al.*, 2004; Taylor *et al.*, 1995a). The mechanism for this effect has been proposed to be the removal of inter-myofibrillar and costameric connections, thereby reducing or removing the linkage between the rigor-induced lateral shrinkage of myofibrils and shrinkage of the whole muscle fibre (Kristensen & Purslow, 2001). Degradation of the structural proteins troponin T, desmin and titin has been shown to be the result of calpain activity (Ertbjerg *et al.*, 1999; Huff-Lonergan *et al.*, 1996; Taylor *et al.*, 1995a). However, Goll *et al.* (1992a & 1991) did not find any effect of calpains on actin and myosin. It is interesting that more recently, the degradation of actin and myosin by calpain has been demonstrated (Lametsch *et al.*, 2004; 2003).

Like the calpain system, the 20S proteasome complex has also been found to contribute to the degradation of structural proteins and intact permeabilized muscle fibrils (Taylor *et al.*, 1995b). The proteasome complex has been suggested to degrade a vast majority of cell proteins (Rock *et al.*, 1994); however, its contribution to the development in specific meat quality attributes is far from fully understood. The proteasome complex has been found to have some substrate specificity to actin, myosin and desmin, which seem to be degraded faster than  $\alpha$ -actinin, troponin T and tropomyosin (Taylor *et al.*, 1995b; Mykles & Haire, 1991). Both the calpain system and the proteasome complex are known to degrade sarcoplasmic proteins (Lametsch *et al.*, 2003; Lamare *et al.*, 2002; Huang & Forsberg, 1998). Cathepsins have been shown to degrade the N2 line and the I-band (Ouali, 1992; 1990; Masanori *et al.*, 1992; Mikami *et al.*, 1987), as well as myosin and  $\alpha$ -actinin (Mikami *et al.*, 1987; Okitani *et al.*, 1980).

The present development in peptide-related analytical techniques in combination with the growing information obtainable via protein databases should ensure that the progress in specific peptide profiles during aging of meat and the origin of these peptides soon will be available. This will contribute to a better understanding of the influence of specific enzyme activities on essential meat quality attributes and to an identification of specific peptides applicable as quality markers in meat quality control. In studies concerning meat quality, 2D-gel electrophoresis has proven to be a useful tool, allowing description of quantitative changes in proteins in relation to meat quality (Hollung *et al.*, 2007; Bendixen, 2005; Hwang *et al.*, 2005; Lametsch & Bendixen, 2001). However, by inclusion of mass

spectrometry using peptide mass fingerprinting, MS/MS spectra and sequence homology search with de-novo sequenced fragments, more detailed information can be obtained (Shevchenko *et al.*, 2001). The most common MS technique in studies concerning meat has been MALDI-TOF, often with an additional MS option. This provides high quality results; however, the limitation of the technique is the interference of the matrix in the range lower than 900 m/z, and the preference of ionization for tryptic peptides (Hjernø & Jensen, 2007). On the other hand, the relative simplicity of managing the apparatus, analyzing the sample and interpreting the data, is the main benefit, compared to other MS techniques.

## Ribonucleotides

Ribonucleotides are non-protein substances in meat. These are compounds in which purine or pyrimidine is linked to ribose, with a base of adenine, guanine, cytosine or uracil. Additionally, one, two or three phosphate groups may be attached to ribose. In relation to flavour development in meat, especially inosine 5'-monophosphate (IMP) and its degradation products are of importance. Its precursor, adenosine 5'-triphosphate (ATP), is used as energy carrier in most living tissues. It consists of an adenine base, connected to the first carbon of ribose, and of three phosphate groups, connected to the fifth carbon atom of the ribose.

### Post-mortem degradation of ATP

ATP post-mortem becomes dephosphorylated step by step to resolve the actin-myosin complex until only AMP is present. Subsequently, adenosine in AMP is deaminated, forming IMP. Further degradation during aging breaks the IMP molecule down to pentose, ribose, and hypoxanthine. The stability of IMP is reported to be both temperature- and pH-dependent (Matoba *et al.*, 1988; Shaoul & Sporns, 1987; Nguyen & Sporns, 1985) due to the residence of weak chemical bonds, for example, glucoside and ester bonds (Matoba *et al.*, 1988). Consequently, pH in the fresh meat is expected to influence IMP degradation through aging and cooking. Several studies have reported a heat-induced increase in ATP metabolites during cooking of different muscle foods, and a significant increase in the concentration of inosine and hypoxanthine during cooking has been demonstrated in goat's and sheep's meat (Arya & Parihar, 1979).

## Meat flavour

The flavour of the meat has been defined as the combination of taste and odour; however, temperature-dependent tactile properties such as mouthfeel and juiciness also affect the flavour perception (Farmer, 1992). Taste has been defined by the five basic tastes, sweet, sour, salty, bitter and umami, and is sensed by the taste buds on the tongue. Moreover, recent studies have suggested the affinity of the fatty acid transporter (CD 36), found also in taste buds, to long-chain fatty acids to be involved in fat perception (Laugerette *et al.*, 2005). Hundreds of volatile compounds are perceived, both through anterior or posterior nares (Farmer, 1994; 1992). Therefore, a slightly more specific approach to describe flavour has been used to get a deeper understanding of flavour perception, referred to as retronasal flavour perception which in addition to basic tastes perceived on the tongue, is perceived in the nose during eating (King *et al.*, 2006; Visschers *et al.*, 2006; Buettner & Mestres, 2005).

The 5'-ribonucleotides, adenosine monophosphate (AMP), inosine monophosphate (IMP) and guanosine monophosphate (GMP), are also important in meat flavour perception, as they hold umami taste characteristics (Durnford & Shahidi, 1998; Spurvey *et al.*, 1998). Umami compounds have a characteristic savory quality, first characterized for monosodium glutamate. Besides the characteristic umami taste, umami compounds have flavour-enhancing properties and are reported to enhance meaty, brothy, mouth-filling, dry and astringent qualities and to suppress sulfurous notes (Kuninaka, 1981). Moreover, brothy taste enhancers have been isolated and identified from beef (Soldo *et al.*, 2004; Ottinger & Hofmann, 2003; Shima *et al.*, 1998).

The flavour of meat develops largely through the cooking process. However, fresh meat contains non-volatile constituents that are essential flavour precursors and contribute to the basic taste of cooked meat (Chen & Ho, 1998; MacLeod, 1986). Consequently, many of the constituents in fresh meat undergo substantial changes during the cooking process and subsequently provide roasted, boiled, fatty, and species-related flavours, as well as the characteristic meaty aromas associated with cooked meats. Model experiments have suggested that the reaction between amino acids and reducing sugars is one of the main pathways in the formation of many of the aroma compounds identified in cooked meat (Mottram & Nobrega, 2002; Farmer & Mottram, 1994; Salter *et al.*, 1989; Mottram, 1985). The importance of the sulfur-containing amino acids cysteine and methionine has been estimated to be largest in flavour development (Werkhoff *et al.*, 1990). Moreover, the biologically active dipeptide carnosine (Hou *et al.*,

2003; Chan & Decker, 1994) has been suggested to influence meat flavour in model systems (Gianelli *et al.*, 2005; Chen & Ho, 2002); however, no effect on broth taste was found by (Nishimura *et al.*, 1988). The activity of proteolytic enzymes in meat has been shown to influence various sensory attributes with the main focus on tenderness development during aging due to the degradation of myofibrillar proteins by calpains (Dransfield, 1992; Koohmaraie, 1992). Even though still far from fully understood, the lysosomal cathepsins are not thought to be a main contributor in tenderness development although some degradation of the structural proteins occurs. In contrast, some of the cathepsins are known to contribute to flavour development in processed meat (Virgili *et al.*, 1998; Toldra *et al.*, 1997). Interestingly, this knowledge has mainly been established through correlation between cathepsin activity and flavour development (Sforza *et al.*, 2001; Virgili *et al.*, 1998). This is why only few studies have concentrated on the identification of the proteolytic-derived peptides and their influence on flavour (Okumura *et al.*, 2004; Tamura *et al.*, 1989; Yamasaki & Maekawa, 1978), of which the results by Tamura *et al.* (1989) found no support from Hau *et al.* (1997) and van Wassenaar *et al.* (1995).

The concentration of thiamine has been shown to be higher in pork than in several other species (Lombardi-Boccia *et al.*, 2005; Leonhardt & Wenk, 1997). The thermal degradation of thiamine to the low threshold compounds 2-methyl-3-furanthiol and the corresponding disulfide (Gasser & Grosch, 1988) are suggested to increase the specific pork flavour characteristics (MacLeod, 1986; Shahidi, 1989). The amount and composition of the lipid fraction in meat influence the fatty taste and the species-specific flavour perception. Moreover, the content of intramuscular fat has been shown to influence the texture properties of meat. Increase in lactate concentration derived from the anaerobic breakdown of glycogen stores has been shown to increase the sour notes in meat (Jeremiah *et al.*, 1990).

Glycogen in muscle has an energy storage function, and together with ribose upon heating results in series of non-enzymatic reactions between named sugars and amino acids. The resulting compounds undergo further reactions with lipid oxidation products, primarily from unsaturated phospholipids (Cameron *et al.*, 2000; Salter *et al.*, 1989; Whitfield *et al.*, 1988), forming numbers of volatile, flavour-active compounds (Gasser & Grosch, 1988).

## Flavour precursors

Flavour precursors of importance in meat are amino acids/peptides, carbohydrates, lipids, vitamins and ribonucleotides. These compounds inherent in meat have a biological function in living tissues; however, during aging of meat they often undergo further degradation giving rise in the concentration of more specific compounds directly influencing flavour or taking part in reactions with other compounds upon heating.

Considering that IMP, ribose and hypoxanthine in this way all are to be considered important constituents in meat flavour formation and development, an understanding of the post-mortem energy metabolism in muscles and the subsequent degradation of the ATP metabolite, IMP, during aging and cooking becomes crucial in the further exploitation of flavour development in meat. Moreover, the 5'-ribonucleotide IMP, may indirectly also contribute to meat flavour through its secondary degradation product, hypoxanthine, which together with several free amino acids and anserine, carnosine and other dipeptides potentially contribute with bitter flavour characteristics. Moreover, increasing cooking temperatures have been found to result in a significant rise in the concentrations of creatinine, IMP and AMP in beef broth, with IMP showing the highest correlation to the sensory data of the broth (Cambero *et al.*, 2000).

Peptides, released from proteins as a result of proteolytic enzymes, are shown to influence flavour development in meat. The multifunctional pool of enzymes and substrates with the changing environmental conditions in meat after slaughter has hampered the identification of the peptides related to taste and/or flavour, which is why only few studies have shown that meat peptides contribute to specific flavour attributes. Meat flavour consists of many compounds and is a result of various reactions, and changes in some flavour or texture characteristics may mask other attributes.

## Meat texture

Variance in meat texture attributes has been found to be more easily distinguished than flavour attributes (Meinert *et al.*, 2008b) and is influenced by many parameters. These are, among others, breed, age, weight, sex of the animal – parameters that are predefined by production (Wood *et al.*, 2004; 1995; Bejerholm & Aaslyng, 2004), and biophysical aspects such as the amount and solubility of connective tissue, intramuscular fat, sarcomere length and the rate and extent of proteolysis, some of which are influenced by the post-mortem conditioning parameters cooling and aging (Hwang *et al.*, 2005). However, with some variation in the specific design/parameters, Meinert *et al.* (2008a) found no or contradictory effects of the

abovementioned production parameters on texture and other sensory parameters. Often these parameters interact with each other, and a more thorough understanding is needed, e.g. of the effect of proteolytic activity on the degradation of the myofibrillar proteins.



## Objectives

The objective of the present thesis was to obtain a more fundamental understanding of the development and importance of the flavour precursors (peptides and ribonucleotides) on flavour development in pork. This will contribute to a better understanding of the influence of specific enzyme activities and production parameters on essential meat quality attributes. Furthermore, the identification of specific flavour precursors applicable as quality markers in meat quality control can be obtained.

The specific aims of the thesis were:

- To develop an analytical approach for monitoring the specific peptide patterns in meat, and to identify the peptides developed as a result of aging.
- To further elucidate the importance of the production parameters (aging, sex, feed and weight class) and the overall meat quality parameters (pH and temperature) on development of the sensory characteristics in relation to specific peptides in pork prepared by using different cooking methods (whole roast loins and pan-fried pork chops).
- To explore the fate of the ribonucleotide IMP during the aging of two qualities of pork (normal and high pH) and the potential relationship between IMP, hypoxanthine and sensory attributes of pork registered both as retronasal and basic taste responses in whole meat, meat juice and the remaining meat residue.



# Material and methods

## Animal material and sampling

### *Paper I*

Twelve crossbred slaughter pigs (Duroc boars and Danish Landrace x Danish Yorkshire sows), half of which were females and the remaining castrates, were reared at the experimental farm at the University of Århus, Faculty of Agricultural Sciences. All pigs were slaughtered at approximately 110 kg live weight after a fasting period of 24 h, during which time the animals had free access to water. On the day of slaughter, the pigs were transported (200 m) from the stable to the slaughterhouse, where they were individually brought up to the stunner. The pigs were stunned by 80% CO<sub>2</sub> for three min, exsanguinated, scalded at 62°C for three min, cleaned and eviscerated within 30 min. Subsequently, the carcasses were placed in a chill room at 4°C. M. longissimus dorsi (LD) was removed from the carcass, and a 2 cm thick sample (35–37 cm from the last rib) was cut out, divided into sub-pieces, vacuum-packed and stored for 3, 15 and 21 days.

### *Paper II*

The animal material used to obtain data in papers I and II was the same.

M. longissimus dorsi (LD) was removed from the carcass and a 2 cm thick sample (35–37 cm from the last rib) was cut out, divided into sub-pieces, vacuum packed and stored for 3 days at 4°C before freezing at -20°C for further peptide analysis. Measurements of pH and temperature is described by Tikk *et al.* (2007).

### *Paper III*

For Paper III, a balanced selection 32 pigs from the animal material from Paper V was made. For peptide analysis the loins (12 cm of the loin, 19–31 cm from the last rib in the cranial direction) were excised and vacuum-packed. 2 cm chops were cut 4 and 15 days after slaughter, vacuum-packed and stored at  $-20^{\circ}\text{C}$  for peptide analysis.

### *Paper IV*

**First Study.** Four pigs (cross-breeds of Duroc boar and Danish Landrace x Yorkshire dams) reared and slaughtered at The Danish Institute of Agricultural Sciences (DIAS), Foulum, Denmark, were included in the experiment. The pigs were stunned by 85%  $\text{CO}_2$  for 3 min, exsanguinated, scalded at  $62^{\circ}\text{C}$  for 3 min, cleaned, and eviscerated within 30 min. *M. longissimus dorsi* was dissected from the area of the last rib. Rectangular meat samples (3 x 3 x 2 cm), from which all visible fat and connective tissue were removed, were cut, vacuum-packed in pairs, and stored at  $4^{\circ}\text{C}$  for 1, 3, 5, and 9 days after slaughter. From all meat samples, 400 mg subsamples were taken at different times of aging to determine inosine monophosphate, inosine, and hypoxanthine in the raw meat. The meat samples were cooked in an oven at  $150^{\circ}\text{C}$  to an inner temperature of either  $70$  or  $90^{\circ}\text{C}$  and subsequently cooled to room temperature. From each cooked sample two subsamples from the outer layer 1 mm and from the centre part, respectively, were obtained. Subsequently, extractions were carried out according to the procedure described below for the determination of inosine monophosphate, inosine, and hypoxanthine.

**Second Study.** Pork carcasses were randomly selected at the slaughter line at Danish Crown, Ringsted, Denmark, according to hot carcass weight (75–79 kg) and meat percent (58.5–63.0%), and 28 carcasses were chosen and grouped according to ultimate pH, with mean values of 5.5 and 5.7 for normal-pH and high-pH groups, respectively (16 carcasses with  $5.5 < \text{pH} < 5.6$ , “normal pH”, and 12 carcasses with  $\text{pH} > 5.7$ , “high pH”). The pH was measured with a Knick Portamess pH-meter 751 (Berlin, Germany) equipped with an Ingold LOT glass electrode type 3120 (Mettler Toledo, Urdorf, Switzerland). Both loins from the carcasses were excised the day after slaughter, vacuum-packed, and aged at  $2^{\circ}\text{C}$  for either 2 days (16 loins with normal pH and 8 loins with high pH), 15 days (8 loins from each pH group), or 21 days (8 loins from each pH group), before they were frozen and stored at  $-20^{\circ}\text{C}$  until further analysis.

### *Paper V*

80 crossbred slaughter pigs (Duroc boars and Danish Landrace x Danish Yorkshire sows), half of which were females and the remaining castrates, were reared at the experimental farm at the University of Århus, Faculty of Agricultural Sciences. Both groups were distributed between high (live weight of 110 kg) and low slaughter weight (live weight of 85 kg) and were fed the standard grower-finishing feed (control feed) or a feed containing low content of digestible starch (experimental feed), resulting in a 2 (feed) x 2 (sex) x 2 (slaughter weight) experimental design. The composition of feeds and the feeding strategy is described by Tikk *et al.* (2006). On the day of slaughter, the pigs were transported (200 m) from the stable to the slaughterhouse, where they were rested for minimum 30 min, before they were brought up individually to the stunner. The pigs were stunned by 80% CO<sub>2</sub> for three min, 122 exsanguinated, scalded at 62°C for three min, cleaned and eviscerated within 30 min. 45 min after slaughter, the carcasses were placed in a chill room at 4°C. Measurements of pH and temperature is described by Tikk *et al.* (2007). For the sensory analysis, whole boneless loins cut from the 4th and 5th rib to the 3rd and 4th vertebrae (counted from the hip) were vacuum-packed and aged at 4°C for 4 and 15 days. Left and right side LD muscles aged for 4 and 15 days were randomised between animals within each raw meat quality group. The loins were subsequently frozen at 20°C until required for sensory analysis. For the precursor analysis, 1 cm thick chops were vacuum- packed individually and aged at 4°C for 4 and 15 days and then frozen at 20°C until needed.

## Methods

### Peptide sampling

#### *Papers I, II and III*

300 mg of frozen LD was homogenized (Polytron PT-MR 2100) at maximum speed on an ice bath with 800 µl of phosphate buffer (8.14 mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O and 58.72 mM KH<sub>2</sub>PO<sub>4</sub>, pH 6) and 1600 µl of water. An internal standard of 100 µl of Gly-Tyr (10 mg/ml) was added to the sample, and subsequently the sample was centrifuged (Multifuge 3 S-R, Heraeus, Germany) at 4000 rpm for 20 min at 4°C. Solid phase extraction (SPE) columns (Strata™ -X 33u, 60mg/3ml, Phenomenex, USA) were conditioned with 3000 µl of acetonitrile and 3000 µl of water, loaded with 2500 µl homogenized LD supernatant, rinsed with 1000 µl of 10%

methanol, and finally eluted with 2x500  $\mu$ l of 80% acetonitrile, 0.1% TFA (trifluoroacetic acid). SPE eluates were all filtered using 0.2  $\mu$ m PTFE membrane filters (Whatman) before further chromatography.

### Separation of peptides

#### *Papers I, II and III*

Separation of peptides from the SPE-eluates was performed on a HPLC (high-performance liquid chromatography) system (Hewlett-Packard 1100 series Germany). Separation was achieved by applying 30  $\mu$ l SPE-eluate onto a C-12 column (Phenomenex Jupiter 4 $\mu$  Proteo 90 $\text{\AA}$ , 250 x 4.60 mm, with preceding SecurityGuard Cartridge, C-12, 4 x 3.0 mm Phenomenex, USA). Subsequently a solvent gradient going from 5 % to 100 % of the organic solvent with the flow of 0.5 ml/min during 25 min was applied. The column was regenerated by elution of 100 % organic solvent for 25 min. Subsequently, the solvent gradient was reduced to 5 % organic solvent within 10 min and equilibrated with 5 % organic solvent for 5 min. Absorbance was measured at 280 nm.

### Identification of peptides

#### *Paper I*

Identification of specific peptides was performed using LC-MS (Liquid Chromatography Mass Spectrometry), combined LC-MALDI-TOF (Liquid Chromatography Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Time-of-Flight Mass Spectrometry) and N-terminal amino acid sequencing.

In the LC-MS approach, an Agilent 1100 Series (LC/MSD, Germany) system was used applying the chromatographic conditions described above; however, with a flow rate of 0.25 ml/min. The system was equipped with a diode array detector, which scanned from 190-400 nm with a bandwidth of 4 nm and the reference fixed at 360 nm. Nitrogen was used as drying and nebulizing gas with a flow of 12 l/min and at a pressure of 30 psig (206.8 kPa) at 330 $^{\circ}$ C. Fragmentor voltage was set to 120 V, gain to 1.0 EMV and step size to 0.20. A potential of 3000 V was used on the capillary. MS spectra were scanned in positive mode from 75-3000 m/z using a MSD (mass spectrum detector).

In the combined LC-MALDI-TOF approach, a capillary Hewlett-Packard HPLC system series 1200 (Germany) was used for separation of peptides, where 0.5  $\mu$ l of sample was injected onto ZORBAX SB C18 5 $\mu$

150 x 0.5 mm using the flow 5  $\mu$ l/min with the solvent gradient for solvent B increasing from 0 to 100% within 10 min, staying at 100% for 30 min, decreasing to 0 within 10 min and staying at 0 for 10 min. Selected fractions were spotted directly to a MALDI target plate using Agilent 1100 Series Micro Fraction Collector (Germany). Prior to MALDI-TOF (Ultraflex TOF/TOF, Bruker Daltonics, Germany) identification of the individual spots, the MALDI plate was added  $\alpha$ -cyano-4-hydroxycinnamic acid and 0.1% TFA.

For N-terminal amino acid sequencing, the peptides were separated according to the chromatographic conditions described above using an injection volume of 100  $\mu$ l and a flow rate of 1.5 ml/min. The individual peaks were collected using Agilent 1100 series equipped with manual injection (Germany) and subsequently up-concentrated by lyophilization before subjection to the N-terminal amino acid sequencing on a Procise Protein Sequencer (Applied Biosystems, Foster City, CA) and subsequent identification by the Fasta3 program (EMBL-EBI) and ProteinProspector Tools (UCSF).

#### Analysis of Inosine 5'-Monophosphate, Inosine, and Hypoxanthine

##### *Papers IV and V*

Pork samples (50 mg) for chemical analysis were homogenized (Polytron PT-MR 2100) for 10 s in 3 ml of ice-cold 0.6 M perchloric acid (PCA) containing a pH indicator (0.004% bromthymolblue and 0.004% phenolphthalein). The samples were left on an ice bath for 15 min before neutralization with 2.7 ml of ice-cold 0.8 M KOH and the addition of 0.125 ml of ice-cold  $\text{KH}_2\text{PO}_4$  buffer. Subsequently, the mixtures were mixed for 10 s (IKA MS 2 Minishaker), and the pH was adjusted to 7-8 using either KOH or PCA. Finally, the mixtures were centrifuged at 4000 rpm for 10 min at 4°C (Multifuge 3 S-R, Heraeus, Germany), and 1 ml of supernatant was transferred to an Eppendorf vial and frozen at -80°C until further analysis.

The samples were thawed and centrifuged at 10000 rpm for 5 min at 4°C (Eppendorf centrifuge 5417R), and the supernatants were transferred to cold HPLC vials and placed in a thermostated autosampler (1-2°C) (G.A.F. 4). Analysis of inosine 5'-monophosphate, inosine, and hypoxanthine was carried out by high-performance liquid chromatography (HPLC) (Hewlett-Packard HPLC system series 1100 Germany) using UV detection (210 nm). A 10  $\mu$ L sample was injected on the column (Lichrospher 250 x 4 mm RP18, Germany) from which the three compounds were separated by

isocratic elution using a solvent based on a buffer containing 10 mM tetrabutylammonium hydrogen sulphate and 215 mM  $\text{KH}_2\text{PO}_4$  to which 7.5 ml methanol/l was added. The following flow gradient was used to obtain optimal separation: 0.5 ml/min for 5 min, increasing to 1.5 ml/min during 1 min, and keeping this flow for 9 min before a final decrease to 0.5 ml/min in 0.5 min. Quantification was based on standard curves using external standards and calculations carried out in the included software (HP Chemstation). In the first study in Paper I, the sample size and extraction volume were increased 8 times.

## Analysis of Ribose

### *Paper IV*

The concentration of ribose was measured on six samples of high-pH meat. Three milliliters of 70% methanol was added to 2 g of minced meat in a 10 ml centrifuge tube. The mixture was homogenized (Ultra Turrax T 25, Janke & Kunkel, Staufen, Germany) for 1 min at moderate speed, followed by centrifugation at 2000g for 3 min. The procedure was repeated three times in all, and the pooled supernatants were evaporated to dryness by air. The centrifugation residues were redissolved in 4 ml of water (Milli-Q, Millipore, Bedford, MA) of which 2 ml was subjected to group separation by ion exchange chromatography as described by Andersen *et al.* (2003). Ribose was eluted with 5 x 4 x 4 ml of water, subsequently evaporated to dryness, and finally redissolved in 200  $\mu\text{l}$  of water before reductive amination and high-performance capillary electrophoresis (HPCE) analysis. The derivatization of carbohydrates by reductive amination was performed as described by Andersen *et al.* (2003) with minor modification. Twenty microliters of internal standard  $\text{D}$ -thymine (12.5 mM) was added to 10  $\mu\text{l}$  of a pure ribose solution (standard) and to 100  $\mu\text{l}$  of the redissolved purified elute from the anion exchanger. Additionally 12.5  $\mu\text{l}$  of 0.15 M tryptamine (dissolved in 10% propanol) was added to the mixtures and heated for 10 min at 90°C. Subsequently, 4.5  $\mu\text{l}$  of aqueous sodium cyanoborohydride solution (0.3 g/ml) was added, and the mixtures were heated at 90°C for 60 min.

Ribose analysis was performed by capillary electrophoresis system ABI 270A-HT (Applied Biosystems, Foster City, CA) using UV detection. For data processing, a Shimadzu (Kyoto, Japan) Chromatopac C-R3A integrator was used. The method is described by Andersen *et al.* (2003).

The concentration of ribose in meat was calculated by the equation

$$C_{\text{ribose}} (\mu\text{mol/g}) = (\text{RNA}_{\text{ribose}} \times C_{\text{internal standard}} (\mu\text{mol}) \times \text{RRF}_{\text{ribose}}) / m_{\text{meat}} (\text{g})$$

where  $\text{RNA}_{\text{ribose}} = \text{NA}_{\text{ribose}} / \text{NA}_{\text{internal standard}}$ ,  $\text{NA}_{\text{ribose}} = (A_{\text{ribose}} / \text{MT}_{\text{ribose}})$ ,  $C$  = concentration,  $\text{MT}$  = migration time,  $\text{NA}$  = normalized area, and  $\text{RNA}$  = relative normalized area.

For the quantification of ribose, relative response factors (RRF) to the internal standard  $\text{D}_5$ -thymine were calculated. RRF was determined as  $\alpha_{\text{D}_5\text{-thymine}} / \alpha_{\text{ribose}}$ , where  $\alpha$  is the slope of the calibration curve of pure ribose solutions (0.0025–0.1M).

#### *Paper V*

The individually packed chops were thawed at 4°C and subsequently trimmed for visible fat and homogenised in a kitchen chopper (Krupps, Speedy pro, Ecully Cedex, France) for 2 x 10 sec at 10,000 rpm and then refrozen. Methanol (70% v/v, room temperature) was used to extract monosaccharides from triplicate samples of homogenised pork (Bjergegaard *et al.*, 2007). The extracted monosaccharides were separated by ion exchange chromatography into neutral and phosphorylated monosaccharides followed by reductive amination and analysis by HPCE (Agilent Technologies, Santa Clara, USA) using UV detection as described by Bjergegaard *et al.* (2007) and Andersen *et al.* (2003).

## Sensory analysis

#### *Paper II*

Pork chops (2 cm) were cut from the left LD and fried in a pan at 160°C and turned every 2 min until inner temperature reached 70°C. The temperature was measured in each chop by a handheld thermometer (Testo 926, Testotherm, Buhl and Bundsoe, Virum, Denmark). LD from the right side was prepared in roasting bags in a convection oven at 90°C until inner temperature reached 65°C. Subsequently, the loins were cut to 1.5 cm slices. For sensory assessment two slices of meat (5 x 5 x 1.5 cm) from both cooking methods were served immediately to each assessor on hot plates with a three-digit number in a randomized design. The panel was familiar with assessment of pork and had been trained according to ISO 4121, ASTM-MNL 13, DIN 10964. Four assessors, two male and two female (aged between 44 and 59 years) were used for evaluation of the pan-fried pork chops. For evaluation of whole roasts eight assessors, all females (aged between 48 and 63 years) were used. The samples were assessed on a 15 cm unstructured scale from nothing to very intense. The attributes for pork chops were: fried meat colour (surface), fried meat odour, sweet odour, sour

odour, sour taste, bitter taste, sweet taste, metal taste, piggy flavour, fried meat flavour, hardness, crumbliness, fibrousness, tenderness and juiciness. The attributes for whole roasts were: boiled meat odour, sweet odour, sour odour, piggy odour, pores, boiled meat flavour, piggy flavour, sourish taste, metal taste, rancid flavour, hardness, juiciness, fibrousness, crumbliness and tenderness.

#### *Papers III and V*

Loins were thawed at 4°C and subsequently cut into 2 cm thick chops and equilibrated at room temperature until a core temperature of 14–17°C was reached. The chops were fried on a pan (Pano Copter Stekbord 9200 cm, Göran Persson Maskin AB, Göteborg, Sweden) smeared with a thin layer of grape seed oil at a pan temperature of 155°C to a core temperature of 70°C. The pan temperature was measured during frying with a surface thermometer (Digitron, 2038 T, Sigfam Instruments Ltd, UK), and the core temperature was measured with a Testo 926 thermometer (Buhl & Bønsøe, Virum, Denmark). The chops were turned after 2, 4, 6 min etc. until 70°C was reached. The cooking loss was determined as the difference in weight before and after frying.

A descriptive sensory analysis was used to study the differences between loins from the 16 treatments. Nine trained assessors (eight females and one male aged between 37 and 64 years, and with 2–17 years of experience in assessing pork) at the Danish Meat Research Institute (DMRI) assessed the pork samples. The panel had previously been trained according to SO4121, ASTM-MNL 13, DIN 10964, and DIN 10952. The panel was trained during three sessions, in which the assessors were presented with the expected sample extremes and a common terminology was defined using different references. Following sensory attributes were used in the study: fried meat odour, roasted nut odour, piggy odour and sour/acidic odour, fried colour, moist appearance and unevenly fried appearance, fried meat flavour, sweet, sour/acidic, salty, piggy and metallic flavour, juiciness, tenderness, hardness, crunchiness, fibrousness, crumbliness, chewing time and chewing remains/rest.

Before serving, the chops were cut into samples measuring 3 x 4 cm taken from the middle of the chops. The samples were served on pre-heated plates covered with an aluminium foil container. The assessors were instructed first to evaluate the odour attributes and then the appearance attributes. The assessors then halved the sample longitudinally and used one slice for the evaluation of flavour attributes and the other slice for the evaluation of texture attributes. At the beginning of each session, the

assessors were given a warm-up sample, which they assessed in the same way as the other samples; however, data were not collected. Each sample was given a three-digit code and served in a randomised order within each session. Randomisation between assessors was not used. The attributes were evaluated on a 15 cm unstructured line scale (from no intensity, 0, to high intensity, 15) using Fizz software (version 2.0, Biosystems, Couternon, France).

#### *Paper IV*

For sensory analysis the loins were thawed at 5°C over a period of 20 h. Samples for chemical analysis were obtained, and subsequently the loins were roasted in an oven at 100°C to a core temperature of 75°C. The roasted loins were allowed to rest for 30 min at room temperature before they were cut into five 1.5 cm thick slices. The meat was served as whole meat, meat juice, and residue. Meat juice and residue were obtained by squeezing two-thirds of the remaining part of the roast in a pneumatic press (~191 kg of pressure). The meat juice was centrifuged (1000 rpm, 14°C, 5 min) to remove dissolved fat globules and subsequently brought to 30°C using a water bath before serving, whereas whole meat and the residue were served at room temperature.

The sensory attributes included in the analysis were salty, sour, sweet, bitter, umami, meaty, brothy, piggy, fatty, and cooked root vegetables. The intensity was evaluated using a 15 cm non-structured line scale. The panel for the sensory analysis received basic training based on ISO, ASTM-MNL 13, DIN 10964, and DIN 10952. The panel consisted of eight assessors—six female and two males—all living in or around Roskilde, Denmark. All assessors were familiar with pork and descriptive analysis. Prior to the analysis, the panelists were given four training sessions on both basic tastes and the samples represented in the experiment. They were also trained in how to distinguish between retronasal flavour perception and basic taste. The intensity was evaluated using a 15 cm nonstructured line scale, end-anchored with 0 = little and 15 = very much. The assessors were served either two identical meat samples or two identical samples of dry matter residue (1 x 2 cm). The meat juice (15–20 ml) was served in small plastic cups. Panelists used nose clips when evaluating the first sample (basic taste perception) and no nose clip when evaluating the second sample (retronasal flavour perception). During training, the following reference compounds were used: sour (citric acid, 2 g/l), sweet (saccharose, 12 g/l), bitter (quinine hydrochloride, 0.01 g/l), umami (L-glutamic acid monosodium salt, 2 g/l), brothy (Knorr, pork stock cube), and greasy (decoction of oxtails). All three

fractions from the same animal were served in the same session in a randomized design.

## Statistical analysis

### *Paper I*

Data analysis was performed using SAS v. 8.02 (SAS Institute Inc., Cary, NC) for analysis of variance with the MIXED procedure and Unscrambler v. 9.6 (Camo Process AS, Oslo, Norway) for partial least square regression analysis (PLS1).

Mean values of the peak areas from three extractions were used for the ANOVA model, where animal was set as random effect, aging and sex as fixed effects. Least-square means (LSM) were calculated for the effect of aging. Interactions of the fixed effects were included if significant ( $p < 0.05$ ).

PLS1 analysis was performed on data from the LC system. The data were handled as spectroscopic data, subjected to prior reduction along the retention time, and aligned according to main peaks on the chromatogram. Aging time as response variable (y) was analysed in relation to peak heights at each time point on the chromatograms (x) using full cross-validation without standardizing of the data.

### *Paper II*

Data analysis was performed using SAS v. 9.1 (SAS Institute Inc., Cary, NC) for partial correlation analysis and analysis of variance, and Unscrambler v. 9.8 (Camo Process AS, Oslo, Norway) for partial least square regression analysis (PLS2). Data analysis was performed separately for both cooking methods – whole roast and chops. Mean values of the peak areas from three extractions were used for partial correlation analysis.

The proc GLM model with MANOVA statement was used for calculating partial correlations between sensory attributes and peptides, after correction for the effect of sex and pH at 45 min post-slaughter. Significant differences between least-square means (LSM) were evaluated using the option pdiff.

For prediction of the sensory data from the peptide data, PLS2 analysis was carried out on the standardized sensory data (Y-variables) and standardized peptide peak areas (X-variables) using full cross-validation.

### *Paper III*

Data analysis was performed using SAS v. 9.1 (SAS Institute Inc., Cary, NC) for partial correlation analysis and analysis of variance, and Unscrambler v. 9.8 (Camo Process AS, Oslo, Norway) for partial least square regression analysis (PLS2). Calculations were carried out separately for both aging times. Mean values of the peak areas from three extractions were used for all methods of statistical analysis.

The proc GLM model with MANOVA statement was used for calculating partial correlations between sensory attributes and peptides. The model contained the fixed effects feed group, weight class and sex. To visually validate the obtained correlations, significant correlations using the output statement of the corrected values were plotted. The proc GLM model for analyzing the influence of production parameters on peptides contained the fixed effects feed group, weight class and sex. Significant differences between least-square means (LSM) were evaluated using the option pdiff.

For prediction of the sensory data from the peptide data, PLS2 analysis was carried out on the standardized sensory data (Y-variables) and standardized peptide peak areas (X-variables) using full cross-validation.

### *Paper IV*

Data analysis was performed using SAS v. 8.02 (SAS Institute Inc., Cary, NC) for analysis of variance with the MIXED procedure and Unscrambler v. 9.1 (Camo Process AS, Oslo, Norway) for principal component analysis (PCA). The ANOVA model for analyzing chemical data contained animal as random effect in the first experiment and animal nested within pH in the second experiment. Aging time and treatment were set as fixed effect in the first experiment and aging time and pH group as fixed effect in the second experiment, and the interactions were set as fixed effects in both experiments. Interactions were kept in the model for calculating necessary least-square means (LSM). The concentrations of IMP and inosine were used in the model as covariates when inosine and hypoxanthine, respectively, were analysed. Data analysis was performed separately on data as to whether a nose clip was used or not and separately for each fraction – meat, meat juice, and residue.

The ANOVA model for analyzing sensory data contained animal nested within pH as random effect, aging time, pH group, and the interaction as fixed effect. The concentrations of IMP and hypoxanthine were used as covariate in the models for analyzing brothy/meaty and bitter/salty/piggy taste, respectively, and removed from the model when non-significant. To

calculate LSM and p values for flavour attributes and different meat fractions, a similar model without separating the fractions, excluding chemical data and including assessors as random effect was used.

Pearson correlation coefficients together with probability values were used for correlation analysis.

PCA was performed on whole meat, meat juice, and the residue fraction, respectively, in combination with the flavour perception method using full cross-validation without standardization of the observations.

#### *Paper V*

The data were analysed by ANOVA mixed models using SAS v. 9.13 (SAS Institute, Cary, USA) (Littell, Milliken, Stroup, & Wolfinger, 1996). In general, the factors feed (control and low glycogen), gender (female and castrate), slaughter live-weight (low and high) and, where appropriate, also side (right and left) and aging (4 and 15 days) were fixed effects, whereas animal, litter and assessors were generally random effects. The models used to analyse the experimental factors differed from each other. Therefore, the data were carefully inspected in each case and the important random effects were identified and included in the model, whereas non-important random effects were excluded. Due to the large number of treatments, and hence even larger number of possible treatment interactions, care was taken in the choice of the so-called random effect part of the models.

The sensory data were further analysed for any possible correlation with flavour precursor concentrations using multivariate data analysis: principal component analysis (PCA) and partial least squares regression (PLS2) using Unscrambler v. 9.2 (Camo AS, Oslo, Norway). Data were autoscaled, and the models were validated using full cross-validation.

## Summary of presented papers

### I Specific peptide profiling during aging of pork

The present study established an analytical approach for monitoring specific peptide patterns in meat. This approach was subsequently used to characterize the development in peptide patterns of porcine *M. longissimus dorsi* during aging. The change in peptide patterns during aging of pork proceeded through continuous increase and decrease of individual peptide fractions, which refer to gradual degradation of individual proteins and peptides throughout aging. Subsequent analysis of selected peptide fractions using Liquid Chromatography Mass Spectrometry, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Time-of-Flight Mass Spectrometry and/or N-terminal amino acid sequencing made it possible to identify numerous specific peptide fragments. Several of those specific peptide fragments containing 8-22 amino acid residues could through subsequent database sequence searches be related to hydrolysis products of glyceraldehyde-3-phosphate, troponin T and myosin heavy chain.

### II The significance of peptide profile on pork flavour

Peptides in meat are the result of proteolytic activity and are known to have a direct effect on flavour development or undergo subsequent reactions and form further flavour compounds. The present study investigated the effect of the peptide pattern in relation to sensory profile in meat using oven roasted pork loins and pan-fried pork chops aged for 3 days. Correlations found between flavour attributes and peptides can be used as predictors in finding the link between specific peptides and sensory properties. Pan-fried pork chops showed some tendency to have more pronounced bitter notes and

reduced juiciness. Finally, the previously reported sourness-suppressing effect of a peptide originating from troponin T is not in accordance with the present study and need further investigation.

### **III The significance of sex, carcass weight and diet on peptide profiles and sensory quality in pork**

Degradation of proteins during aging of meat is known to increase the tenderness of meat and moreover, increases in the fractions of amino acids and peptides have also potential to influence the flavour development. The present study explored the changes in peptide profile as influenced by production parameters and the subsequent influence on flavour development during aging of pork.

A decreased rate in the development in peptide pattern in meat was formed when glycogen-reducing finishing feeding was applied and when the animals were slaughtered at low weight. A previously described troponin T-derived sourness-suppressing peptide was found to suppress sour odour in the pork at 4 days of aging, supporting the theory that it binds to sour eliciting substances. Finally, it can be concluded from the present study that further identification of specific peptides will give rise to a deeper knowledge about the origin of the peptides and explain the potential of specific peptides on both texture and flavour development.

### **IV Development of inosine monophosphate and Its degradation products during aging of pork of different qualities in relation to basic taste and retronasal flavour perception of the meat**

Inosine monophosphate (IMP) and its degradation products, ribose and hypoxanthine, are all considered to be important constituents in meat flavour formation and development. The present study explored the fate of IMP during the aging of two qualities of pork ( $5.5 < \text{pH} < 5.6$  and  $\text{pH} > 5.7$ ) and the potential relationship between IMP, hypoxanthine, and sensory attributes of pork registered both as retronasal and basic taste responses in whole meat, meat juice, and the remaining meat residue. During aging the concentration of IMP decreased with a simultaneous increase in the concentrations of inosine, hypoxanthine, and ribose. The rates at which IMP was degraded to inosine and inosine to hypoxanthine during aging were found to be in agreement with the known rate constants of the dephosphorylation of IMP and the hydrolysis of inosine, respectively. Moreover, high-pH pork resulted in a significantly higher concentration of

hypoxanthine throughout storage compared with low-pH pork due to an initially higher concentration of IMP in high-pH meat. The sensory analysis showed increasing intensity in bitterness and saltiness of pork as a function of aging, with the intensity being most pronounced in the meat juice. The increasing bitterness of the pork as a function of aging coincided with the higher content of hypoxanthine in these samples, thereby suggesting that degradation of IMP to hypoxanthine might influence pork flavour. In contrast, IMP was associated with non-aged meat and the sensory attributes meaty and brothy.

#### **V Flavour development in pork; influence of flavour precursor concentrations in longissimus dorsi from pigs with different raw meat qualities**

Flavour development and overall eating quality of pan-fried pork chops of *M. longissimus dorsi* from eight different raw meat qualities aged for 4 and 15 days were assessed by a trained sensory panel. The raw meat qualities were obtained through combinations of strategic feeding/fasting (control vs. low glycogen concentration), slaughter live-weight (85 kg vs. 110 kg), and gender (female vs. castrate). The flavour development was investigated for possible correlation with the concentrations of selected individual flavour precursors present in the raw meat: monosaccharides, IMP and degradation products, fatty acids, lactate and thiamine. Differences in precursor concentrations between the raw meat qualities were observed with feeding/fasting and aging as the main factors with the largest influence of all experimental factors. However, the concentrations of the precursors could not explain the differences in sensory perception of the pan-fried pork chops. Overall, the differences were small.



## General discussion

Flavour precursors are defined as chemical compounds inherent in fresh meat, which through post-mortem degradation either directly or through subsequent reactions upon cooking form numerous volatile or non-volatile taste compounds (Chen & Ho, 1998; Shahidi, 1989). Many of these flavour precursors have been reported to develop/degrade during aging and cooking of meat. Moreover, the variation in meat quality, caused by e.g. pre-slaughter stress, or derived from the specific parameters in animal production has been found to influence the rates of these post-mortem processes and the subsequent acceptability of meat.

In relation to the present thesis, two groups of flavour precursors, peptides and ATP metabolites were investigated in relation to flavour development. Subsequently, the influence of primary production parameters and of aging and cooking methods on the formation of specific flavour compounds was investigated.

Initially, an analytical method for peptide analysis was developed to monitor the changes taking place in the peptide fraction of meat during aging. Further analysis of separated peaks resulted in identification of 12 specific peptides from 6 peaks from the peptide pattern (Paper I). Subsequently, the flavour development and production parameters in relation to peptide development were investigated (Papers II and III). Next the development of IMP and its degradation products was followed in meat of normal and high pH during aging in relation to basic taste and retronasal flavour development (Papers IV and V).

An analytical approach to follow the peptide pattern was developed and described in Paper I. Combining the initial solid phase extraction of homogenized meat samples with reversed phase liquid chromatography resulted in appropriate fractionation of peptide fractions, which were

subsequently able to be further characterized using either mass-spectrometric techniques or more straightforward N-terminal amino acid sequencing.

The characterization of the five selected peptide fractions (peaks 10, 11, 12, 13 and 15) was successful (Paper I) and revealed by direct LC-MS analysis that peak 11 contained mass fragments analogous to a tri-peptide that contains two tryptophan residues and one serine residue. However, further analysis is needed to determine the exact sequence of this tri-peptide. MALDI-TOF analysis of peaks 10, 12, 13 and 15 revealed that they contain peptide fragments with the following sequences TDK/QEK/QRK/QK/Q, APPPPAEVHEVH, APPPPAEVHEVHEE, APPPPAEVHEVHEEVH, DNEFGYSNR, TQYKFGHT, APPPPAEVHEVHEEVHEVHEPE and EAPPPAEVHEVHEEVHEVHEPE.

A database search of peptide fragments created with MALDI-TOF showed significant hits of APPPPAEVHEVH, APPPPAEVHEVHEE, APPPPAEVHEVHEEVH, APPPPAEVHEVHEEVHEVHEPE and EAPPPAEVHEVHEEVHEVHEPE to fast skeletal muscle troponin T (gi|46389773, NCBIInr). The 32 kDa fragment of fast troponin T has previously been reported to form during aging of pork (Hwang *et al.*, 2005; Okumura *et al.*, 2003; Lametsch *et al.*, 2002). Based on in vivo studies the calpains are shown to be responsible for specific fragmentation of peptide APPPPAEVHEVHEEVH from troponin T. Moreover, the cleavage to obtain peptide APPPPAEVHEVHEE during aging in meat is suggested to be the result of another proteolytic system (Kitamura *et al.*, 2005). Furthermore, the degradation of troponin T has been found to improve tenderness of pork (Hwang *et al.*, 2005; Melody *et al.*, 2004), as also settled in early studies on peptides of importance for tenderness in beef (Penny and Dransfield, 1979). Peptide fragments TQYKFGHT match with the sequence from pig myosin heavy chain (gi|157279731, NCBIInr) and DNEFGYSNR with glyceraldehyde-3-phosphate dehydrogenase (UniRef100\_P00355, UniProtKB/Swiss-Prot). Moreover, a blast search of the N-terminal amino acid sequencing of peak 12 resulted in a significant match to a fragment of glyceraldehyde-3-phosphate dehydrogenase (G-3-PD), residues 247-257. G-3-PD catalyzes the conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate in the oxidation process of glucose (Alberts *et al.*, 2008). MALDI-TOF analysis gives a possible continuation of the sequence until residue 265, matching the theoretical molecular weight 2189.56 Da, based on the database-derived sequence and a peak from MALDI-TOF. Hydrolytic degradation of G-3-PD in pork during aging has previously been reported in studies by Lametsch *et al.* (2006) and Okumura *et al.* (2003). Moreover, G-3-PD fragments, residues 258-265, have

previously been identified in trichloroacetic-acid-soluble extracts of bovine *M. longissimus dorsi*, which shows that hydrolysis of G-3-PD appears to be a generic process taking place during aging of meat (Stoeva *et al.*, 2000).

Even though the MALDI-TOF analysis was successful in identifying peptide fragments in four peaks, clear mass spectrum was not obtained from several of the other peaks. This is allegedly due to the limited mass range of the MALDI instrument in the region below 900 m/z, where the matrix used for ionization of peptides is known to affect the obtained spectra (Hjernø & Jensen 2007). Moreover, the fact that the analysed peaks most probably contain two or more peptides in an unknown concentration suggests a limit to functionality of the MALDI instrument. This is because of the specificity of the MALDI instrument for peptides from tryptic digests, and occasionally the ionization of the peptides depends on specific sequences and amino acids that can suppress ionization of other peptides in the sample.

The effect of aging on peptide development was studied in Paper I. The combined chromatographic approach of meat homogenates from pork aged for different periods clearly showed a development in peptide patterns during aging. In addition, further statistical analysis of the obtained data showed an overall tendency to a continuous increase in peptide formation throughout aging, as also reported previously (Moya *et al.*, 2001a; 2001b; Feidt *et al.*, 1998). However, this more thorough analysis of the progress in peptide fractions throughout aging also showed that a number of peptide fractions, which appeared during the early stages of the aging period, decreased during the later stages of the aging period. This clearly shows that hydrolytic products of meat proteins are continuously degraded throughout the aging period, as previously reported by Lamare *et al.* (2002). No specific patterns in the progress in peptide profiles were evident from these data. However, the most pronounced change took place between day 12 and day 21 of aging, even though significant changes also took place between days 3 and 12, and throughout the aging period (day 3 to 21). Post-mortem proteolytic activity in meat has been contributed to a large extent to calpain activity. The effect of calpains on myofibrillar proteins in relation to tenderness development during aging has been suggested to take place during the first 3 to 4 days post-mortem; however, cathepsins are expected to become active at low pH and after release from lysosomes, contributing to the proteolysis after 6 to 7 days post-mortem (Hwan & Bandman, 1989; Bandman & Zdanis, 1988). Moreover, the effect of the proteasome complex has been found on several proteins in various conditions, and their broad range of substrates (Lamare *et al.*, 2002; Taylor *et al.*, 1995b; Rock *et al.*,

1994; Mykles & Haire, 1991) has shown their potential on peptide development. Thus the development of peptides in the current study can be assigned to a multi-component pool of proteases (Houbak *et al.*, 2008; Ouali, 1992).

Paper IV showed a decrease in the concentration of IMP and a simultaneous increase in the concentrations of inosine and hypoxanthine in fresh pork during aging and, hereby, resemble data previously reported by Kato & Nishimura (1987). However, the temporary accumulation of IMP in the meat during the first day post-mortem was not confirmed unlike the results from Dannert & Pearson (1967) on beef.

The maximum concentration of IMP and its degradation products, inosine and hypoxanthine, was reached at 72 h after slaughter, which is in agreement with data by Lindahl *et al.* (2004), who found that the post-mortem metabolism proceeds up to 2 days post-slaughter.

The data show that inosine accumulates without noticeable increase in the concentration of hypoxanthine during 9 days of aging. This can be explained by the fact that the rate at which IMP is dephosphorylated to inosine is much more rapid than the hydrolysis of inosine to hypoxanthine (Durnford & Shahidi, 1998).

Additionally, the effect of cooking end-temperature on IMP and its degradation products in relation to aging and sampling was investigated. The degradation of IMP proceeded more rapidly at relatively high temperatures, whereas only small or no differences were found in the concentrations of inosine and hypoxanthine upon increase in heating temperature from 70 to 90°C. This indicates that during heating both inosine and hypoxanthine go into subsequent reactions with other constituents in meat.

A highly significant difference was found between the concentrations of IMP, inosine and hypoxanthine in the centre and at the surface of the pork samples, with a higher concentration at the surface. This might be explained by a pronounced dehydration of the outer layer during cooking, although it cannot be completely ruled out that a thermally induced dephosphorylation of residual ATP/ADP and AMP may also take place. However, additional studies are needed if this aspect has to be elucidated further.

The effect of animal production parameters on peptides was studied in Paper III. The experimental feed did not have any effect on pH early after slaughter; however, a higher ultimate pH was found. Moreover, changes in the metabolism resulted in an accelerated rate of temperature decrease at cooling early after slaughter and resulted in lower temperature at 24 h after slaughter in the experimental feed group. Calpains have been reported as the

most active proteases after slaughter, and their activity contributes to the degradation of proteins at higher pH and at the first 3 to 4 days post-mortem (Koochmaraie *et al.*, 2002; Veiseth *et al.*, 2001), which is expected to increase the rate of peptide development. Interestingly, a higher concentration of peptides was found in the control feed group. This can be explained by the compensation of lower energy intake by up-regulation of the calpain inhibitors, calpastatins, during the strategic feeding, leading to a slower post-mortem peptide development. Similarly, Therkildsen *et al.* (2002b) found a decreased calpain activity in pork in a compensatory growth study when a short compensatory growth period was used, suggesting calpain activity to be dependent on the length of the subsequent ad libitum feeding after the restricted feeding.

Peptide development in meat was more pronounced in the heavy weight class animals. This can be contributed to the higher temperature in heavy weight class animals at 45 min and 24 h post-mortem, which has been shown to support calpain activity (Hwang & Thompson, 2001; Koochmaraie, 1996; Dransfield, 1994). On the other hand, a lower enzyme activity in pig *M. transversus abdominis* has been reported to be the result of a more pronounced protein denaturation due to fast post-mortem pH decline (Claeys *et al.*, 2001).

The degradation of IMP in the second study in Paper IV was found to be weakly dependent on the meat quality (normal or high pH), with pork of normal pH having lower IMP concentration after 2 days of aging. However, meat quality was not found to influence the degradation of IMP further during aging. This influence of meat quality might be explained by the fact that the stability of IMP, containing weak chemical bonds, for example glucoside and ester bonds (Matoba *et al.*, 1988), is both temperature- and pH-dependent. This is why low pH might accelerate the dephosphorylation of IMP in the period early post-mortem. The degradation of IMP showed a similar pattern in Paper V; however, no significant effect of production parameters on any of the ATP metabolites was found. Longer aging time used in Paper V in relation to Paper IV may be sufficient to level out the concentration of IMP between the feed groups. Moreover, even if higher levels of IMP are expected in meat with higher pH, created by the glycogen-reducing experimental feed, the level of IMP may be influenced by the overall lower energy content of the muscle in animals given the experimental feed.

As expected, the concentration of ribose increased during aging in the samples analysed in Paper IV. Absolute values of ribose equal to the concentration of hypoxanthine thereby support the stoichiometry of the

degradation of IMP. In contrast, the concentration of ribose in the samples analysed in Paper V was found to be rather low, and did not follow the same rule.

The correlation between peptide pattern and flavour attributes in whole roasts and pan-fried pork aged for 3 days was investigated in Paper II. The pan-fried chops are generally expected to have pronounced fried notes derived from the Maillard reaction products between volatile compounds created at the higher temperatures between amino acid and reducing sugars. The obtained results showed a few differences between cooking methods in peaks identified in Paper I. Peak 11 was positively correlated to boiled meat odour and meat flavour in whole roasts, whereas pork chops had a positive correlation to crumbliness. Crumbliness has previously been reported to be strongly correlated to tenderness by Bejerholm & Aaslyng (2004). Moreover, the same authors report on higher correlation coefficients between tenderness and drip loss in pan-fried pork chops and lower correlation in oven-roasted loins. Peptide identification in this peak by LC-MS analysis and N-terminal amino acid sequencing resulted in two fragments with no success in the subsequent blast search from the protein sequence database. However, these results give reason to assume that the identified peptides originate from structural proteins. Peak 13 in Paper II was positively correlated to sour taste; however, this result is not in accordance with the negative correlation of peak 13 to sour odour in Paper III. Identified peptides in peak 13 originate mainly from troponin T and from G-3-PD. Degradation of troponin T is found to be a generic process taking place post-mortem in several species (Muroya *et al.*, 2006; Lametsch *et al.*, 2002; Geesink & Koohmaraie, 1999; Claeys *et al.*, 1995) and the appearance of the 32 kDa fragment in pork during aging has been found to be correlated to tenderness development. A further increase in smaller fragments originating from this protein produced during aging has been found in beef (Stoeva *et al.*, 2000) and pork (Okumura *et al.*, 2003), and also found in ready-to-eat beef meat (Bauchart *et al.*, 2006). Moreover, the effect of this fragment on flavour is supported by the results from Okumura *et al.* (2004) and Nishimura *et al.* (2004) showing the sourness-suppressing properties of a peptide APPPPAEVHEVHEEVH originating from troponin T. This also supports the negative correlations of peptides to undesired flavour attributes. However, with a small difference in the sequence in a similar peptide from beef, the potential of the sourness-suppressing peptide is emphasized by its temperature resistance found by Bauchart *et al.* (2006), who suggested that the temperature resistance was achieved by the large number of proline

residues. Nishimura *et al.* (2004) suggests the mechanism of the sourness-suppressing peptide to be either covering the entrance of the sour taste ion channel or the binding of the peptide to sour taste substances. The latter can explain the reduced sour impression in the obtained result.

The reduction in significant correlations between peptides and sensory attributes upon prolonged aging can partly be contributed to an overall lack of difference in flavour attributes at day 15 of aging as found in the overall sensory analysis in Paper V. However, masking the flavour attributes with a number of other substances and flavour precursors developing during aging can be a possible explanation. Moreover, based on the results from Paper I, which shows that the development in specific peptides originating from structural proteins to large extent takes place in the later stage of aging, where according to the results from Paper V changes in tenderness and other texture-related attributes take place, the development in the peptide pattern cannot readily be related to the development in texture attributes in cooked pork.

The variations in data from both studies analysed by PLS2 analysis confirm the results obtained from partial correlation analysis. PC1 in whole roast pork expand texture characteristics, and PC2 expand the flavour characteristics. PC1 in pork chops in Paper II expand texture attributes and also flavour attributes such as fried meat odour, sweet taste and meat flavour against metal and bitter taste. This more pronounced flavour can be due to the Maillard reaction products formed during pan-frying compared to oven roasts; however, in Paper III PC1 in meat aged for 4 days clearly expand the texture attributes with almost no correlation between flavour attributes. The overall tendency to more pronounced texture attributes is in accordance with the results from Meinert *et al.* (2008b), who showed that the differences in texture attributes are more easily detectable. Moreover, the effect of peptides on flavour attributes seems to disappear with prolonged aging.

Depending on whether sensory analysis was performed with or without nose clip, the difference in perception of salty, sour, bitter, and umami attributes (basic tastes) and the other sensory attributes (e.g. meaty, brothy, and fatty flavour) suggests that this approach fulfils the required distinction between basic taste perception and retronasal flavour perception (Paper IV).

The difference in pH of the two meat quality groups influenced the sour flavour attribute, resulting in higher scores of sourness of the meat with normal pH, as expected from previous results (Bryhni *et al.*, 2003). The source of the sour taste might mainly be lactic acid from an extensive buildup of lactic acid from post-mortem glycolysis of meat with low pH

(Jeremiah *et al.*, 1990). Schlichtherle-Cerny & Grosch (1998) showed that lactic acid is the most active ion in beef broth and that 83% of the assessors in a sensory triangle test could recognize the omission of lactic acid in a model system with the broth. Lactic acid is water-soluble, and the low scores for the sour flavour attribute in the residue fraction might thus be a result of the combination of the facts that most of the lactic acid is no longer in this fraction, but is in the meat juice, and that it is more easily masked by other flavour compounds present in the residue fraction.

The bitter taste can be elicited by hypoxanthine, hydrophobic amino acids, either free or bound in smaller peptides (Tanimoto *et al.*, 1992; Matoba & Hata, 1972), and certain Maillard reaction products, for example, pyrazines (Belitz & Wieser, 1985), which might be perceived by basic taste perception as well as by retronasal flavour perception. The flavour attribute bitter increased as a function of aging in both the meat and the residue fraction as assessed by retronasal flavour perception, with the increase being most pronounced from day 15 to 21.

Brothy flavour in beef and pork broth has been shown to increase with time (Cambero *et al.*, 2000; Kato & Nishimura, 1987), and it is believed to be influenced by proteolytic breakdown of proteins to free amino acids and peptides. However, in the present study development in the brothy flavour attribute as a consequence of aging time was not found.

Compounds contributing to a specific pork flavour, such as 2-methyl-3-furanthiol, and the corresponding disulfide from the thermal degradation of thiamine have been reported to have an extremely low odour threshold (Gasser & Grosch, 1988). This was confirmed by the high scores for the pork flavour attribute for all fractions in the present study. The fact that the pork flavour attribute was mainly assessed by retronasal flavour perception indicates that volatile compounds mainly contribute to this flavour attribute. This is in accordance with the general principles of the meat flavour development, as Maillard reaction products and lipid oxidation products, which are mainly volatiles, are thought to be responsible for both the meaty and the species-specific flavour of meats (Mottram, 1998; Farmer, 1992).

The pork flavour was not influenced by the pH of the meat (Paper IV). This is in contrast to the results by Bryhni *et al.* (2003), who reported lower intensities of pork flavour in meat with a high pH. This discrepancy might be due to the rather small pH difference and to the low cooking temperature, which results in only low intensities of the pork flavour attribute.

No significant relationship was established between ribose and flavour attributes in neither Papers IV nor V. This might be due to the relatively

small number of samples analysed in both papers, and the relatively low cooking temperature in Paper IV, or the fact that the flavour attributes included in the sensory analysis do not reflect ribose or ribose-related flavour compounds. Further studies are needed to exploit this.

To illustrate the connection between sensory attributes of the two meat qualities during aging and IMP and its degradation products, a PCA of each of the three meat fractions was carried out. The obtained PCs clearly distended the time of aging and the quality of the meat in the loading plots. The data showed that the sensory attributes brothy and meaty correlated significantly with non-aged meat and IMP, being most pronounced in whole meat, and thereby they support previous data showing that IMP is a desirable flavour enhancer in meat and fish (Madruga, 1997; Murata & Sakaguchi, 1989; Maga, 1987). Moreover, the correlation between the sensory attribute bitter and hypoxanthine after aging for 21 days indicates that the formation of hypoxanthine upon degradation of IMP might contribute to flavour deterioration during storage of pork. This was previously suggested to be the case during prolonged storage of fish (Bremner *et al.*, 1988). Finally, the PCA data confirmed the result from the sensory analysis that the sensory attribute sour is highly correlated to the pH of meat.



## Conclusions

The developed approach has proven to be an appropriate tool to follow post-mortem changes in the peptide profile of pork during aging and hereby makes it possible to identify specific peptides that may be of potential importance for meat quality development and final meat quality. Moreover, changes in peptide patterns during aging of pork are shown to proceed through continuous increase and decrease of individual peptide fractions, which refer to gradual degradation of individual proteins/peptides throughout aging.

A decreased formation of meat peptides took place if the animals were given glycogen-reducing finishing feeding and when the animals were slaughtered at low weight. Pan-fried pork chops showed some tendency to more pronounced bitter notes and reduced juiciness, whereas whole roasts showed a positive relationship between a peptidic peak containing identified peptides. The effect of the troponin T-derived sourness-suppressing peptide was confirmed on sour odour suppression in pork at 4 days of aging.

Finally, the obtained results clearly demonstrate that aging and cooking are important for the development of essential meat flavour precursors. In contrast, the difference in meat quality (pH) within the limits studied here had only negligible effect on the development of the investigated flavour precursors. The flavour enhancer inosine monophosphate was found to contribute to the sensory attributes brothy and meaty, whereas its degradation product hypoxanthine was related to the sensory attribute bitter. Finally, the change in sensory attributes from brothy/meaty to bitter taking place during the storage of pork was found to coincide with the continuous degradation of inosine monophosphate to hypoxanthine.



## Future research

The investigations performed in connection with the present thesis have shown the effect of flavour precursors in relation to flavour development in pork pertaining to production parameters and cooking methods. Further studies are suggested to explore the following:

- Further identification and quantification of specific peptides in relation to environmental conditions and proteolytic activity will contribute to a deeper understanding of post-mortem processes.
- Specific peptides were correlated to sensory attributes; however, knowledge about the origin of the peptides might explain the potential of specific peptides in both texture and flavour development.
- The study of peptide patterns in cooked meat would contribute with knowledge of the stability of peptides in fresh meat upon thermal treatment, and the development of additional flavour-active peptides.
- Combining information about IMP and peptides as flavour precursors could help to reveal their interaction in flavour development.
- Model studies on both peptides and ribonucleotides might help to develop specific references in relation to their concentration/ratio in future sensory analysis for specific flavours.



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