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The importance of meat enzymes in ripening and flavour generation in dry fermented sausages. First results of a European project

K. Molly,[a]* D. Demeyer,[a] G. Johansson,[b] M. Raemaekers,[a] M. Ghistelinck[c] & I. Geenen[c]

[a] *Vakgroep Dierlijke Produktie, Universiteit Gent, Melle, Belgium*
[b] *Swedish Meat Research Institute, Kävlinge, Sweden*
[c] *Ter Beke N.V., Waarschoot, Belgium*

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The importance of bacterial and meat enzymes in lipolysis and proteolysis was evaluated during dry sausage ripening. The data suggest that lipolysis is to a great extent brought about by muscle and fat tissue. Polyunsaturated fatty acids are liberated from the polar lipid fraction and their specific liberation is higher than for monounsaturated and saturated fatty acids. Lipolysis seemed to be more pronounced in sausages with pork than with beef. Initial protein degradation seemed to be originating from cathepsin D like muscle enzymes. In a later stage, bacterial enzymes become more important in further degradation of the protein fragments formed. © 1997 Elsevier Science Ltd

INTRODUCTION

Fermentation is one of the oldest technologies used to store food for longer times and it is an area where microbes, meat and technology gather. The technique allows a low energy conservation of meat and remains to be considered as yielding a high quality product. Besides the valuable protein and fat nutrients, flavour compounds are abundant and can be varied by meat, starter cultures and process conditions. Optimisation and standardisation of both flavour and safety requires detailed knowledge of the relative contribution of these three factors.

Quality, linked with flavour as well as safety, is determined by the formation of end products, originating from the breakdown of proteins, lipids and carbohydrates. This process is determined by both endogenous muscle enzymes and bacteria. Therefore, it would be worthwhile to determine the relative role of muscle and bacterial enzymes in hydrolysis and further metabolism of proteins and lipids during meat fermentation and ripening. Such knowledge will allow the choice of both meat and bacteria to improve flavour and safety of fermented meat products.

This contribution will deal with the relative importance of endogenous meat and bacterial enzymes, both in lipolysis and proteolysis during dry sausage production. Two experimental model systems were used:

- Industrially produced sausages with and without added antibiotics. The sausages were produced using a short fermentation period (3 days) followed by a longer drying period (3–4 weeks).
- Muscle and fat tissue prepared under sterile conditions with or without bacterial inoculation (i.e. meat model system).

Lipids form the major fraction in fermented sausages and are considered, with proteins, to be the main substrate for production of flavour compounds, including aldehydes, ketones, alcohols, short chain fatty acids (Berger *et al.*, 1990; Stahnke & Zeuthen, 1992; Motilva *et al.*, 1993). During ripening, the lipid fraction undergoes hydrolytic and oxidative changes, involving liberation of free fatty acids (FFA) and oxidation of unsaturated fatty acids, particularly polyunsaturated acids, with production of carbonyl compounds (Demeyer *et al.*, 1974). Traditionally, lipolysis has been mainly related to bacterial lipase activity whereas oxidative changes of unsaturated fatty acids, resulting in the production of lipid peroxides and carbonyl compounds, have been related to both chemical reactions and bacterial metabolism (Demeyer *et al.*, 1974).

Accordingly, selection and identification of microbial species with lipolytic activity for use as starter cultures to guarantee flavour development associated with shorter ripening times, have often been described (Hammes *et al.*, 1985; Comi *et al.*, 1992).

Recently, the importance of endogenous meat and fat lipases in lipolysis during dry sausage ripening has been

Present address: N.V. Vitamex, Booiebos 5, B-9031 Drongen, Belgium.

suggested by Talon *et al.* (1992) and Garcia *et al.* (1992). The first group reported that free fatty acid (FFA) production in sausages with or without added mixtures of *Streptococcus* and *Lactobacillus*, could not be explained by bacterial numbers. The same group (Montel *et al.*, 1993) found that lipolysis not only occurred in samples inoculated with *S. carnosus*, but also in the controls, although the highest activity was found with inoculation. In several experiments, we have found that the FFA production was not lowered by addition of antibiotics (Molly *et al.*, 1996).

In its turn, proteolysis is considered to be one of the major processes involved in aroma development in dry sausages (Lucke, 1985; Bacus & Brown, 1985). It is reflected in a significant increase of the non-protein nitrogen (NPN) fraction during dry sausage production. This fraction can reach up to about 20% of the total nitrogen content of the product (Dierick *et al.*, 1974). The breakdown of meat proteins during meat conditioning is studied extensively because of the relation between protein degradation and meat tenderness (Claeys *et al.*, 1995; Uytterhaegen *et al.*, 1994; Ouali *et al.*, 1995). As for fermented meat products, however, the knowledge of proteolysis occurring during the fermentation and ripening process is only limited. Our objective is to identify the proteins degraded and degradation products formed during dry sausage fermentation, as a function of processing conditions. This may result in a better knowledge of the enzyme systems involved in the proteolytic process in dry sausages, which in turn can help to steer the aroma development in fermented meat products.

Another objective of our work is to establish the relative role of endogenous meat proteinases and bacterial proteinases in proteolysis in dry sausages. Again, this problem is approached using sausages with and without added antibiotics. Also, experiments with specific proteinase inhibitors are used to establish the nature of the proteinases involved. If endogenous proteolytic systems were shown to be of any importance in proteolysis, it will be worthwhile to investigate the effects of origin, age, storage time, and other properties of meat on proteolysis in dry fermented sausages.

MATERIALS AND METHODS

Preparation of dry sausages

For each experiment, a batch of basal sausage mix was prepared on a different day. Mixes were prepared by blending in sequence: pork back fat (30.6%), pork meat (30.6%), beef meat (30.6%), 3 days old back inoculation sausage (1%), glucose (0.7%), pepper (0.9%), nutmeg (0.07%), sodium ascorbate (0.03%) and colouring salt (NaCl containing 0.6% sodium nitrite) (2.85%). The mix was blended in a 45 L Kramer-Gorbe cutter. The inoculum starter contained atypical *Streptobacteriaceae* resembling *Lactobacillus sake*, *L. plantarum* and *L. curvatus*. According to the experiment, various *Micrococcaceae* were added to the sausage mix, as a suspension at a dose of 6 log colony forming units (CFU) g^{-1} fresh sausage. *Micrococcus* sp. M74 was obtained from the collection of Ter Beke N.V. (Belgium), *Staphylococcus xylosus* 863 and *S. xylosus* 350 originated from the culture collection of INRA (Theix, France). *S. xylosus* 361 was obtained from the Swedish Meat Research Institute Culture Collection (Kävlinge) and *S. xylosus* 107 from the culture collection of IATA-CSIC (Valencia, Spain). The antibiotic-antimycotic cocktail, dissolved and/or suspended in 20 ml of water, contained 200,000 I.U. penicillin, 200 mg streptomycin and 500 mg amphotericin per 10 kg of sausage mix. Proteinase inhibitors were added in the following amounts (in 20 ml per 6 kg of sausage mix): 2 mg of pepstatin, 5 mg of leupeptin, and 10 mg of E64. Where indicated, glucono-delta-lactone (GDL) was added (1%) to compensate for the decreased or absent microbial acid production and corresponding drop in pH. Per series, six sausages (1 kg, diameter 9 cm) were vacuum-filled into naturin casing. Sausages were fermented at 22°C and a relative humidity (R.H.) decreasing from 94 to 90% during 3 days. After 3 days, sausages were transferred to drying chambers (15°C, 82% R.H.). Half a sausage was obtained after 0, 3 and 21 days, ground using a meat grinder, vacuum-packed and kept at −80°C until analysis.

Preparation of meat model systems

In order to prepare sterile meat and fat tissues, the exterior of beef entrecote and pork loins were sterilised by searing using a gas burner. Surface tissues were removed using sterile instruments. The remaining meat and fat were cut into pieces and minced through 6 mm or 3 mm plate under sterile conditions. All operations were carried out in a sterile bench. The meat and fat were carefully mixed and 1.8% NaCl, 0.5% glucose, 0.01% $NaNO_2$ and 0.02% Na-ascorbate were added. Sodium chloride was heat sterilised and a solution of glucose was sterilised by autoclaving. Solutions of nitrite and ascorbate were filter sterilised. The sterility of the meat–fat mixtures was verified before and after storage.

Three different strains of *Staphylococcus xylosus* were used: SX361 (Culture Collection of SMRI), M350 (Culture Collection of INRA, Theix, France) and SX107 (Strain BS107, Instituto de Agroquímica y Tecnología de Alimentos, CSIC, Valencia, Spain). The cultures were added at a level of 10^6 CFU g^{-1} mixture. An antibiotic-antimycotic mixture (A7292, Sigma) was added to all non-inoculated mixtures (0.007 ml g^{-1}).

Samples of 100 g of the meat–fat mixtures were put as a 1 cm thick layer in glass jars (10 cm diameter, 5 cm height). A loose fitting lid was used, where the atmosphere could freely diffuse along the lid into the jar. The jars were wrapped with an oxygen permeable polyethylene film to avoid drying during storage. The

mixtures were stored at 25°C for 12 days. All the meat–fat mixtures were vacuum-packed in Lamofoil-bags (Curevac, Gothenburg, Sweden) and stored at −70°C until analysis.

Methods of analysis

Dry matter and crude fat (ethyl ether extract) were analysed according to E.U. standard methods, ISO 1442–1973 and ISO 1444–1973 respectively. Total viable counts in dry sausage samples were enumerated on Plate Count Agar (Difco) after 72 h of incubation at 37°C. Results were expressed in log CFU g^{-1} fresh sausage. Total viable counts in meat model samples were determined on APT agar (BBL, Cockeysville, Maryland, USA.) after 3 days incubation at 25°C. The final pH was measured on every sausage sample before grinding, using a Knick Portamess 654 equipped with an Ingold electrode LoT406-M6-DXK-S7/25. The pH in the meat model systems was measured with a Knick Portamess 751 instrument with a xerolyte electrode (W. Ingold Ltd, Urdorf, Switzerland). Frozen samples (5g) of dry sausages were homogenised twice (Ultra-turax, 60 s) in a chloroform/methanol mixture (2/1 v/v) (50 ml) for lipid extraction. The extracts were filtered (S and S $589^{1/2}$), washed with distilled water and the chloroform fraction made up to volume for colorimetric FFA determination according to Koops and Klomp (1977). Palmitic acid was used as a standard. Polar lipids (PL), and the neutral lipid classes, free fatty acids (FFA), triglycerides (TG) and mono- and diglycerides (MDG) were separated and quantified as fatty acids by a combination of thin-layer and gas chromatography using margaric acid (heptadecanoic acid) as internal standard as described earlier (Demeyer *et al.*, 1974). Two sausages were sampled per time and every sausage was analysed in triplicate. Results are shown as mean values ± S.D. of these six determinations. In the meat model samples, the FFA were extracted with chloroform and quantified by titration with potassium hydroxide (Anon., 1954). FFA were calculated as oleic acid and expressed as % of total fat.

Proteins were extracted from 2 g of the ground dry sausage using 50 ml sodium dodecyl sulphate buffer (SDS buffer) containing 2% (w/v) sodium dodecyl sulphate, 2% mercapto-ethanol and 0.0068% (w/w) imidazol. After 24 h of extraction at room temperature, the mixture was filtered on S and S $595^{1/2}$, heated to 100°C for 5 min, filtered again on 45 μm and stored at −18°C. After analysing the protein content of the extracts (Kjeldahl), the protein concentration was brought to 0.25 mg nitrogen ml^{-1} sample with SDS buffer. Sucrose (10%) and bromophenol blue (0.01%) were added to the sample solution prior to electrophoresis.

SDS-PAGE electrophoresis was performed according to the separation method file N° 110 (Gradient gel 8–25) and development method file N° 200 (Coomassie staining) of PhastSystem Pharmacia. The relative density of the colour development was measured with a densitometer (Ultrascan XL, Pharmacia) at 600 nm. Bovine serum albumin (BSA), was run as an external standard. The protein concentrations were expressed in BSA-equivalents, as described by Buts *et al.* (1986).

Perchloric acid extracts (0.6 N) of dry sausages were used to determine the amount of free amino acids, peptides and ammonia according to Vandekerckhove (1978). Perchloric acid extracts were neutralised using NaOH (0.6 N). Consequently, 2 ml of picryl sulfonic acid solution (0.05%) was added to 0.25 ml of the neutralised extract and the extract was incubated for 60 min at 50°C. Next, 4 ml of 0.1 N HCl was added and the extract was allowed to cool. Finally the solution was measured at 340 nm wavelength, resulting in the determination of free amino acids.

For the determination of α-NH_2-nitrogen, the perchloric acid extract was hydrolysed by heating 30 ml of the extract for 24 h at 142°C, after the addition of 60 ml HCl (6 N). After this, the amount of free amino acids was quantified colorimetrically as outlined above. Peptides were calculated using the difference between α-NH_2-nitrogen and free amino acid contents.

Ammonia nitrogen was distilled from the perchloric acid extract and absorbed in a 4% boric acid solution. The solution was titrated with a standard solution of 0.01 N HCl and the amount of ammonia was calculated accordingly.

RESULTS AND DISCUSSION

Lipid metabolism

In two experiments, we confirmed the importance of endogenous lipase activity in the sausage batter: the use of antibiotics clearly prevented normal development of bacteria but did not affect the extent of lipolysis (Table 1). In the first experiment, addition of lipolytic *Micrococcaceae* did not increase overall lipolytic activity in the presence of antibiotics. Moreover, the addition of antibiotics did not change the amount of FFA formed in the presence of lipolytic *Micrococcaceae* or not. The latter result suggests that release of lipases from bacteria lysed by addition of antibiotics does not explain lipolytic activity observed in the presence of antibiotics. However, the addition of antibiotics may increase FFA production, indicating the selection of lipolytic bacteria from the natural flora resistant to antibiotics and as such increasing the FFA content. However, in the absence of antibiotics, FFA may be degraded and consumed as a substrate by bacteria. Such utilisation may vary from batch to batch and has to be further investigated. Results should however be interpreted with care, as lipolytic *Micrococcaceae* are sensitive to a pH drop and probably die or are non-active during the drying period (pH < 5.0) (Leistner, 1992). In a second experiment, the addition of other *Micrococcaceae* increased in

Table 1. Effects of addition of antibiotics (AB) and lipolytic *Micrococcaceae* (Mic) on production of free fatty acids (FFA) (mean ± S.D.), pH (mean ± S.D.) and total aerobic counts (TAC) (mean ± S.D.) in sausages after 21 days of ripening

AB	Mic	Formed after 21 days FFA	After 21 days of ripening pH	TAC
Exp. 1				
–	+ (M74)	82 ± 3.1^a	4.71 ± 0.01^a	8.15 ± 0.08^a
+	+ (M74)	97 ± 2.6^b	5.19 ± 0.01^b	6.65 ± 0.08^b
+	—	99 ± 3.6^b	5.18 ± 0.01^b	4.72 ± 0.23^c
Exp. 2				
–	—	80 ± 4.3^a	4.78 ± 0.03^a	8.17 ± 0.13^a
–	+ (M74)	93 ± 4.4^b	4.78 ± 0.02^a	8.29 ± 0.09^a
+	+ (M74)	72 ± 3.3^c	5.01 ± 0.02^b	5.26 ± 0.07^b
–	+ (863)	81 ± 2.6^a	4.81 ± 0.03^a	7.46 ± 0.20^c
+	+ (863)	70 ± 4.3^c	4.98 ± 0.03^b	4.46 ± 0.20^d

FFA are expressed as μmoles palmitic acid g^{-1} fat and TAC as log CFU g^{-1} fresh material.
[a,b,c,d] different superscripts indicate significant differences (the two experiments should be considered separately), calculated according to a Student's *t*-test ($\alpha = 0.05$) for means (FFA) or to a one-factor ANOVA for paired series (pH and TAC). M74 = *Micrococcus* sp., 863 = *Staphylococcus xylosus* 863. (Experiment 1 from Molly *et al.*, 1996)

one case (M74) the concentration of FFA by about 14%. Addition of antibiotics lowered the amount of FFA in both cases by 12 to 20%.

Similar results were obtained by the Swedish partner: using an inoculated and non-inoculated sterile meat–fat mixture, it was shown that lipolysis is mainly (60–80%) brought about by muscle enzymes (Table 2). The lipolytic activity seemed to be higher in pork than in beef, and this was related to differences in endogenous lipolysis. The bacterial lipolysis was relatively smaller and was not influenced by the type of meat.

The differences between the relative contribution of *Micrococcaceae* to lipolysis in pilot trials (Belgium - Table 1) and in inoculated sterile meat model systems (Sweden - Table 2) could be explained by the differences in pH or by a natural lipolytic flora resistant to antibiotics. The sausages in Belgium had a rapid pH decline from ±5.6 to ±4.8, whereas the pH in the Swedish model system remained at 5.2–5.8. Muscle lipases seemed to be favoured at a lower pH (Toldrá, 1992) while the *Micrococcaceae* are more active at higher pH values (Leistner, 1992).

Table 2. Formation of free fatty acids (FFA), final pH and final amount of total aerobic counts (TAC) in meat–fat mixtures of beef and pork after 12 days storage at 25°C

Inoculation		None	SX361	SX350	SX107
Beef	pH	5.7	5.7	5.3	5.3
	TAC	< 1.0	6.5	4.8	4.8
	FFA	1.1	1.4	1.9	1.9
Pork	pH	5.6	5.8	5.2	5.2
	TAC	< 1.0	4.9	4.4	5.7
	FFA	3.2	4.4	4.0	4.0

TAC are expressed as log CFU g^{-1} fresh material; FFA are expressed as % of total fat. SX361 = *Staphylococcus xylosus* 361; SX350 = *Staphylococcus xylosus* 350; SX107 = *Staphylococcus xylosus* 107.

In the assumption that the total FFA content in the non-inoculated dry sausages (control) is entirely from endogenous lipolytic action, it can be calculated that lipolysis is mainly brought about by endogenous muscle enzymes (60–80%), varying from batch to batch and depending on the *Micrococcaceae* added.

Table 3 shows FFA production in sausages differing in degree of comminution and origin of meat. Experiments were again carried out using the sterile meat model system (Sweden) and the dry sausage model (Belgium). Both approaches indicate that lipolysis with pork is more important than with beef, whereas degree of comminution enhances lipolytic activity irrespective of meat origin.

Detailed analysis of the FA composition of the lipid classes revealed that antibiotic treatment did not affect the pattern of lipolysis. Mean FA values were calculated and shown in Table 4. It is clear that in absolute amounts, the degree of lipolysis was higher in the triglyceride fraction (71.1 mg g^{-1}) than in the polar lipid fraction (0.5 mg g^{-1}) (Table 4). However, preferential specific release (21%) of polyunsaturated fatty acids from the polar lipid fraction was observed, mainly consisting of n-6 linoleic acid. These results could be repeated, confirm earlier data from our laboratory (Demeyer *et al.*, 1974) and are in agreement with the predominant degradation of polar lipids by muscle lipases as observed in cured ham (Buscailhon *et al.*, 1994).

Toldrá (1992) has summarised the muscle and adipose tissue lipase systems present in animal and meat products. At this moment it is not clear which

Table 3. Free fatty acids (FFA) content of meat–fat mixtures of beef and pork (Sweden) and of sausages containing beef or pork (with lard) (Belgium). Details on the meat–fat mixtures can be found with Johansson (1995) and on the sausages with Demeyer *et al.* (1996)

	Particle size (mm)	Days of incubation		
Sweden		0	12	Formation (day 12–day 0)
Beef	3	1.6	2.7	1.1
	6	1.0	1.8	0.8
Pork	3	1.4	4.2	2.8
	6	1.5	3.4	1.9
Belgium		0	5	Formation (day 5–day 0)
Beef	3	28 ± 5	90 ± 5	52 ± 5^a
	6	33 ± 3	88 ± 5	45 ± 3^b
Pork	3	26 ± 2	119 ± 15	86 ± 13^c
	6	24 ± 3	113 ± 13	74 ± 6^d

The FFA contents of the Swedish experiment are expressed as % of total lipid content. The data of the Belgian partner are expressed as μmol palmitic acid g^{-1} fat.
[a,b,c,d] different superscripts indicate significant differences, calculated according to a Student's *t*-test ($\alpha = 0.05$) for means.

Table 4. Degree of lipolysis expressed as the decrease of polar lipids (PL), triglycerides (TG), and expressed as the increase of mono- and diglycerides (MDG) and free fatty acids (FFA) in dry sausages after 21 days of ripening, as fatty acids in mg/g total fat (Molly *et al.*, 1996)

	SFA	MUFA	PUFA	Total
Decrease of				
PL	0.1	0.1 (3)[a]	0.5 (21)[a]	0.5 (6)[a]
TG	36.1 (11)[a]	26.7 (7)[a]	8.3 (10)[a]	71.1 (9)[a]
Total	36.0 (11)[a]	26.8 (7)[a]	8.8 (11)[a]	71.6 (9)[a]
Increase of				
MDG	6.9	11.1	2.0	20.0
FFA	7.8	13.5	6.1	27.5
Total	14.7 (41)[b]	24.6 (92)[b]	8.1 (92)[b]	47.5 (66)[b]

[a]( ) = % of fatty acids initially present;
[b]( ) = % total increase verus total decrease, as a measure of recovery; SFA = saturated fatty acids; MUFA = mono-unsaturated fatty acids (mainly palmitoleic and oleic acid); PUFA = poly-unsaturated fatty acids (mainly linoleic, linolenic and arachidonic acid).

lipase system mentioned by the author is responsible for the biochemical changes in sausage ripening. However, it seems most likely that muscle lipases are relatively more important, as the pH in the sausages is around the optimal pH of these lipases (4.5–5.5) and our results indicate that the polar lipid fraction is degraded more intensely. Swedish results show that the enzymes in the fat tissue are more important for the total lipolysis than the enzymes in the muscle tissue. This was shown in experiments with sterile melted fat (with no enzyme activity left) *contra* fat tissue. The lipases in the fat tissue are probably most important for the total lipolysis, while the muscle lipases are responsible for the lipolysis of the polar lipid fraction, which only constitutes a minor fraction of the total fat in fermented sausages.

Protein metabolism

Using the model system with antibiotics, earlier results on sausage proteolysis were confirmed (Tables 5 and 6), although the data presented in Table 5 contrast with earlier work from our laboratory (Verplaetse, 1992; Demeyer, 1992). They do suggest a considerable contribution of bacterial enzymes to myosin degradation. However, in the experiment reported here, pH was lowered to 5.2 in the sausages treated with antibiotics, whereas the final pH was 4.7 in the control sausages. Verplaetse (1992) demonstrated a clear depressing effect of higher pH values on proteolysis. His results, showing the relation between pH and resulting endogenous proteolytic activity, allow us to correct the actual results for this pH effect (Table 5). Nevertheless the corrected data also suggest that, in contrast to earlier experiments (Verplaetse, 1992; Demeyer, 1992), addition of antibiotics had a minor inhibitory effect on myosin degradation. Actin degradation was not affected by the addition of antibiotics. The antibiotic effect was also reflected in the increased formation of peptides and the considerable decrease of free amino acid (50%) and ammonia production.

In agreement with earlier results, the proteinase inhibitor pepstatin inhibited myosin and actin degradation, as well as peptide, free amino acid and ammonia production to the same extent. The latter effects probably reflect the limitation of substrate for bacterial

Table 5. Effect of antibiotics on proteolysis in fermented sausages

	Formed after 21 days[a]			Myosin[b]	Actin[b]
	Ammonia	α-NH_2-N			
		Free	Peptide		
Control	8.1	55.6	61.1	74.5	56.9
Control + antibiotics	5.7	46.8 (27.0)	55.1 (74.0)	39.9 (60.0)	45.5 (60.0)

[a]in mmoles kg^{-1} D.M.;
[b]% of initial amount degraded as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) and densitometry using bovine serum albumin as an internal standard. The antibiotic treatment received glucono-delta-lactone. ( ) Corrected data, showing imaginary proteolytic activity at pH 4.7 (see text).

Table 6. Effect of proteinase inhibitors on proteolysis in fermented sausages

			Control	Pepstatin	Leupeptin	E64
Formed after 21 days[a]	Ammonia		8.1	5.7	10.2	7.6
	α-NH2-N	free	55.6	11.3	37.9	41.1
		peptide	61.1	27.7	68.0	67.7
Degraded (%)[b]						
	Myosin (188 kDa)		74.5	36.0	70.0	66.0
	Actin (42.5 kDa)		56.9	30.0	37.0	40.1
Formed (%)[c]						
	HMM (122 kDa)		40.5	20.3	34.6	38.1
	Protein 38 kDa		22.5	9.5	11.4	11.9
	Protein 29 kDa		17.7	6.2	5.9	6.0
	Protein 13 kDa		26.5	3.4	12.2	15.2

[a]in mmoles/kg D.M.;
[b]% of initial amount degraded as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) and densitometry using bovine serum albumin as an internal standard.
[c]amounts expressed as μg bovine serum albumin equivalents mg^{-1} sausage protein as determined by SDS-PAGE. Glucono-delta-lactone was added at 1% to compensate for the lack of microbial acid production.

proteolysis and de-amination in the presence of pepstatin. The absence of a clear inhibitory effect on myosin degradation during leupeptin addition reinforces the hypothesis that a large part of myosin hydrolysis during dry sausage ripening is due to cathepsin D (-like) activity. This is also reflected in Table 6, showing the formation of myosin and actin degradation products. Results illustrate that the inhibitor pepstatin causes, in comparison to other inhibitors, radical changes in the protein degradation pattern. The production of HMM (Heavy Mero Myosin) decreases with 50% compared to the control sausage, which corresponds to an analogous inhibition of myosin degradation. An analogous decrease of the formation of the 38 kDa and 29 kDa protein was noticed, but only a 10% formation of the 13 kDa protein was found. In contrast with these results, leupeptin and E64, which both inhibit cystein proteinases as cathepsin B, H and L, did not change the HMM formation. The production of the 38 kDa and 29 kDa proteins were similar as with the pepstatin treatment. No effect on the degradation of myosin and actin and the formation of smaller proteins (13–38 kDa and HMM) was found with the inhibitors bestatin, PMSF, APMSF and phosphoramidon (data not shown). It can be concluded that proteolysis during dry sausage ripening is predominantly determined by muscle cathepsin D-like enzymes, activated by the drop in pH (Demeyer, 1992; Verplaetse *et al.*, 1992). Cathepsin B, H and L only degrade actin and its degradation products. Moreover, it seems that a cooperation exists between cystein proteinases and acid proteinases for the formation of the 29 kDa and 13 kDa products. The results prove furthermore that trypsin-like-proteinases, serin proteinases and metallo-proteinases are not important in proteolysis during dry sausage ripening.

Analogous to proteolysis in cheese ripening, it can be assumed that both bacterial and muscle proteinases contribute to the process (Verplaetse, 1994):

PROTEIN ⟶ PEPTIDES ⟶ AMINO ACIDS ⟶ AMMONIA AMINES

|— muscle enzymes —||— bacterial enzymes —|

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