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Characterization of proteolysis during the ripening of semi-dry fermented sausages

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Abstract

The respective contribution of indigenous enzymes and enzymes from starter bacteria to proteolysis in fermented sausages were determined by comparing the proteolytic changes occurring in sausages resulting from the presence of a proteolytic strain of *Staphylococcus carnosus*, i.e. *S. carnosus* MC 1 to the proteolytic changes occurring in control sausages containing glucono-δ-lactone (GDL) and an antibiotic mixture. Proteolysis was quantified by assaying for non-protein nitrogen (NPN) and free amino acids. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and reversed phase high performance liquid chromatography (RP-HPLC) were used to qualitatively assess the proteolytic changes in the sarcoplasmic and myofibrillar proteins as ripening progressed. The concentration of NPN and free amino acids increased in both sausages initially, but subsequently decreased towards the end of ripening in sausages inoculated with the starter culture. SDS-PAGE showed a similar pattern of proteolysis of sarcoplasmic proteins in both sausages, while of the two sausage types; the *S. carnosus* MC 1 inoculated sausages exhibited the most intense degradation of myofibrillar proteins, especially myosin and actin. RP-HPLC profiles of 2% trichloroacetic acid (TCA)-soluble peptides for the two sausage types were similar, with the production of numerous hydrophilic peptides. N-Terminal amino acid sequence analysis and sequence homology with proteins of known primary structure showed that six of the TCA-soluble peptides were released from the sarcoplasmic (myoglobin and creatine kinase) and myofibrillar (troponin-I, troponin-T and myosin light chain-2) proteins. In addition, the initial degradation of sarcoplasmic proteins was due to the activity of indigenous proteinases, while both indigenous and bacterial enzymes contributed to the initial degradation of myofibrillar proteins. Furthermore, indigenous enzymes were responsible for the release of TCA-soluble peptides, which, were further hydrolysed by bacterial enzymes.

Keywords: Proteolysis; Proteolytic enzymes; Peptides

1. Introduction

Proteolysis is one of the most important biochemical changes occurring during the ripening of fermented sausages. It influences both texture and flavour development due to the formation of several low molecular weight compounds, including peptides, amino acids, aldehydes, organic acids and amines, which are important flavour compounds, or precursors of flavour compounds (Demeyer et al., 1995; Díaz, Fernández, García de Fernando, de la Hoz, & Ordóñez, 1993; Naes, Holck, Axelsson, Anderson, & Blom, 1995).

The pattern of proteolysis in fermented sausages is influenced by variables such as product formulation, starter culture and processing conditions. Johansson, Berdagué, Larsson, Tran, and Borch (1994) monitored the changes in sarcoplasmic proteins in sausage fermented for 7 days at 25 °C and dried for 42 days at 4 °C. They found that sarcoplasmic proteins with molecular weights of between 20 and 30 kDa had almost disappeared by the end of the fermentation period. Díaz, Fernández, García de Fernando, de la Hoz, and Ordóñez (1997) found that sarcoplasmic proteins with molecular weights of ~40, 44, 84 and 100 kDa were completely degraded in sausages fermented for 24 h at 22 °C and ripened for 26 days at 12 °C. Polypeptides with molecular weights of 8, 10, 11, 16, 38 and 49 kDa appeared over the same time period, while the intensities of polypeptide bands corresponding

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to molecular weights of 13, 36 and 69 kDa increased (Diaz et al., 1997).

Verplaetse, DeBosschere, and Demeyer (1989) monitored the changes in the myofibrillar proteins throughout the fermentation (22 °C for 3 days) and drying (15 °C for 18 days) of fermented sausage. They detected an increase of 80% in the concentration of polypeptides with molecular weights from 14 to 36 kDa during ripening, while myosin heavy chain (MHC), actin and troponin (TN)-T were degraded by 49, 33 and 27%, respectively. A slight increase in the intensity of peptides with molecular weights of between 10 and 13 kDa was also observed. García de Fernando and Fox (1991) reported that the intensity of α-actinin, MHC, and actin bands decreased during the ripening of dry-fermented pork sausage at 10–15 °C for 40 days. The bands corresponding to desmin, TN-C, TN-I, TN-T, tropomyosin and myosin light chain (MLC)-1, 2 and 3 were found to be stained more intensely after fermentation and drying, possibly due to their co-migration with degradation products of larger proteins, e.g., myosin. Johansson, Molly, Geenen, and Demeyer (1996) reported that myosin and actin were degraded by 75 and 57%, respectively, in sausages fermented at 24 °C for 3 days and dried at 15 °C for 18 days. Polypeptides with molecular weights of 13, 29, 38 and 122 kDa not present on day 0 were formed throughout ripening (Johansson et al., 1996).

Detailed studies on the characterization of low to medium molecular weight peptides present in the water-soluble nitrogen (WSN) and non-protein nitrogen (NPN) fractions of fermented sausages are rare. However, a study by García de Fernando and Fox (1991) using gel permeation chromatography fractionated the peptides present in the permeate obtained by ultrafiltration of the WSN fraction (nominal molecular weight cut off of 10 kDa) throughout the ripening of a dry-pork sausage. They found that a peak present in the void volume representing peptides with molecular weights of between 5 and 10 kDa remained static during fermentation, but decreased markedly during the first half of the drying period and thereafter remained constant throughout the rest of ripening at 10–15 °C for 40 days. Peptides with molecular weights of less than 5 kDa were also shown to have decreased appreciably by the end of ripening. Henriksen and Stahnke (1997) isolated low molecular weight water-soluble peptides from retail Danish salami, Italian sausage and Spanish chorizo by gel filtration chromatography and found the molecular weight of the majority of the peptides to be less than 4.2 kDa. In addition, studies determining the amino acid composition of low molecular weight water or trichloroacetic acid (TCA)-soluble peptides by acid hydrolysis and subsequent amino acid analysis revealed the presence of mainly hydrophilic peptides in fermented sausages (Henriksen & Stahnke, 1997; Zapelena, Zalacain, De Peña, Astiasarán, & Bello, 1997).

The respective roles of indigenous and bacterial enzymes in protein degradation during sausage ripening have been a source of controversy (Verplaetse et al., 1989). However, numerous studies over the last decade have concluded that indigenous proteinases, particularly cathepsin D, are primarily responsible for proteolysis during fermentation, while bacterial enzymes are more important during the latter stages of ripening (Demeyer, et al., 1995; Demeyer, Claeys, Otles, Caron, & Verplaetse, 1992; Molly, Demeyer, Johansson, Raemaekers, Ghistelinck, & Geenen, 1997; Verplaetse, Demeyer, Gerard, & Buys, 1992).

The objectives of this study were two fold. Firstly, to determine the respective contribution of indigenous and bacterial enzymes to proteolysis by comparing proteolytic changes in sausages made with a known proteolytic starter culture to that of a control chemically acidified using glucono-δ-lactone (GDL) and an antibiotic mixture. Secondly, to identify some of the parent proteins of TCA-soluble peptides released throughout ripening by amino acid sequence analysis.

2. Materials and methods

2.1. Salami manufacture and sampling

Two trials were performed, in which two batches of fermented sausages were prepared. Results of Trial 1 will be presented. A commercially available frozen meat starter culture: LS-25 (Gewürzmüller, Germany) consisting of *Lactobacillus sake* LAD and *Staphylococcus carnosus* MC 1 was used for the production of one batch of salami. A second batch of salami was prepared in the presence of GDL (1.35%) and antibiotics (20,000 I.U. penicillin, 20 mg streptomyocin and 50 mg amphotericic/kg sausage) and was deemed the control. Salamis were produced using a standard recipe consisting of 55% beef (chilled), 30% pork (chilled), and 15% pork backfat (frozen). Other ingredients were added as follows: NaCl (25.0 g/kg), $NaNO_2$ (0.13 g/kg), KNO_3 (0.13 g/kg), glucose (5.0 g/kg) and lactose (3.0 g/kg). Ingredients were mixed in a bowl chopper and appropriate starter cultures were added during mixing. The mixture was then stuffed into 50 mm diameter RZ casings (Naturin, Germany) using a piston filler. The sausages were placed in a fermentation chamber (Ness and Co. GmbH, Remshalden, Germany) equipped with a Ness digitronic rf 3 d-n process control system. The following conditions of relative humidity (RH) and temperature were applied: day 0 until day 3, 88–94% RH and 24 °C; day 3, until day 7, 84–90% RH and 18 °C; day 7 until day 10, 80–88% RH and 18 °C. The sausages were smoked after 3 and 6 days in a Sümann (Ness and Co. GmbH, Remshalden, Germany) smoking chamber (15–30 °C) for 30 min. After reaching 30%

weight loss (day 10), the sausages were removed from the fermentation chamber, vacuum packaged and stored at 15 °C for a further 25 days. At each sampling period (days 0, 1, 2, 3, 7, 21 and 35) three sausages from each batch were taken for microbiological analysis and determinations of pH and a_W. The casing was removed from the remainder of the sausages and they were ground in a meat grinder, vacuum packaged and stored at −18 °C pending further analysis.

2.2. Microbiological analysis

Sausage samples (10 g) were homogenized with 90 ml maximum recovery diluent (LAB M, Bury, UK) using a laboratory blender, (Stomacher 400, Seward, London, UK) for 2 min and decimal dilutions prepared. Lactic acid bacteria (lactobacilli) counts were determined by the over-lay technique using MRS agar (LAB M, Bury, UK) and colonies counted after incubation at 30 °C for 5 days. The numbers of staphylococci were determined on Baird-Parker agar (LAB M, Bury, UK) supplemented with Egg Yolk Tellurite Emulsion (Oxoid, Hampshire, UK) after incubation at 37 °C for 2 days; and black colonies were counted.

2.3. pH, a_W and compositional analysis

All analyses were carried out on triplicate samples from each batch at various sampling times. The pH was determined using a pH 320 meter (WTW, 82362 Weilheim, Germany) fitted with a Mettler Toledo spear probe (Lot 406-M6-Dxk-57/25). Water activity measurements were carried out using an Aqua lab CX-2 water activity meter (Decagon Devices, Inc., Pullman, WA). Moisture, fat and ash were determined according to AOAC methods (Cuniffe, 1995). Total protein and NPN were determined by the Kjeldahl method (Cunnife, 1995).

2.4. Preparation of myofibrillar proteins, NPN and phosphotungstic acid-soluble extracts

Myofibrillar proteins were prepared according to the method of Diaz et al. (1997), by homogenizing 5 g of sausage in 40 ml of 0.03 M potassium phosphate buffer (pH 7.4) for 2 min using an Ultra Turrax T25 (Janke and Kunkel, Staufen, Germany) at 13,500 rev/min. The homogenate was centrifuged for 20 min at 10,000 ***g*** at 4 °C and the myofibrillar proteins were extracted from the resultant pellet by homogenizing with an extractant solution containing 8 M urea and 1% (w/v) β-mercaptoethanol for 2 min. The homogenate was recentrifuged as above and the supernatant, containing the myofibrillar proteins, was dialyzed against water prior to electrophoresis. NPN was extracted by homogenizing 10 g of sausage with 20 ml of 2% TCA for 1 min using an Ultra Turrax at 13,500 rev/min. The homogenate was centrifuged at 10,000 ***g*** for 20 min and the supernatant was retained for determination of NPN by Kjeldahl, quantification of free amino acids and peptide profiling by RP-HPLC. Phosphotungstic acid (PTA), i.e. 5% PTA-soluble extracts were also prepared on sausages ripened for 35 days according to the procedure described for the preparation of the 2% TCA-soluble extracts.

2.5. Free amino acids

Free amino acids were determined by RP-HPLC according to the Waters AccQ·Tag method (Millipore Co-Operative, Milford, MA, USA). This method involved pre-column derivatization and conversion of the amino acids to stable fluorescent derivatives by reaction with AccQ·Fluor reagent (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate) (Cohen & Michaud, 1993). RP-HPLC was performed using a Waters liquid chromatography system consisting of a Waters™626 pump, Waters™ 600 S controller, Waters™ 717 S autosampler (Millipore Co-operative, Milford, MA, USA) and a Shimadzu RF-551 fluorescent detector (Shimadzu, Kyoto, Japan). RP-HPLC was performed using a Nova-Pak™ C_{18} column (4 μm, 3.9 × 4.6 mm) heated to 37 °C in a column oven (Shimadzu model CTO-10AC column oven). Elution was by means of a gradient of solvent A (Waters AccQ·Tag eluent A), solvent B (acetonitrile: Aldrich Chemical Co., Milwaukee, WI) and solvent C (20% methanol in Milli-Q water). The gradient was formed as follows: initial eluent 100% A; 99% A and 1% B at 0.5 min; 95% A and 5% B at 18 min; 91% A and 9% B at 19 min; 83% A and 17% B at 29.5 min; 60% B and 40% C at 33 min and held under these conditions for 20 min before returning to 100% A. The concentration of A was maintained at 100% up to 65 min, after which the gradient was changed to 60% B and 40% C for a further 35 min, before returning to the starting conditions. The eluent was monitored at an excitation wavelength of 250 nm and an emission wavelength of 395 nm. Individual amino acids were identified by comparison of their retention times with those of calibration standards. Peak areas were processed using Millennium 32 software and the concentration of individual amino acids expressed as mg/100 g dry-matter.

2.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein concentrations of the myofibrillar protein extracts were determined by the Biuret assay (Gornall, Bardawill, & David, 1949). The protein concentration was adjusted with water to give a final concentration of 6 mg/ml and diluted 1:1 with SDS-PAGE sample buffer to give a final concentration of 3 mg/ml. The samples were heated at 100 °C for 5 min prior to electrophoresis.

SDS-PAGE was performed according to the method of Laemmli (1970) in gels containing 12.5% T (acrylamide plus bisacrylamide), 4% C (bisacrylamide as a percentage of T). After electrophoresis, the gels were stained using Coomassie Brilliant Blue R-250 (0.1%) in fixative (40% methanol, 10% acetic acid). Destaining was performed using 40% methanol and 10% acetic acid. The molecular weights of the products of proteolysis were estimated by reference to the relative mobilities of standard proteins.

2.7. Reversed phase high performance liquid chromatography (RP-HPLC)

RP-HPLC was performed on the 2% TCA and 5% PTA-soluble extracts from sausage. Prior to analysis the extracts were filtered through 0.45 μm filters (SRP15, Sartorius, Germany). RP-HPLC was performed using a Symmetry C_{18} column (300 Å, 5 μm, 250 × 4.6 mm) on a Shimadzu liquid chromatograph consisting of a model LC-9A pump, SIL- 9A autosampler and a SPD-6A UV spectrophotometric detector (Shimadzu, Kyoto, Japan). Elution was by means of a gradient of solvent A (0.1% trifluoroacetic acid, TFA, in H_2O) and solvent B (0.1% TFA in acetonitrile, Aldrich Chemical Co., Milwaukee, WI). The gradient was formed as follows 0 to 20% B at a rate of 0.31% B min^{-1} followed by an increase in B of 0.15% min^{-1} to a concentration of 26% B. Acetonitrile concentration was then increased to 95% at a rate of 3.5% min^{-1} and held at this concentration for 5 min before returning to the starting conditions. The eluent was monitored at 214 nm and flow rate was at 1.2 ml min^{-1}. Peptides were collected from the control sample 2% TCA-soluble extracts on day 35, freeze-dried and identified by N-terminal sequencing.

2.8. Identification of peptides

Peptides were sequenced at the National Food Biotechnology Centre, University College, Cork, Ireland by Edman degradation on an automated pulsed liquid-phase protein–peptide sequencer (Applied Biosystems Inc., Foster city, CA 94404, USA; model 477A). Liberated amino acids were detected as their phenylthiohydantoin derivatives by means of a model 120A analyser (Applied Biosystems Inc.). Amino acid sequence homology of isolated peptides with muscle protein of known primary structure was obtained by searching CRS-Blast sequence database, search tool (Worley, Wiese, & Smith, 1995).

2.9. Statistical methods

The data obtained for pH, moisture, water activity (a_w); NPN expressed as a% of total nitrogen (TN), individual and total amino acids, was assessed statistically to determine if significant differences occurred for these parameters as a result of individual treatments during ripening. Analyses of each parameter for the two sausage treatments were carried out in triplicate at various sampling times. As the data for all parameters was not normally distributed, data for individual days were ranked. One-way ANOVA was subsequently carried out on the ranked data employing a significance level of $P < 0.05$. Where statistically significant differences were noted, a post-hoc test (Tukeys pairwise multiple comparison procedure) was carried out. Statistical analyses were performed using Minitab version 11 for Windows (Minitab Inc., State College, PA).

3. Results and discussion

3.1. Microbiological analysis

Initial counts of lactobacilli and staphylococci were $\sim 10^7$ CFU/g in the *S. carnosus* MC 1 inoculated sausages. *Lactobacillus* counts reached maximum levels of $> 10^8$ CFU/g after 3 days of ripening and thereafter decreased to $\sim 10^7$–10^8 CFU/g, while *Staphylococcus* counts decreased to $\sim 10^6$ CFU/g in the sausages inoculated with starter by the end of ripening. This may be due to the staphylococci being poor competitors (Roca & Incze, 1990). The initial counts of lactobacilli and staphylococci in the sausage containing GDL and antibiotics were 10^4 and $< 10^1$ CFU/g, respectively and by day 2 of ripening, the counts of lactobacilli had decreased to $< 10^1$ CFU/g, while staphylococci were non-detectable due to the presence of antibiotics.

3.2. pH, a_W and compositional analysis

Results for analysis of pH and moisture are shown in Table 1. The initial pH of both sausages was pH 5.85; thereafter, it decreased rapidly in the control to pH 4.99, after 24 h. However, it took the sausages inoculated with *S. carnosus* MC 1, 3 days to reach a similar pH value (pH 5.03). From day 3, a slight increase in pH was

Table 1
Changes in pH and moisture (%) content during the ripening of fermented sausages[a]

Day	pH		Moisture (%)	
	Control	*S. carnosus* MC 1	Control	*S. carnosus* MC 1
0	5.85a	5.85a	62.81a	63.39a
1	4.99a	5.88b	61.56a	62.86a
2	5.01a	5.20a	60.00a	62.95b
3	5.05a	5.03a	57.98a	58.88b
7	5.12a	5.07a	51.43a	54.49b
21	5.12a	5.09a	46.90a	49.86b
35	5.12a	5.20a	45.88a	48.99b

[a] Values in a row followed by the same letter are not significantly different ($P < 0.05$).

observed in both sausages. This may be due to production of ammonia and biogenic amines as a result of enzymatic activity (Lücke, 1998). Statistical analysis of pH values recorded throughout ripening revealed significant differences between treatments on day 1 (Table 1). The moisture content decreased from initial values of 62–63 to 45–49% during the ripening process, with significant differences between treatments (Table 1). On the basis of final moisture content the salamis can be classified as semi-dry (Campbell-Platt, 1995). Water activity decreased from an initial value of $\sim$0.96 to $<$0.915 in both sausages over the ripening period, with no significant differences between treatments ($P > 0.05$; results not shown).

3.3. NPN

Changes in NPN content expressed as % total nitrogen (TN) throughout ripening is shown in Fig. 1. The NPN concentration increased in both sausages, primarily during the initial 3 days of ripening (Fig. 1). The concentration of NPN reached maximum levels of $\sim$9.3 and 8.8% of TN in the *S. carnosus* MC 1 and control sausages, respectively. In agreement with the results of this study, several other workers have shown that proteolysis during the ripening of fermented sausages is reflected by an increase in NPN concentration (De Masi, Wardlaw, Dick, & Acton, 1990; Dierick, Vanderkerckove, & Demeyer, 1974; García de Fernando & Fox, 1991). De Masi et al. (1990) reported a similar increase in NPN as that obtained in this study for the starter-inoculated sausages, i.e. $\sim$1.5–2.0% of TN. On the other hand, García de Fernando and Fox (1991) noted a much larger increase in NPN, i.e. from an initial level of $\sim$8 to $\sim$15% of TN during the ripening of pork sausage. Statistical analysis showed no significant differences in the NPN content between treatments ($P < 0.05$).

3.4. Free amino acids

The changes in the concentration of free amino acids during ripening are shown in Table 2. The total free amino acid concentration of the initial sausage mix constituted between 352 and 357 mg/100 g of dry-matter. An increase in the concentration of most amino acids was observed as ripening progressed giving rise to final concentrations of total free amino acids of 753 and 1292 mg/100 g of dry-matter in the control and *S. carnosus* MC 1 inoculated sausages, respectively. The amino acids primarily responsible for the increase in total free amino acids during ripening were Ala, Leu and Ile followed by Glu, Arg, Lys Phe, Val, Thr, Tyr, His, Ser and Gly. High levels of Met were also found in the *S. carnosus* MC 1 inoculated sausage. The concentration of most amino acids decreased in the sausages inoculated with the starter culture during the last 2 weeks of ripening. The most significant decreases occurred in the concentrations of Ala, Arg, Glu, His and Lys. The decrease in the concentrations of amino acids may indicate that

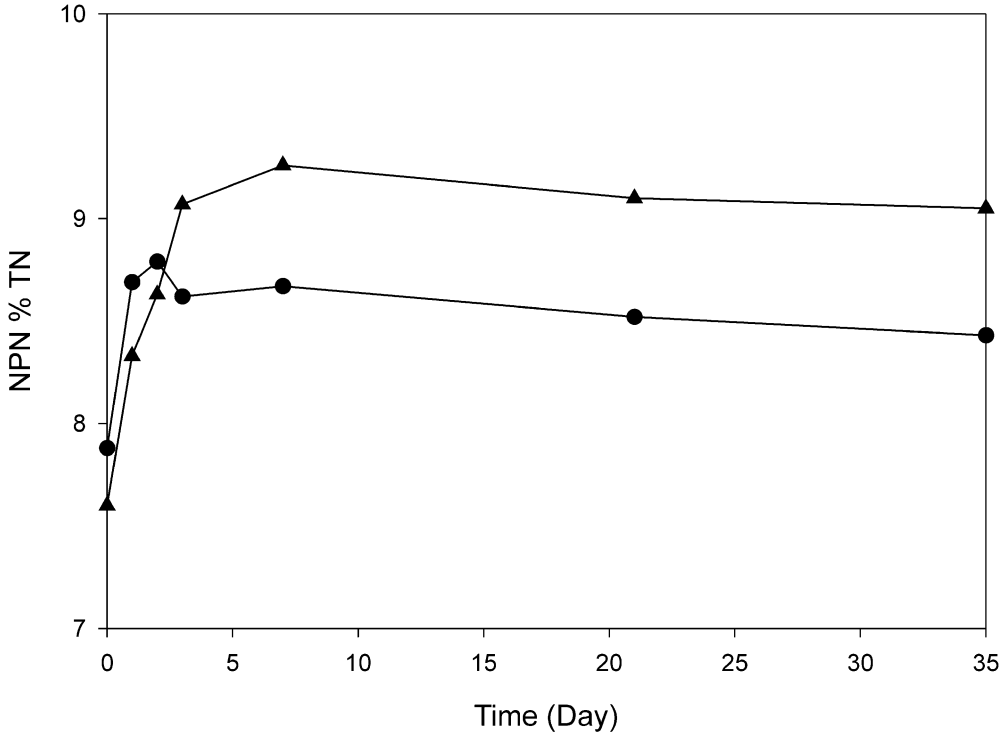

Fig. 1. Changes in NPN content (% of total nitrogen) throughout the ripening of fermented sausages; (●), Control and (▲), *S. carnosus* MC 1.

Table 2
Changes in free amino acid content during the ripening of fermented sausages[a]

Amino acid	Day 0		Day 7		Day 21		Day 35	
	Control	*S. carnosus* MC 1	Control	*S. carnosus* MC 1	Control	*S. carnosus* MC 1	Control	*S. carnosus* MC 1
Non-polar								
Gly	10.3±2.5a	10.4±1.3a	24.2±1.4a	22.6±1.4a	52.3±14.2a	103.7±112b	53.9±2.8a	68.8±3.7b
Ala	80.5±8.7a	68.4±8.2a	100.3±14.0a	103.3±5.9a	108.4±15.5a	214.1±158b	107.7±13.5a	134.5±13.1b
Val	16.1±1.3a	24.2±2.2b	19.2±2.7a	76.9±6.0b	26.3±1.5a	88.6±9.0b	42.3±5.8a	80.1±14.8b
Leu	24.1±0.5a	20.2±0.8a	42.4±8.6a	61.6±2.5b	56.3±5.7a	122.3±33.0b	53.8±2.7a	100.0±29.3b
Ile	23.0±0.6a	36.1±1.1b	30.8±3.9a	88.5±8.6b	48.7±7.5a	117.0±5.7b	46.2±7.6a	114.5±17.9b
Pro	NDa	4.1±0.3b	11.8±0.5a	19.3±1.0b	12.3±0.8a	39.1±68b	19.3±2.2a	30.5±5.9b
Phe	27.0±2.4a	17.7±0.5b	23.7±1.0a	73.0±64b	36.2±4.8a	80.0±4.0b	50.1±9.5a	80.2±24.7a
Trp	ND	ND	ND	ND	ND	ND	ND	ND
Met	ND	ND	4.1±0.3a	19.4±0.6b	11.9±1.5a	88.6±12.6b	19.3±3.5a	113.2±27.4b
Polar								
Ser	3.4±0.7a	4.4±0.7a	34.6±2.5a	42.0±1.1b	32.2±19a	97.6±8.3b	34.6±3.0a	61.3±3.6b
Thr	28.1±2.4a	13.6±1.0b	67.7±11.0a	58.9±7.4a	54.4±5.4a	97.7±23.4b	69.3±4.5a	45.8±3.9b
Cys	ND	ND	ND	ND	ND	ND	ND	ND
Tyr	16.1±0.9a	16.1±0.7a	15.9±0.3a	73.1±9.3b	40.2±3.9a	96.6±5.9b	30.8±5.8a	76.3±8.9b
Charged								
Arg	42.6±7.7a	48.8±6.0a	209.1±31.7a	229.1±56.0a	56.5±8.4a	222.8±76b	51.3±8.9a	125.9±10.2b
Asp	6.8±0.7a	4.7±0.6b	4.3 ±0.3a	8.6±1.4b	8.3±1.6a	24.7±6.4b	8.9±1.2a	16.7±4.0b
Glu	40.1±10.8a	46.7±2.4a	48.2±5.4a	113.3±10.1b	68.4±10.7a	169.1±13.6b	57.7±2.9a	91.6±4.1b
His	13.3±2.1a	22.6±0.5b	96.9±5.0a	96.9±5.0a	36.2±3.3a	110.7±34.3b	57.8±6.6a	57.2±11.8a
Lys	20.1±1.8a	18.9±1.4a	26.9±3.1a	80.7±2.7b	28.1±2.0a	120.5±15.7b	50.0±6.6a	95.0±14.0b
Total	352.0±32.31a	356.9±24.0a	760.1±46.6a	1151.7±35.9b	676.7±41.6a	1793.1±10.6b	753.0±21.7a	1291.6±25.7b

[a] Results are expressed as mg/100 g dry-matter. ND, not detected. Values in a row followed by the same letter are not significantly different ($P<0.05$).

their metabolism by bacteria was more intense than their production during the latter stages of ripening (Bover-Cid, Schoppen, Izquierdo-Pulido, & Vidal-Carou, 1999; Ordóñez, Hierro, Bruna, & de la Hoz, 1999). Statistical analysis revealed significant differences in the concentrations of Ala, Val, Ile, Phe, Tyr, Glu and Lys between treatments ($P<0.05$).

The findings of this study indicate that significant proportions of the amino acids released during sausage ripening are hydrophobic. Similar findings have been reported by a number of research groups. Henriksen and Stahnke (1997) found that the most predominant free amino acids in retail Danish salami, Italian sausage and Spanish chorizo were Pro, Ala, Val, Ile, Leu and Phe. Ansorena, Zapelena, Astiasarán, and Bello (1998) reported significant increases in the concentration of Glu, His, Lys, Ser, Ala, Pro, Val, Met, Ile, Leu and Phe and insignificant increases in the concentration of Asp, Gly, Thr and Tyr throughout the ripening of a dry fermented Spanish sausage. Demeyer et al. (2000) in addition to finding large increases in the concentration of hydrophobic amino acids also noted significant increases in the concentration of the basic amino acids, Arg and Lys in Norwegian, Belgian and Italian sausages by the end of ripening.

3.5. SDS-PAGE

SDS-PAGE electrophoretograms of the sarcoplasmic and myofibrillar proteins at various stages of ripening are shown in Figs. 2 and 3, respectively. Sarcoplasmic proteins with molecular weights of ~16, 29, 50, 97 and 150 kDa disappeared within the first 3 days of ripening in each preparation due to proteolysis or acid and salt induced denaturation. Polypeptides with molecular weights of 14 and 42 kDa were produced by day 2 in both sausages. Furthermore, the intensity of a polypeptide with a molecular weight of ~36 kDa progressively increased throughout ripening of the two sausage types. From the pattern of proteolysis observed on SDS-PAGE, it can be concluded that indigenous muscle enzymes were primarily responsible for the initial degradation of sarcoplasmic proteins.

SDS-PAGE electrophoretograms of myofibrillar proteins showed an intense degradation of the major myofibrillar proteins, myosin and actin, throughout ripening in all sausages (Fig. 3). The intensity of the MHC band decreased over time, with the simultaneous appearance of a degradation product with a molecular weight of ~116 kDa. MHC was completely degraded by 7 and 35 days of ripening in the sausages inoculated with *S. carnosus* MC 1

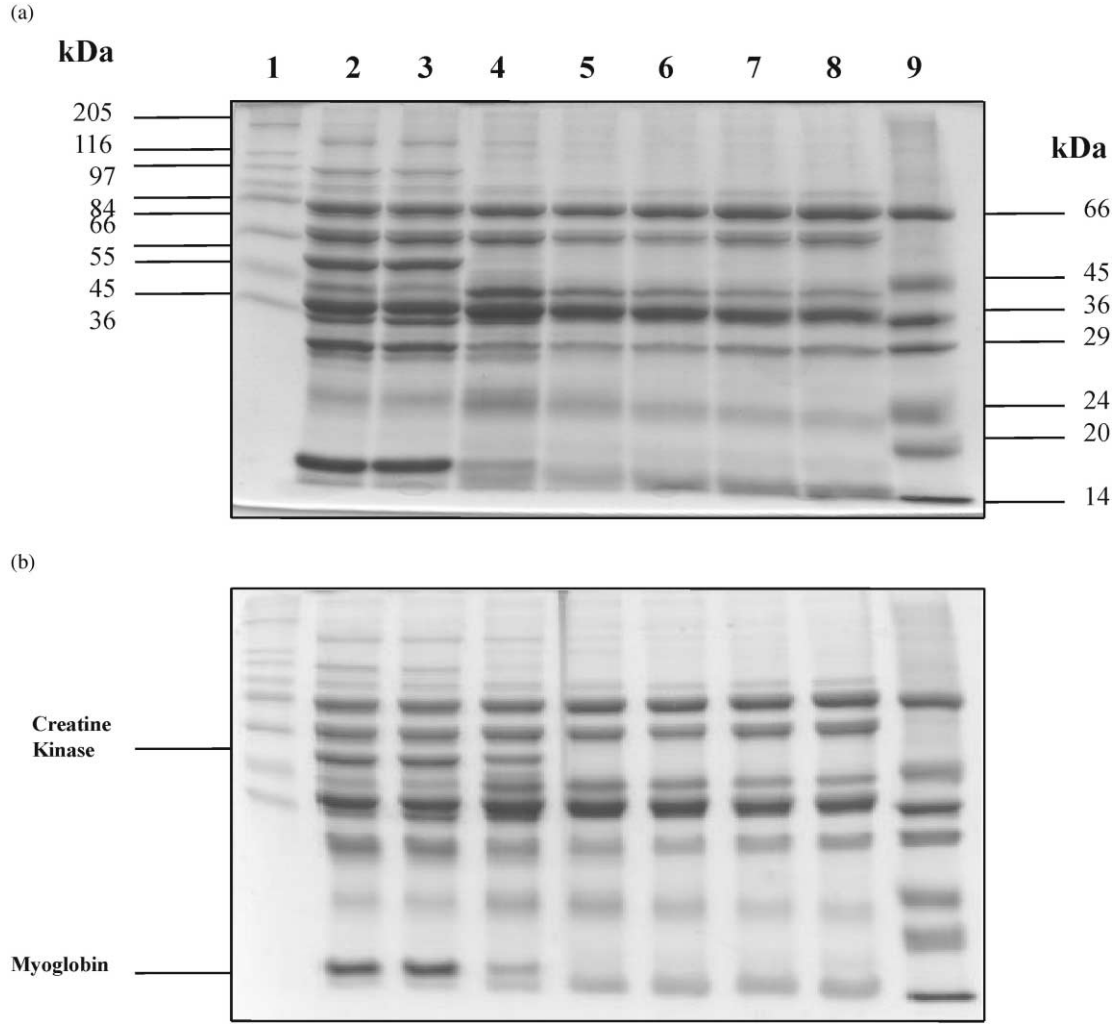

Fig. 2. SDS-PAGE electrophoretograms of sarcoplasmic proteins throughout the ripening of fermented sausages: (a) Control and (b) *S. carnosus* MC 1. A protein load of 30 μg was applied to the gel. Lanes 1 and 9, molecular weight standards ranging from 36 to 205 kDa and 14 to 66 kDa, respectively. Lanes 2–8, sarcoplasmic proteins after 0, 1, 2, 3, 7, 21 and 35 days of ripening.

and the control, respectively. The degradation of actin was most rapid in the sausage inoculated with *S. carnosus* MC 1, with almost complete degradation of this protein by the end of ripening. α-Actinin was rapidly degraded, while there was a slight decrease in the intensity of the MLC-1 and TN-I bands in both sausages. In addition to the 116 kDa polypeptide, two other polypeptides with molecular weights of ~50 and 85 kDa also accumulated over time. The TN-T and MLC-3 bands became more intensely stained as ripening progressed due to their possible co-migration with degradation products of larger proteins such as myosin. Similar findings have been reported by Verplaetse et al. (1989) and García de Fernando and Fox (1991). The pattern of myofibrillar protein degradation detectable by SDS-PAGE suggests that both indigenous and bacterial proteinases contributed to the initial degradation of these proteins.

3.6. RP-HPLC

RP-HPLC profiles of the 2% TCA-soluble peptides produced throughout ripening are shown in Fig. 4. The profiles were complex and preliminary experiments were necessary to select a gradient, which gave adequate separation of peptides with retention times of 35–65 min to facilitate their isolation and characterization. Attempts to further separate a cluster of peptides with retention times of between 60 and 80 min were unsuccessful. The RP-HPLC elution profiles show the production of numerous peptides by day 3 of ripening, which increased in concentration over time. However, no new peptides were produced as ripening progressed. The RP-HPLC profiles showed minor differences between the control and the sausages inoculated with *S. carnosus* MC 1. The most notable difference was the reduction in the concentration of a number of peaks in

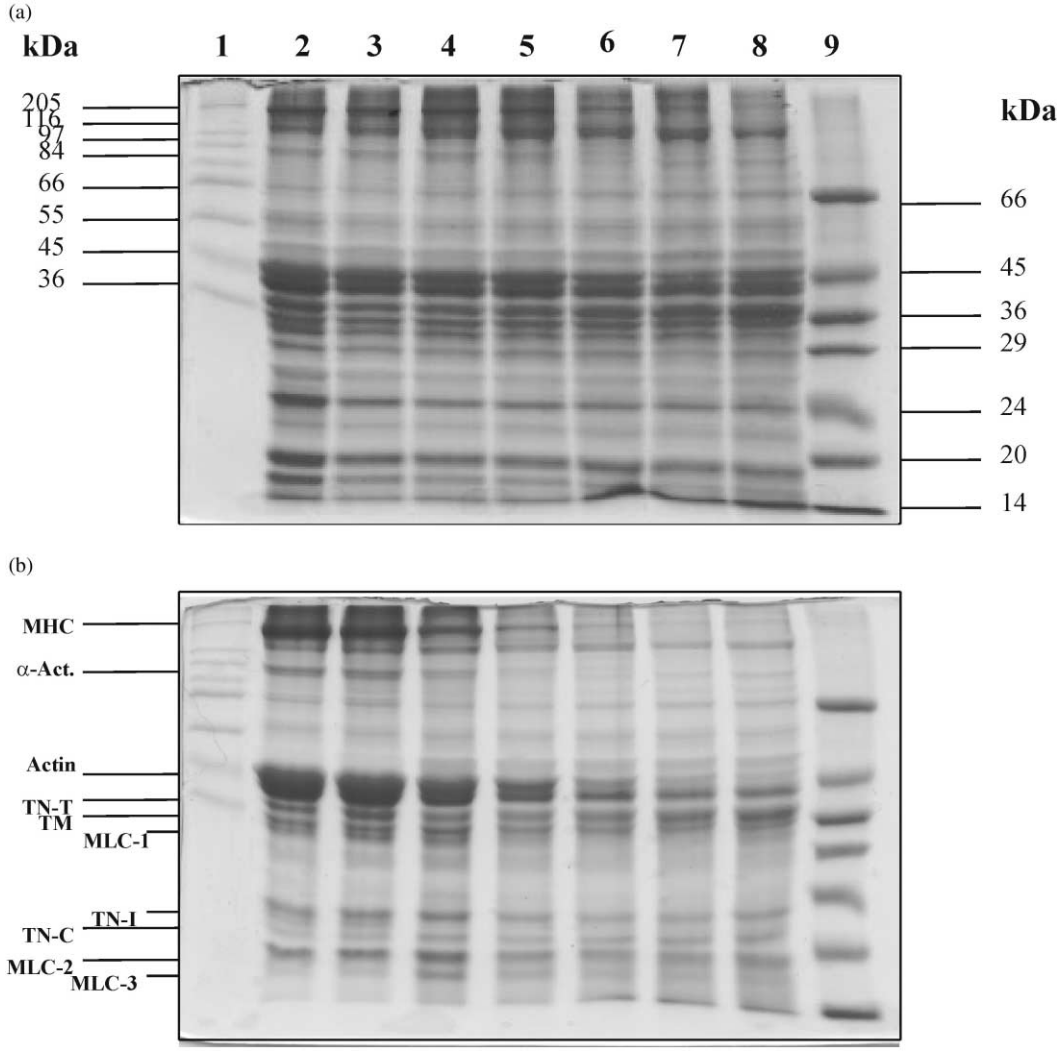

Fig. 3. SDS-PAGE electrophoretograms of myofibrillar proteins throughout the ripening of fermented sausages: (a) Control and (b) *S. carnosus* MC 1. A protein load of 45 μg was applied to the gel. Lanes 1 and 9, molecular weight standards ranging from 36 to 205 kDa and 14 to 66 kDa, respectively. Lanes 2–8, myofibrillar proteins after 0, 1, 2, 3, 7, 21 and 35 days of ripening.

the sausages inoculated with *S. carnosus* MC 1 as ripening progressed, suggesting that these peptides were further hydrolysed by peptidases of starter culture.

In an attempt to determine if the peaks observed on the 2% TCA-soluble RP-HPLC profiles were peptides, 5% PTA-soluble extracts were prepared on the sausages on day 35 and RP-HPLC analysis was carried out on these extracts. There is no distinct cut-off point in relation to peptide size and solubility in TCA, but all the peptides studied by Yvon, Chabanet, and Pelissier, (1989) containing less than seven amino acid residues were soluble in 12% TCA. On the other hand, PTA (5%) precipitates all peptides with molecular weights of >0.6 kDa as tungstates (Jarrett, Aston, & Dualley, 1982). Comparison of the RP-HPLC profiles obtained for the 2% TCA and 5% PTA-soluble extracts on day 35, revealed that the peaks which eluted before 35 min and a peak with a retention time of $\sim$55 min comprised of free amino acids and peptides with molecular weights of <0.6 kDa, which are unidentifiable by N-terminal amino acid sequencing. The RP-HPLC profiles of the 2% TCA-soluble extracts showed that the majority of the peptides eluted between 35 and 75 min, i.e. between $\sim$8 and 21% acetonitrile, indicating that they were relatively hydrophilic in nature. The presence of predominantly hydrophilic peptides in this study is in agreement with the findings of other research groups. Henriksen and Stahnke (1997) and Zapelena et al. (1997) using acid hydrolysis and amino acid analysis, concluded that the small peptides released throughout the ripening of fermented sausages contained a high proportion of hydrophilic amino acids.

3.7. Identification of peptides

Studies to date have concentrated on characterization of proteolysis in fermented sausages by SDS-PAGE and determination of total NPN. At present there are no

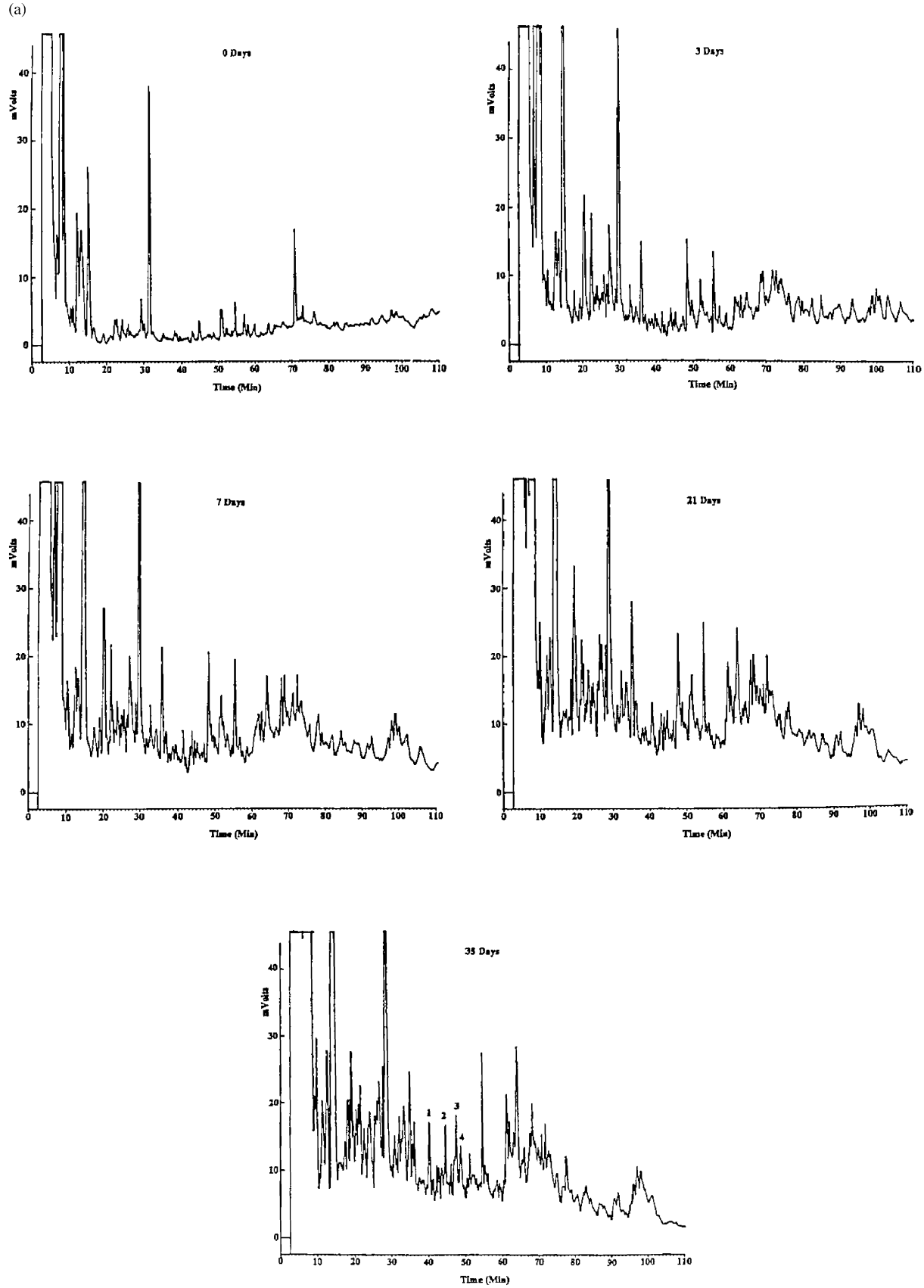

Fig. 4. (a) Reverse-phase RP-HPLC (C_{18}) elution profiles of 2% TCA-soluble peptides produced throughout the ripening of the control sausages. (b) Reverse-phase RP-HPLC (C_{18}) elution profiles of 2% TCA-soluble peptides produced throughout the ripening of sausages inoculated with *S. carnosus* MC1.

(b)

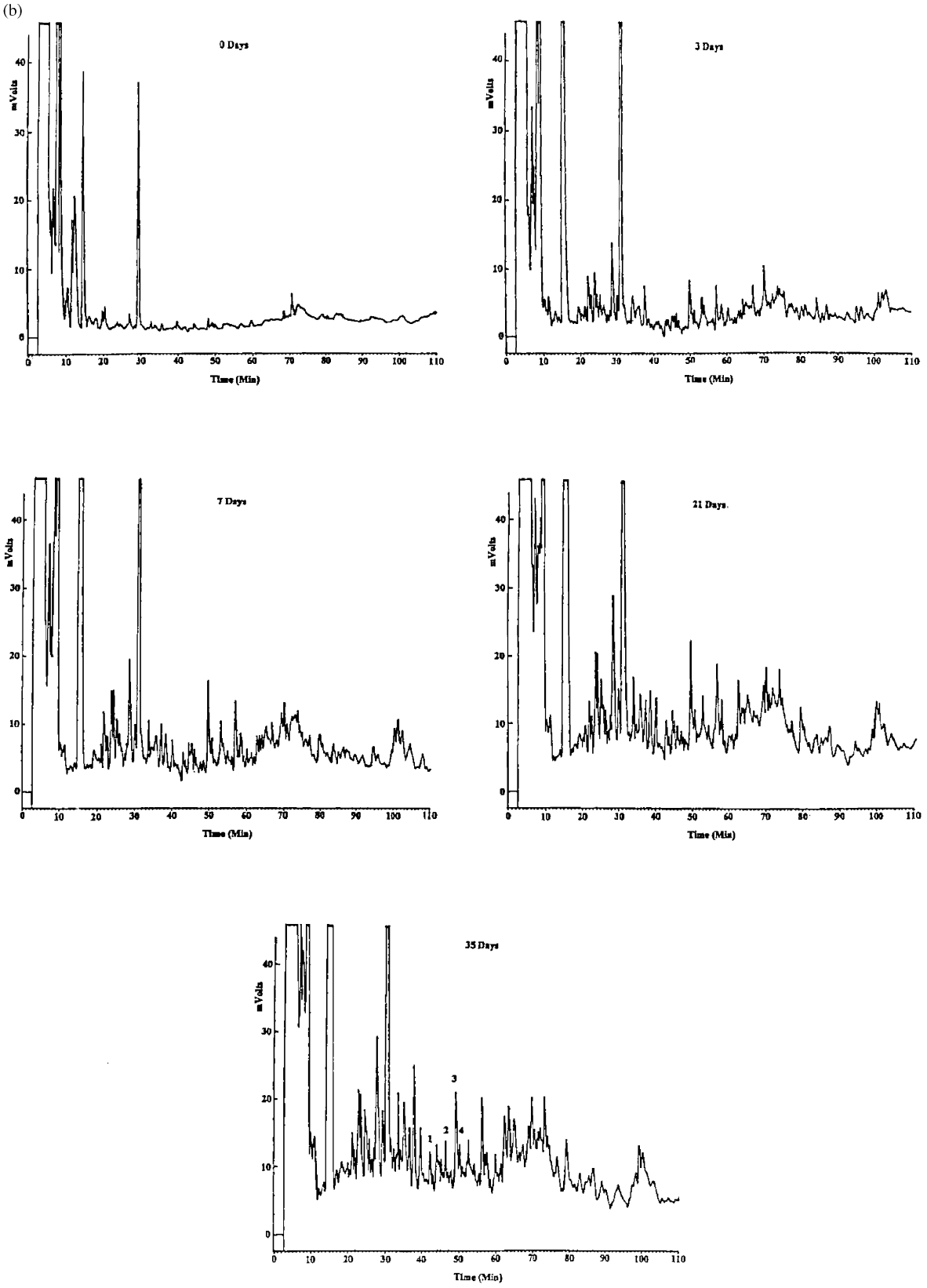

Fig. 4. (continued)

Table 3
Identity of peptides isolated by RP-HPLC produced during the ripening of fermented sausages

Peak no.	N-Terminal sequence	Parent protein	Species/muscle	No. amino acids in 1° structure	Location on protein	N-Terminal cleavage site	% Homology
1	VGGRWK	Troponin-T	Rabbit skeletal muscle	259	Val_{254}-Lys_{259}	Lys_{253}-Val_{254}	100
			Chicken skeletal muscle	251	Val_{246}-Lys_{251}	Lys_{245}-Val_{246}	100
2(A)	GKVEADVAGH	Myoglobin	Bovine heart muscle	154	Gly_{16}-His_{25}	Trp_{15}-Gly_{16}	100
			Porcine heart muscle	154	Gly_{16}-His_{25}	Trp_{15}-Gly_{16}	100
2(A)	PFGNTHNKY	Creatine kinase m-chain	Human skeletal muscle	381	Pro_2-Lys_9	Met_1-Pro_2	88
			Rabbit skeletal muscle	381	Pro_2-Lys_9	Met_1-Pro_2	88
3	DVGDWRKNV	Troponin-I	Human skeletal muscle	183	Glu_{139}-Asn_{146}	Val_{138}-Glu_{139}	88
			Rabbit skeletal muscle	179	Asp_{154}-Asn_{161}	Arg_{153}-Asp_{154}	100
4(A)	VHIITHGEEK	Myosin light chain 2	Human cardiac muscle	165	Val_{155}-Lys_{164}	Leu_{154}- Val_{155}	100
			Mouse skeletal muscle	166	Val_{156}-Lys_{165}	Leu_{155}- Val_{156}	100
4(B)	HAKHPSDFGA	Myoglobin	Porcine cardiac muscle	154	Gln_{117}-Ala_{126}	Leu_{116}-Gln_{117}	70
			Bovine cardiac muscle	154	His_{117}-Ala_{126}	Leu_{116}-His_{117}	100

reports identifying the parent proteins of the peptides present in the NPN fraction of fermented sausages. In this study, the identities of peptides present in four RP-HPLC peaks were ascertained by N-terminal amino acid sequence analysis (Table 3). Two peaks when isolated and sequenced were found to contain two peptides. The amino acid sequence homologies of the isolated peptides with muscle proteins of known primary structure from various mammalian sources were found to be high (70–100%). Two peptides were found to have originated from myoglobin, while creatine kinase was the parent protein of a third peptide. Amino acid sequence analysis revealed that TN-T, TN-I and MLC-2 were the parent proteins of peptides represented by RP-HPLC peaks 1, 3 and 4, respectively. The peptides released from TN-T, TN-I and MLC-2 originated from close to the N-termini of the protein molecules. The RP-HPLC profiles of all sausages showed that the peptides present in the starter inoculated sausages were also present in the control and therefore it can be concluded that indigenous muscle proteinases are responsible for the production of small peptides including those identified in this study throughout the ripening of fermented sausages.

The peptides produced as a result of enzymatic degradation of proteins have an important influence on meat flavour development (Spanier & Edwards, 1987; Spanier, Flores, McMillin, & Bidner, 1997). Hydrophilic peptides are associated with desirable flavour, while high levels of hydrophobic peptides give rise to off-flavours in fermented dry sausage (Verplaetse, 1994). The length of the peptides is also considered to influence taste development. Small peptides impart a spicy taste to the product, however too high a concentration may give a negative taste impression. Low concentrations of small peptides give rise to beefy and sweet-flavoured sausages (Verplaetse, 1994). The peptides identified in this study were both hydrophilic and relatively short, however further study is required to ascertain any significance that these peptides may have to flavour development of these products.

4. Conclusions

The findings of this study show that indigenous enzymes are primarily responsible for the initial degradation of the sarcoplasmic proteins. However, bacterial enzymes also contributed to the initial breakdown of myofibrillar proteins, particularly myosin and actin. Furthermore, the RP-HPLC profiles showed that the release of hydrophilic 2% TCA-soluble peptides from both protein fractions was due to indigenous proteinases, while bacterial peptidases contributed significantly to the release of free amino acids. This study has also conclusively shown that many small hydrophilic peptides produced in fermented sausages originate from both the sarcoplasmic and myofibrillar proteins.

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