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Effects of environmental conditions on microbial proteolysis in a pork myofibril model system

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P.M. KENNEALLY, N.G. FRANSEN, H. GRAU, E.E. O'NEILL AND E.K. ARENDT. 1999. A number of bacterial strains used for meat fermentations were screened for proteolytic activity. A strain of *Micrococcus* which was found to be proteolytic was evaluated for the effects of environmental conditions on its proteolytic activity against pork myofibrillar proteins using response surface methodology. Three strains of micrococci were also tested for the ability to produce free amino acids from pork myofibrils. Analysis of the effects of environmental conditions showed that proteolytic activity would be minimal under conditions normally found in fermented sausages, thereby suggesting that proteolysis in these products is largely due to endogenous meat enzymes. The three strains of micrococci were shown to produce free amino acids from pork myofibrils, thereby demonstrating the presence of peptidase activity in these strains.

INTRODUCTION

The proteolysis occurring during the ripening of fermented sausages is considered to be a result of the action of endogenous meat enzymes, such as cathepsins, as well as proteases of microbial origin (Diaz *et al*. 1993, 1997). The result of this proteolysis is that polypeptides, peptides and free amino acids, amongst other compounds, are produced (Demeyer *et al.* 1986; DeMasi *et al.* 1990; Diaz *et al*. 1993). The production of these end-products has a positive effect on the flavour of dry sausages both directly (Demeyer *et al*. 1986; Hagen *et al.* 1996) and indirectly through the production of many odour and flavour compounds from free amino acids (Deng and Lillard 1973). Among the bacteria used as starter cultures for fermented sausages are the lactobacilli and micrococcaceae (micrococci and staphylococci), with greater proteolytic activity mainly being attributed to the micrococcaceae and some peptidase activity to lactobacilli (Sanz and Toldra 1997). Microbial enzymes are deemed to be effective for the breakdown of oligopeptides and small peptides with endogenous meat proteinases acting in the initial stages of protein degradation (Verplaetse 1994).

The effects of different levels of curing ingredients and processing parameters on muscle proteases and peptidases involved in the production of fermented meat products have previously been reported (Toldra *et al*. 1992, 1993). However, there have been very few reports on the effects of sausage conditions on the proteolytic activity of starter bacteria, although a recent report by Sanz and Toldra (1997) examined the effect of curing ingredients and processing parameters on the activity of aminopeptidases from *Lactobacillus sake*. Low acid fermented sausages have been shown to have a low proteolytic activity with none of the major meat proteins being broken down while in medium and high acidity sausages myosin and actin are degraded to fragments of 135 and 38 kDa, respectively (Verplaetse 1994). This proteolytic pattern is similar to that of endogenous cathepsins. This observation, coupled with experiments where microbial growth and metabolism were inhibited, seems to suggest that proteolysis mostly arises out of endogenous cathepsin activity. However, it is possible to accelerate the ripening of fermented sausages by the addition of proteinases of microbial origin, such as the serine proteinase of *Lact. paracasei* ssp. *paracasei* NCDO151 (Naes *et al.* 1995) and the pronase E of *Streptomyces griseus* (Diaz *et al*. 1993).

The purpose of this study was to examine the proteolytic activity of bacteria used for meat fermentation and to model, using response surface methodology, the effect of environmental factors relevant to meat fermentations, so as to determine the relative effects of conditions found in a fermented

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sausage on the proteolytic activity of a selected bacterial strain and a commercial enzyme.

MATERIALS AND METHODS

Preparation of myofibrils

Myofibrils were extracted from lean pork lap muscles using the method of Etlinger *et al*. (1976), as modified by Wang (1982). Samples of muscle frozen at −18 °C were allowed to thaw at room temperature. The meat was cut into dice measuring approximately 1 cm^3 and divided into portions weighing 12·5 g. Samples were then homogenized in a blender (Waring, New Hartford, CT, USA) in 8 volumes (100 ml) of pyrophosphate relaxing buffer (2 mmol l^{-1} $Na_4P_2O_7$, 2 mmol l^{-1} $MgCl_2$, 2 mmol l^{-1} EGTA, 10 mmol l^{-1} Trizma-maleate, 0·5 mmol l^{-1} dithiothreithol and 0·1 mmol l^{-1} phenylmethylsulphonyl fluoride, pH 6·8) for two 30 s bursts at low speed with a 15 s resting step in between. The homogenate was then transferred to 250-ml centrifuge containers and centrifuged at 10 000 rev min^{-1} (10 000 *g*) for 10 min at 4 °C (Beckman J2–21; Beckman Instruments, High Wycombe, UK). After decanting the supernatant fluid the pellet was resuspended in 100 ml extraction buffer (pyrophosphate relaxing buffer without $Na_4P_2O_7$) and centrifuged as before. The resultant pellet was resuspended in extraction buffer (100 ml) and filtered through synthetic cheese-cloth to remove connective tissue and large fat particles and then centrifuged as described previously. The resultant pellet was then washed for another step with extraction buffer and the resultant pellet resuspended in 100 ml Triton X-100 buffer (extraction buffer supplemented with 1·5% w/v Triton X-100) and centrifuged at 10 000 rev min^{-1} (10 000 *g*) for 6 min (to remove fat micelles). The samples were then washed a further three times with extraction buffer as described previously. All buffers were kept at 4 °C. The pellet was then transferred to a petri dish and lyophilized in a freeze drier. The freeze-dried samples were ground to a fine powder using a mortar and pestle and stored in an airtight container in a dessicator.

Growth of strains for agar tests and spectrophotometric enzyme test

Strains of lactobacilli were grown in de Man, Rogosa and Sharpe broth and strains of lactococci in M17 broth (Oxoid, Basingstoke, UK) supplemented with 0·5% glucose (BDH Laboratory Supplies, Poole, UK), while strains of micrococci and staphylococci were grown in PM broth, which consisted of 1% tryptone (Difco Laboratories, Detroit, MI, USA), 0·5% yeast extract (Difco), 0·5% NaCl (Merck, Darmstadt, Germany) and 0·1% glucose (BDH), pH 7·2. All strains were grown at 30 °C for 24 h.

Growth and harvesting of strains for response surface methodology

Strains of micrococci were grown in PM broth at 30 °C for 36 h. Strains were then harvested by centrifugation at 10 000 rev min^{-1} (10 000 *g*) for 10 min (Beckman) and resuspended in a mixture (1:1) of 80% glycerol (Merck) and 0·1 mol l^{-1} phosphate buffer (pH 7·0) to give a final cell count of approximately 5×10^9. The culture suspensions were distributed into stock bottles (Sarstedt, Nümbrecht, Germany), which had been sterilized by autoclaving at 121 °C for 15 min, and then frozen and stored at −18 °C until further use.

Determination of proteolytic activity using agar methods

Proteolytic activity was determined as described by Fransen *et al*. (1997), using a medium which consisted of 1·5% agar (Difco), 0·5% tryptone (Difco), 0·25% yeast extract (Difco), 0·1% glucose (BDH), 1·0% freeze-dried myofibrils and $CaCl_2.2H_2O$ (20 ml l^{-1} of a 1 mol l^{-1} solution; BDH) in a 0·015 mol l^{-1} trisodium citrate solution (Prolabo, Manchester, UK), pH 6·9. After autoclaving the medium at 121 °C for 15 min plates were poured while swirling the medium regularly and vigorously to disperse the proteins. After solidification of the medium, three wells (diameter 0·6 cm) were made in each plate using sterile pasteur pipettes. Each well was then inoculated with 20 μl of a 24-h culture of the appropriate strain. Proteolytic activity was also tested in caseinate agar whose composition was identical to that of the myofibrillar agar except that 1·0% sodium caseinate was substituted for the myofibrils. All plates were incubated at 30 °C for 48 h. After incubation, the agar layers were removed from the petri dishes and stained for 5 min in a solution which consisted of 0·05% (w/v) coomassie brilliant blue R-250 (BDH), 50% (v/v) methanol (BDH) and 9·2% (v/v) acetic acid (BDH) in distilled water. Samples were subsequently destained overnight using a solution containing 25% (v/v) ethanol and 5% (v/v) acetic acid in distilled water. Proteolytic activity was determined by the presence of a clear zone around the inoculated wells. Each strain was evaluated in two independent tests.

Detection of proteolysis by a spectrophotometric method

A confirmatory test for proteolytic activity, as described by Fransen *et al*. (1997), was carried out on all strains. This was based on the detection of tyrosine and tryptophan residues in the peptide/amino acid fraction of a substrate solution. The ability of the strains to break down casein and myofibrillar proteins was measured over a period of 6 h. The test procedure was as follows: 100 μl of the overnight culture was

added to 0·5 ml of a substrate solution (solution I) containing 5% 1 mol l^{-1} Tris-HCl (Sigma, St. Louis, MO, USA), pH 7·5, 2 ml 4% Na-azide (Sigma), 0·074% $CaCl_2.2H_2O$ (BDH) and 0·8% (w/v) substrate (sodium caseinate or freeze-dried myofibrils) in distilled H_2O (make up to 100 ml) which had been shaken at 37 °C and stored at 4 °C in an Eppendorf tube. Samples were incubated at 37 °C in a shaking water-bath (SS40 D; Grant, Cambridge, UK). At the appropriate sample times (immediately after inoculation and every hour for 6 h), the enzymatic reaction was stopped by the addition of 0·5 ml solution II (composed of 1·634% (w/v) trichloroacetic acid (BDH), 1·804% (w/v) sodium acetate (BDH) and 1·886% (v/v) acetic acid (BDH) in distilled water (stored at 4 °C)). The samples were then left to stand at room temperature for at least 35 min, after which they were centrifuged at 14 000 rev min^{-1} (15 800 *g*) for 15 min (centrifuge 5415C; Eppendorf, Engelsdorf, Germany). The absorbance of the supernatant fluid was subsequently measured in a Beckman DU 640 spectrophotometer (Beckman, Fullerton, CA, USA) at 275 nm (u.v.) in a quartz cuvette with a 1-cm lightpath. A negative control (reagent blank without culture added) was also included. Each strain was evaluated in two independent tests.

Examination of the effects of pH, NaCl concentration and temperature on proteolytic activity in *Micrococcus varians* 4 and papain

A full three-factorial design, as described by Box and Draper (1987), was chosen to determine the effects of temperature, pH value and NaCl concentration on the proteolytic activity of *Micrococcus varians* 4 and papain. The tests were carried out in a model system containing pork myofibrils as a substrate. A combination of three temperatures (18, 24 and 30 °C), three NaCl concentrations (1, 2 and 3% w/v) and three pH values (4·5, 5·75 and 7·0) were selected which resulted in 27 different experiments being performed. The conditions selected were chosen to represent those found in a fermented sausage (where the starting pH can be $>5{\cdot}75$ with the possibility of decreasing to approximately 4·5, temperatures employed are normally in the range of 12–24 °C and salt can be found at a level of $>3\%$ (w/w)) and conditions of optimal growth (30 °C and pH 7·0).

Substrate solutions for this assay were prepared by adding specific volumes of 0·1 mol l^{-1} citric acid (BDH) and 0·2 mol l^{-1} Na_2HPO_4 (BDH) to give the appropriate pH to 0·8% (w/v) freeze-dried pork myofibrils, 0·074% (w/v) $CaCl_2.2H_2O$ (BDH) and 2 ml (per 100 ml) 4% Na-azide (Sigma), to which the appropriate level of NaCl had been added (1, 2 or 3% (w/v)).

Samples were prepared by adding 25 ml of the appropriate substrate solution (with the desired pH and % NaCl) to sterile Mc Cartney bottles and adding 250 μl of the culture, which had been prepared as described previously, or 250 μl of a solution of papain (Sigma) containing 4·5 units proteolytic activity ml^{-1}. Blanks were also prepared by omitting the addition of the culture or papain and the samples then incubated at the appropriate temperature in a shaking water-bath set at 230 strokes min^{-1} (Grant). Measurement of proteolytic activity was then carried out using a similar procedure to the spectrophotometric method described previously except that 1 ml of solution II (described above) was added to 1 ml of the inoculated substrate. The rest of the method was similar to that described in Detection of proteolysis by a spectrophotometric method. The absorbance of each sample at 275 nm (u.v.) was determined in triplicate and the values of the blanks subtracted. Proteolytic activity was then monitored as absorbance at 275 nm over a period of 6 h. The value of maximum absorbance was regarded as being an indicator of the degree of proteolytic activity. The resultant maximum absorbance values multiplied by 10 000 (for the 27 combinations) were then selected as response variables and evaluated using the 'Design Expert' statistical programme (Stat-Ease Inc., Minneapolis, MN, USA) and the response surface plots obtained.

Analysis of proteolysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis

Myofibrils were extracted as previously described (but not freeze dried) and resuspended in a phosphate buffer (pH 7·0) composed of 0·5 mol l^{-1} Na_2HPO_4 and 0·5 mol l^{-1} NaH_2PO_4 containing 0·1 mol l^{-1} NaCl and 1 mmol l^{-1} NaN_3. The protein content of the resultant solution was measured by the Kjeldahl method. The myofibril solution was distributed in 25-ml portions in sterile Mc Cartney bottles and to each was added 1 ml of the appropriate *Micrococcus* culture (prepared as for RSM, response surface methodology) or 1 ml of either 0·45 or 4·5 units ml^{-1} papain solution. Uninoculated myofibril solution was used as a control. All samples were incubated at 37 °C in a shaking water-bath set at 230 strokes min^{-1}. Samples were taken immediately after inoculation and after 6 and 24 h of incubation, in the case of those inoculated with the strains and 4·5 units ml^{-1} papain, and after 10 min, 30 min, 1, 2, 3, 4 and 6 h, in the case of those inoculated with 0·45 units ml^{-1} papain. A portion of sample (1 ml) was diluted with sample buffer (0·0625 mol l^{-1} Tris-HCl (pH 6·8), 2% sodium dodecyl sulphate (SDS), 10% glycerol, 5% 2-mercaptoethanol and 0·01% bromophenol blue) in a bijou bottle to give a protein concentration of approximately 4 mg ml^{-1}. Samples were then boiled for 2×2 min with vortexing in between (Laemmli 1970). Samples (10 μl) were subsequently run on an SDS polyacrylamide gel consisting of a 3% stacking gel and a 10% resolving gel, in a MINI PROTEAN IITM electrophoresis unit (Biorad, CA, USA) powered by a PowerPac 3000 power unit (Biorad). Conditions of the run were

set at a voltage of 170 V and power of 80 W. The gels were subsequently stained for 1 h in a 0·125% Coomassie brilliant blue R250 staining solution and then destained in a solution of 25% methanol, 10% acetic acid and 65% water.

Determination of free amino acid production

Myofibril solutions were prepared, inoculated with culture and incubated as previously described in Analysis of proteolysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis. In the case of papain, 1 ml of a solution containing either 0·45 or 4·5 units ml^{-1} proteolytic activity was inoculated. A control was also prepared by adding 1 ml distilled water to the myofibril solution. Culture blanks (to account for any contribution of the inoculated culture to the free amino acids (FAA)) were also prepared by inoculating 1 ml of the appropriate culture into a buffer whose composition was identical to the myofibril solution, except for the absence of myofibrils. Samples of the inoculated solutions were taken immediately after inoculation and after 3, 6, 24 and 48 h and immediately frozen at −18 °C. Samples were then thawed when required and deproteinized by mixing with equal volumes of 24% trichloro acetic acid (12% in final volume) and allowing to stand for 10 min. Samples were centrifuged at 14 400 *g* (Microcentaur; MSE, UK) for 10 min. Supernatant fluids were then removed and diluted with 0·2 mol l^{-1} sodium citrate buffer (pH 2·2) to give approximately 25 nmol of each amino acid residue per 50 μl injection volume and then analysed on a 120 × 4 mm cation exchange column (Na^+ form) using a Beckman 6300 amino acid analyser (Beckman Instruments).

RESULTS

Detection of proteolytic activity by agar methods

The results of the analysis of proteolytic activity by starter strains (Table 1) clearly showed that, of the strains tested, proteolytic activity in caseinate agar was largely confined to strains of the genus *Micrococcus*, with strains of staphylococci and one of the strains of *Lactobacillus* (*Lact. pentosus* 03 A) showing slight activity as detected by the presence of clear zones. None of the strains of *Lactococcus* showed proteolytic activity against casein. Proteolytic activity against pork myofibrillar proteins was confined to strains of micrococci, with none of the other strains showing proteolytic activity against this substrate.

Detection of proteolytic activity by spectrophotometric means

The results of the spectrophotometric determination of the proteolytic activity of the strains were in line with those of the agar method (Fig. 1a,b) in relation to casein as a substrate, with the micrococci again showing the greatest level of activity. One strain of *Lactobacillus* (*Lact. sake* LAD) was also positive for proteolytic activity as measured by this method, suggesting that this strain may have had a peptidase activity. However, none of the other strains showed appreciable amounts of proteolytic activity. Performance of this test with myofibrillar proteins as a substrate showed that proteolytic activity against pork myofibrils was confined to strains of the genus *Micrococcus* (results not shown).

Response surface methodology design experiment

Model summary statistics. The results of the model summary statistics showed that, in both the cases of papain and MCV4, the measured values fitted a quadratic model better than a linear model (Table 2). In the case of MCV4, 77·2% and of papain 81·3% of values fitted the model. When these values were adjusted for degrees of freedom, these values decreased to 65·1 and 71·4%, respectively, which is still quite high. In all cases, the actual R2 value was higher than the R2 values predicted for the model.

RSM model for Micrococcus varians *4*. The response surface plots of the combined effects of % NaCl, pH and temperature for *Mic. varians* 4 (MCV4) showed that pH had the greatest effect on proteolytic activity, followed by salt concentration, with temperature in the measured range having the least effect on activity. Results showed that there were no significant interactions between the variables in relation to their effect on protease activity (Table 3). The maximum absorbance values for MCV4 increased when the pH was increased and also when the temperature was increased (Fig. 2). These values also decreased when the NaCl concentration was increased (results not shown). The response surface plots for the various salt concentrations all showed similar trends. Overall highest maximum absorbance values, which are indicative of the highest proteolytic activity, were reached at pH 7·0, 30 °C and 1% NaCl, and lowest values (lowest protease activity within the measured range) were reached at pH 4·5, 18 °C and 2% NaCl.

RSM model for papain. The response surface plots of the combined effects of % NaCl, pH and temperature for papain also showed that pH had the greatest effect on proteolytic activity, followed by temperature, with salt concentration again having the least effect. A significant interaction ($P < 0·05$) between pH and temperature (Table 4) was also found. The RSM plots showed that maximum absorbance values for papain increased when the pH was increased until a maximum was reached at a pH of approximately 6·0–7·0 (Fig. 3). The plots also showed that proteolytic activity increased with increasing temperature, with optimum activity (within the measured range) being reached at 28–30 °C (Fig. 3). The results of the effect of % NaCl showed that

Table 1 Proteolytic activity of starter strains as determined on agar plates

Strain	CA	MA	Origin of strain
Micrococcus varians 4 (MCV4)	+ + +	+ + +	Nestec
Mic. varians 13 (MCV13)	+ + +	+ + +	Nestec
Mic. varians 20 (MCV20)	+ + +	+ + +	Nestec
Staphylococcus carnosus M17	+	−	Christian Hansen
Staph. carnosus MIII	+	−	Christian Hansen
Lactobacillus pentosus 03A	+	−	Rüdolf Müller
Lact. sake LAD	−	−	Gewürzmüller
Lactococcus lactis UC 317	−	−	UCCmd
L. lactis UC 509	−	−	UCCmd
L. lactis T303	−	−	UCCftd

CA, Caseinate agar; MA, myofibrillar agar; + + +, strong activity (zone > 1·5 cm); +, weak activity (zone < 1·0 cm); −, no activity (no zone); UCCmd, Department of Microbiology, University College Cork; UCCftd, Department of Food Technology, University College Cork.

protease activity was not greatly affected by NaCl concentrations in the range 1–3%. However, increasing the NaCl concentration within this range did result in a decrease in proteolytic activity (results not shown), although this decrease was not significant ($P > 0{\cdot}001$). Overall, highest maximum absorbance values for papain were attained at a pH of about 7·0, a temperature of approximately 30 °C and between 1 and 2% NaCl, which illustrates conditions where optimum proteolytic activity would occur within the constraints of the experimental design. Lowest values of proteolytic activity for papain were reached under less severe conditions than MCV4, when combined conditions were pH 4·5, 24 °C and 1% NaCl.

Analysis of proteolysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis

Electrophoretic patterns showed that there was breakdown of the myosin heavy chain after 24 h in all samples including the control (results not shown). However, there was no difference in the patterns between the control and the samples inoculated with micrococci and lactobacilli, suggesting that the breakdown was due to some endogenous enzyme activity remaining in the myofibrils after extraction. Samples inoculated with 4·5 units ml^{-1} papain showed almost immediate destruction of myofibrillar proteins, while the addition of 0·45 units ml^{-1} papain resulted (after 6 h) in a more pronounced breakdown of proteins than samples inoculated with cultures and the control (results not shown).

Production of free amino acids

Analysis of FAA clearly showed that the inoculation of any of the micrococci strains or the addition of two different concentrations of papain resulted in the production of FAA from pork myofibrils (Table 5) after 24 and 48 h. An increase in FAA was also observed for the control, which was not as great as the increase observed for inoculated samples, indicating that there was possibly some enzyme activity retained in the myofibrils after extraction. It was also evident that the inoculation of the cultures affected the values of FAA, which could be seen by the different starting values for each sample (Table 5). This was possibly due to the presence of some of the growth culture media. Overall, over time, the contribution of culture media to the level of FAA was found to be negligible (results not shown). The results of the analysis clearly show that, over a period of 48 h, the sample inoculated with *Mic. varians* 20 produced the greatest amount of FAA, followed by *Mic. varians* 13, 4·5 units ml^{-1} papain and the solution of 0·45 units ml^{-1} papain. The samples inoculated with *Mic. varians* 4 gave the lowest increase in free amino acids. It was also evident that the different strains produced various FAA to different degrees (Table 6a, b).

DISCUSSION

The typical flavour of dry sausage is due to products originating from the fermentation of carbohydrates, lipolysis and lipid oxidation, proteolysis, seasonings and curing salts (Verplaetse 1994). Proteolysis occurring in these sausages is considered to be mainly due to endogenous meat enzymes, although it is possible that starters could have an effect. Of the starter cultures used for meat fermentations, it is considered that proteolytic activity is mainly associated with staphylococci and micrococci rather than the lactobacilli whose presence is essential for the production of fermented sausages. The results of the screening process clearly showed that, of the strains tested, proteolytic activity against pork

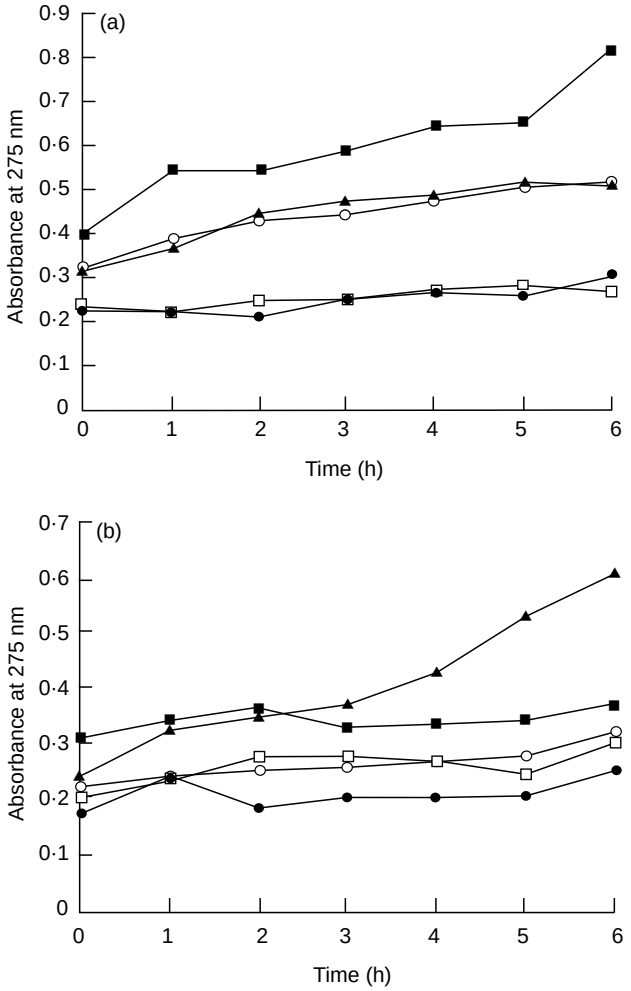

Fig. 1 Determination of proteolytic activity by spectrophotometric means. (a) ■, MCV4; ▲, MCV13; ○, MCV20; □, M17; ●, MIII. (b) ■, O3A; ▲, LAD; ○, T303; □, UC 317; ●, UC 509

Table 2 Model summary statistics

	R^2	R^{2*}	Predicted R^2
MCV4			
Linear	0·651	0·606	0·507
Quadratic	0·772	0·651	0·345
Papain			
Linear	0·502	0·437	0·306
Quadratic	0·813	0·714	0·506

MCV4, *Micrococcus varians* 4.
* Fitted for degrees of freedom.

Table 3 Regression model fitted for proteolytic activity of *Micrococcus varians* 4

	RC	S.E.	SL
Constant	318·67	68·55	—
Linear			
A	192	31·74	0·00
B	58·56	31·74	0·083
C	−92·56	31·74	0·01
Quadratic			
A × A	−55	54·96	0·331
B × B	−55·67	54·96	0·325
C × C	−105	54·96	0·073
Interaction			
A × B	58·5	38·87	0·151
A × C	−34.25	38·87	0·391
B × C	−20·92	38·87	0·597

A, pH; B, temperature (°C); C, level of NaCl (%); RC, regression coefficient; S.E., standard error; SL, significance level.

myofibrils was confined to strains of the genus *Micrococcus*, suggesting that these strains could have an effect on the proteolysis in fermented sausages. While the screening process demonstrated the inherent ability of the strains to degrade protein, the conditions of the test differed greatly from conditions found in fermented sausages where the pH can be <5, the temperature can be as low as 12 °C and NaCl concentration can be $>3\%$. Therefore, it was decided to examine the effects of environmental conditions on the proteolytic activity of one of the strains, with papain as a positive control. While previous studies have shown that the addition of starter culture has little effect on proteolysis in fermented sausages (Verplaetse 1994; Molly *et al*. 1997) the effect of the various environmental factors on microbial proteolysis has not been studied in detail, in contrast to endogenous meat enzymes which have been examined extensively (Toldra *et al*. 1992, 1993). Examination of the effect of environmental conditions on proteolytic activity clearly showed that, under conditions normally found in fermented sausages, activity was drastically reduced in the case of both MCV4 and papain. In the case of MCV4, it was shown that the highest proteolytic activity (within the constraints of the experimental design) was achieved at pH 7·0, 30 °C and 1% NaCl and the lowest activity was achieved at pH 4·5, 18 °C and 2% NaCl, which reflect conditions close to those found during the production of fermented sausages. This clearly suggests that microbial proteases are not very active during the production of dry sausages therefore adding further strength to suggestions that proteolysis is due mostly to endogenous meat enzymes. Results of RSM plots for papain also showed that maximum activity was achieved at pH 7·0, 30 °C and 1% NaCl which

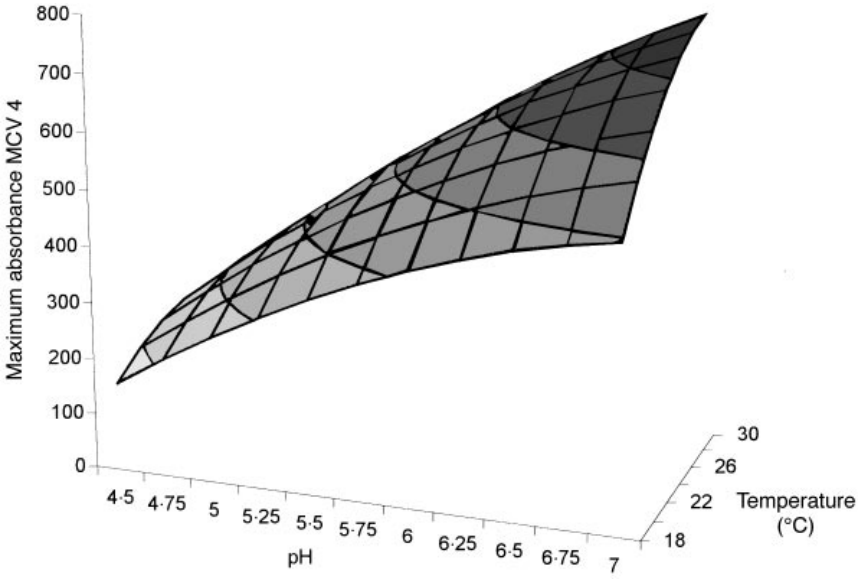

Fig. 2 Effect of combined environmental conditions on the proteolytic activity of MCV 4 (1% NaCl). □, 0–100; □, 100–200; □, 200–300; □, 300–400; □, 400–500; □, 500–600; □, 600–700; □, 700–800

Table 4 Regression model fitted for proteolytic activity of papain

	RC	S.E.	SL
Constant	1005·67	135·29	—
Linear			
A	293·5	62·63	0·000
B	285·22	62·63	0·000
C	−107·11	62·63	0·105
Quadratic			
A × A	−401·167	108·47	0·002
B × B	167·67	108·47	0·141
C × C	−129·67	108·47	0·248
Interaction			
A × B	196·83	76·7	0·02
A × C	−155·67	76·7	0·058
B × C	−24·17	76·7	0·757

A, pH; B, temperature (°C); C, level of NaCl (%); RC, regression coefficient; S.E., standard error; SL, significance level.

therefore shows a similar trend to MCV4. This result is in agreement with a report by Diaz *et al.* (1996) where the pH optimum was reported as being within the range 6·0–7·5. It was also seen that the proteolytic activity of papain seemed to be greatly affected by changes in temperature as the lowest activity was already observed when the temperature was 24 °C. Overall, the minimum activity for papain was observed at pH 4·5, 24 °C and 1% NaCl, again suggesting that the activity of this enzyme would be reduced under conditions found in fermented sausages, although it can be added to meat at levels of 1 mg kg^{-1} (Sargeant *et al.* 1993) which could counteract this reduction in activity. It can also be seen that, under the conditions of the experiment, the proteolytic activity of papain was well in excess of that of MCV4 (the maximum value of papain was more than twice the value for MCV4). Sodium dodecyl sulphate polyacrylamide gel electrophoretograms also showed that the addition of lactobacilli or micrococci to the myofibril solution did not result in an improvement in the breakdown of myofibrillar proteins in comparison to the control. The fact that there was breakdown of myosin heavy chain in all samples including the control suggested that some endogenous calpain and cathepsin activity remained in the myofibrils after extraction. The resultant breakdown products of protein by proteases include polypeptides, peptides and FAAs. These FAAs are important flavour precursors and may also be beneficial from a health viewpoint due to the better bioavailability of some of the essential amino acids (Toldra and Aristoy 1993). Micrococci have previously been shown to have peptidase activity (Bhowmik and Marth 1998; Hinrichsen *et al.* 1994), as have lactobacilli (Sanz and Toldra 1997), and therefore these microorganisms may have an effect on the flavour of fermented sausages. A recent report (Rodriguez *et al.* 1998) has shown that, although a number of strains of staphylococci and micrococci as well as yeasts and moulds isolated from dry-cured ham could completely break down myosin in a broth system, small amounts of FAAs were produced in a model cured ham system. However, this work did not specifically examine the ability of micrococci to produce FAA, but concentrated on strains of *Staphylococcus*, *Penicillium* and *Debaromyces*. It can clearly be seen from the results that the three strains of micrococci evaluated all had peptidase activity, which mani-

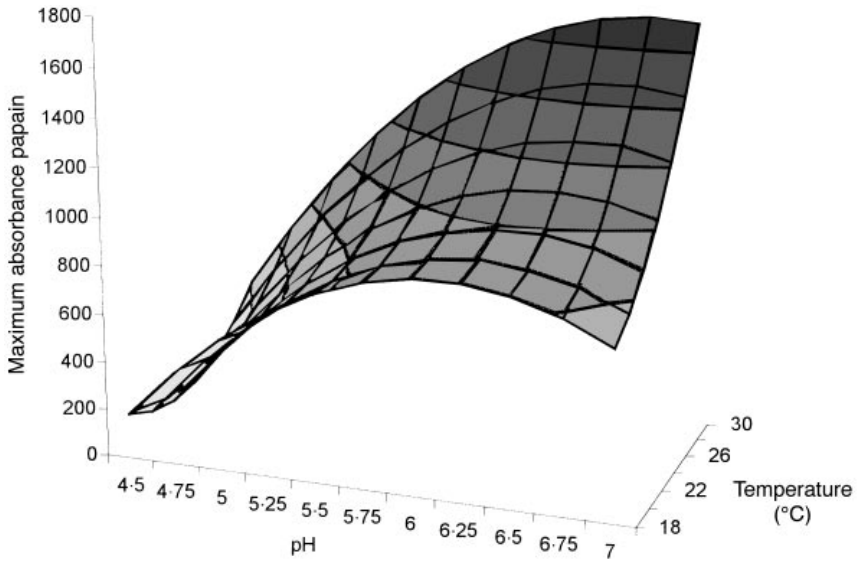

Fig. 3 Effect of environmental conditions on the proteolytic activity of papain (1% NaCl). □, 0–200; □, 200–400; □, 400–600; □, 600–800; □, 800–1000; □, 1000–1200; □, 1200–1400; □, 1400–1600; □, 1600–1800

Table 5 Total free amino acid production (μg g^{-1} protein)

	24 h	48 h
Control	1555 ± 4	2606 ± 36
MCV4	7221 ± 222	16 847 ± 166
MCV13	11 225 ± 255	56 913 ± 1048
MCV20	4550 ± 112	594 991 ± 900
Papain (4·5 units ml^{-1})	24 427 ± 997	34 761 ± 1766
Papain (0·45 units ml^{-1})	14 678 ± 807	32 307 ± 1609

MCV, *Micrococcus varians.*

fests itself in the production of FAAs. It was observed that *Mic. varians* 20 produced the greatest level of FAA followed by MCV 13 and MCV4, and that the levels of FAA produced by these strains compared favourably with the levels produced by the addition of papain. The three strains also produced different amino acids to varying degrees, as did the two different concentrations of papain. The fact that no breakdown of myofibrillar proteins was observed for the micrococci strains using sodium dodecyl sulphate polyacrylamide gel electrophoresis suggests that the FAAs acids resulted from degradation of low molecular weight proteins or peptides by a peptidase system, possibly in conjunction with minor breakdown of some of the larger proteins. Therefore, the presence of a peptidase system in the three micrococci strains was demonstrated. The activity of these peptidases under sausage conditions was not evaluated definitively, although the response surface methodology experiment measured total proteolytic activity (including peptidases). Hence, it appears that these enzymes are not particularly active under sausage conditions, which could point to the involvement of tissue peptidases in the production of FAA.

CONCLUSION

The proteolytic activities of a microbial strain and papain were found to be at a minimum under conditions reflecting those found in fermented sausages, thereby highlighting the major role of endogenous meat enzymes in the proteolysis occurring in these products. The presence of peptidase activities in the micrococci strains was also shown, which could be significant from a flavour and health point of view in fermented meats, although their activity under the environmental conditions associated with fermented sausages has not been examined and may merit further investigation.

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Table 6a Free amino acid production (μg g^{-1} protein)

	Control T24	Control T48	MCV4 T24	MCV4 T48	Papain T24	Papain T48
Cysteic acid	318·2 ± 3·8	352·7 ± 2·9	403·3 ± 75·3	674·7 ± 5·5	2486 ± 524	3226·5 ± 5·5
Aspartic acid	147·6 ± 0·8	186·9 ± 0	233·3 ± 14·3	455·9 ± 9·3	650·6 ± 3·4	806·6 ± 8·8
Threonine	23·5 ± 0·4	28·6 ± 2·5	309·2 ± 7·1	439·5 ± 10·5	153·9 ± 2·9	230·1 ± 1·3
Serine	17 ± 2·9	7·7 ± 2·5	54·2 ± 4·2	122 ± 51·3	120·5 ± 9·7	138·1 ± 7·6
Glutamic acid	40·2 ± 5·5	30·6 ± 7·1	1486·9 ± 79·1	3861·6 ± 0·4	220·5 ± 4·6	722 ± 21
Glycine	0 ± 0	12·5 ± 0	115·8 ± 2·1	316·4 ± 3·8	67·9 ± 96	117·3 ± 165·8
Alanine	9·8 ± 5·5	34·2 ± 1·3	464·3 ± 1·7	1292·9 ± 1·7	272 ± 183·5	605·6 ± 489·5
Cysteine	351·2 ± 26·9	397·6 ± 11·8	507·4 ± 101·4	595·2 ± 27·8	6882·1 ± 1547·2	8765·5 ± 2605·3
Valine	48·5 ± 2·1	182·4 ± 8	515·8 ± 24·8	1316·4 ± 21·5	2025·9 ± 5·5	2878·9 ± 27·4
Methionine	0 ± 0	87·8 ± 5·5	267·6 ± 10·5	983·3 ± 22·7	2049·1 ± 10·5	3193·4 ± 19·4
Isoleucine	30·1 ± 1·3	101·2 ± 0·8	298·8 ± 10·9	703·9 ± 15·6	676·5 ± 16·4	1120·8 ± 30·3
Leucine	75·6 ± 0·8	203·9 ± 0·4	802·1 ± 29·9	2009·2 ± 20·6	2132·4 ± 45·9	3971·1 ± 119·1
Tyrosine	106·5 ± 4·2	170·5 ± 1·3	244 ± 3·4	0 ± 0	1224·1 ± 14·7	1081·8 ± 54·3
Phenylalanine	73·5 ± 8	162·2 ± 3·8	359·8 ± 4·6	1350·9 ± 27·4	1024·1 ± 83·8	1876·8 ± 164·1
Histidine	49·4 ± 0·8	102·7 ± 3·8	75 ± 0·8	211·6 ± 299·3	2756·2 ± 380·1	3283·6 ± 229·4
Lysine	264 ± 1·3	554·3 ± 15·6	1083 ± 34·1	2513·4 ± 40	1685·1 ± 26·1	2742·9 ± 27·8

MCV, *Micrococcus varians.*

Table 6b Free amino acid production (μg g^{-1} protein)

	MCV13 T24	MCV13 T48	MCV20 T24	MCV20 T48	Papain* T24	Papain* T48
Cysteic acid	3598·5 ± 9·3	1330·4 ± 75·8	426·2 ± 31·1	1508·6 ± 368·3	1666·7 ± 218·9	2633·9 ± 117
Aspartic acid	440·5 ± 6·7	1334·2 ± 1·3	197·9 ± 7·1	2134·8 ± 30·7	360·1 ± 24·4	733 ± 34·9
Threonine	469·3 ± 1·3	1501·8 ± 23·6	173·8 ± 4·2	2094·9 ± 29·9	90·8 ± 14·7	61·6 ± 16·4
Serine	240·8 ± 3·8	187·5 ± 5·1	72·9 ± 8	623·5 ± 11·4	30·4 ± 9·3	49·4 ± 7·6
Glutamic acid	1833·9 ± 10·1	100019·6 ± 189·4	1469·1 ± 22·7	8312·8 ± 104	61·6 ± 13·9	716·7 ± 29·5
Glycine	319·6 ± 4·2	173·8 ± 39·6	26·2 ± 0·8	1232·1 ± 26·9	48·2 ± 4·2	101·8 ± 10·1
Alanine	820·5 ± 38·3	4800·6 ± 351	164·6 ± 54·3	3630·6 ± 1103·2	366·1 ± 18·5	941·4 ± 34·9
Cysteine	1042·9 ± 172·6	2308·3 ± 729·8	533·6 ± 296·7	4897·3 ± 3864·2	2934·8 ± 98·9	4968·4 ± 271·9
Valine	750·9 ± 8·8	4770·5 ± 46·7	230·9 ± 13·5	4661·9 ± 64	1358 ± 2·1	3996·1 ± 193·2
Methionine	503 ± 0·8	4231·8 ± 9·7	119·3 ± 52·6	4560·7 ± 57·2	1339 ± 4·6	3559·8 ± 131·7
Isoleucine	411 ± 3·8	3864·6 ± 49·2	110·1 ± 19·4	3935·1 ± 86·7	511·3 ± 36·2	1533·3 ± 86·7
Leucine	1289 ± 16·4	8233·3 ± 150·7	317·3 ± 10·1	8618·1 ± 242	1359·8 ± 11·4	5155·4 ± 151·5
Tyrosine	307·4 ± 3·8	0 ± 0	0 ± 0	0 ± 0	810·1 ± 68·2	422·6 ± 4·2
Phenylalanine	690·5 ± 4·2	5291·7 ± 88·4	149·1 ± 45·9	6081·5 ± 228·1	665·8 ± 24	2409·5 ± 42·9
Histidine	196·7 ± 85·4	683 ± 816·1	0 ± 0	1093·1 ± 480·2	1958·9 ± 819·1	1780·6 ± 145·2
Lysine	1549·1 ± 22·3	6581·5 ± 96	558·6 ± 13·9	6113·1 ± 131·3	1116·4 ± 61·9	3242·9 ± 677·6

* 0·45 units ml^{-1} papain.
MCV, *Micrococcus varians.*

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