Fermentation of Dry Sausage - The Importance of Proteolytic and Lipolytic Activities of Lactic Acid Bacteria.

H. NÆS[1], J. CHRZANOWSKA[2], J. NISSEN-MEYER[3], B.O. PEDERSEN[1] and H.BLOM[1].

1) MATFORSK, Norwegian Food Research Institute, Oslovn. 1, N-1430 Ås, Norway. 2) Agricultural University of Wroclaw, C.K. Norwida 25/27, 50-375 Wroclaw, Poland. 3) Laboratory of Microbial Gene Technology, N-1432 Ås-NLH, Norway.

SUMMARY: By adding lipase and proteinase to sausage mixtures, significant differences in off-odour, meat whiteness, bitter taste, hardness and stickiness were observed during fermentation. Lipases from 3 different lactic acid bacteria have been partly purified and characterized. Optimal lipase activity was detected at pH 5.5-6.0. The enzymes were active at a temperature range of 14-45 °C, with an optimum at 30-37 °C. A proteinase isolated from a lactobacillus strain showed optimal activity at 35-37 °C and at pH 4.8 when using haemoglobin as substrate. Consequently, the enzymes can be used in fermentation productions.

INTRODUCTION: Much attention has been given to the mechanism of lactic acid production and nitrate and nitrite reduction by lactic acid bacteria during dry sausage fermentation (LIEPE 1982, LÜCKE 1984). However, microorganisms may also play an important role in flavour formation as a result of proteolysis and lipolysis (SELGAS et al. 1988). The aim of this study was to evaluate by sensoric analysis the aroma developed in fermented salami after addition of lipase and proteinase. The present paper also describes the lipolytic activity towards various substrates of 3 different lactic acid bacteria originating from meat sources. Lipases from the same strains have been partly purified and characterized. A cell wall bound proteinase from *Lactobacillus casei* NCDO151, has been purified and characterized.

MATERIALS and METHODS:

Preparation of sausages: The basic initial sausage mixture contained (% w/w): beef (51.3), pork (18.7), lard (25.6), salt (3.3), ascorbate (0.4), dextrin (0.3) and glucose (0.4). Smoking and addition of spices were excluded. *Lactobacillus sake* L45 was used as starter culture and supplied at $5 \cdot 10^5$ /g sausage mixture. The components were mixed and divided into 4 batches. To batch 1 lipid esterase (Boeringer Mannheim, cat. no 414590) was added to give a final concentration of 2 units (U)/g sausage. Proteinase extracted from *L. casei* NCDO151 was added to batch 2 and 3 to a final concentration of 1 U/g and 10 U/g sausage, respectively. Batch 4 represented the control without any enzyme added. Each batch was divided into sausages of 1 kg portions. After the initial fermentation phase (2 days at 24 °C and 92 % relative humidity (rh), 2 days at 20 °C and 88 % rh, 2 days at 18 °C and 85 % rh) the sausages were ripened at 15 °C and 85 % rh for 30 days. Thereafter they were vacuum-packed and stored at 4 °C until sensory analysis. Total viable counts and pH was measured throughout the whole experimental period.

Sensory analysis: Standard procedures for sensory descriptive profiling according to AMERINE et al. (1965) and RISVIK (1985) were used. Three replicates were served to 10 trained assessors and evaluated for the following attributes: odour intensity, acidic odour, off-odour, whiteness of fat, colour strength, colour tone, taste intensity, meat taste, acidic taste, bitter taste, rancid taste, maturity taste, off-taste, hardness, fattiness, juiciness and stickiness. Sensory characteristics were scored on an open ended intensity scale from low (1) to high (9) intensity for each of the characteristics. Statistical analyses were performed using the SYSTAT-program for univariate analysis (WILKINSON, 1990).

Cultivation and preparation of lipase extract: The organisms were cultivated in MRS-broth (Difco) at 30 °C over night. The culture was harvested , centrifuged at 8000 x g for 10 min. and washed twice in 50 mM Na-phosphate buffer, pH 7.1. The pellet was resuspended in the

Na-phosphate buffer (1% of the culture volume) and disrupted by ultrasonic treatment (30 sec. disrupture and 60 sec. cooling on ice, repeated for 1 to 3 h depending on the bacterial strain). The sample was then centrifuged (18000 x g, 50 min) and filtered through h 0.22 µm filter. This supernatant was designated the crude lipase extract.

Determination of lipase activity in enzyme extracts: Lipase activity was tested by applying 0.1 ml enzyme extract in wells on agar plates containing tributyrin and Rhodamine B and supplied with Tween-20,-40,-60 and -80, respectively. The agar plates were made according to KOUKER and JAEGER (1987). Lipase activity was also analyzed against pork fat. These agar plates had the following composition : 200 ml nutrient agar (Difco), 0.2 g pea fiber (Grinsted P-fibre 150, Danisco, Denmark), 0.5-1.0 ml fat/leaf fat and 2 mg Rhodamine B. The plates were incubated at 37 °C over night and the diameter of the zones of hydrolysis measured.

Effect of pH and temperature on lipase activity: The effect of pH from 4.5 - 7.0 was determined using agar plates containing tributyrin and Tween 80 without Rhodamine B. The effect of temperature was measured by incubation in the range of 14-57 °C, at pH 7.0.

Purification of lipase: The crude lipase extract was purified using Fast Protein Liquid Chromatography (FPLC, Pharmacia) and an anion exchange column (Mono Q HR 5/5) that was equilibrated with 5 mM Na-phosphate buffer, pH 7.1. The enzyme was eluted with a gradient of NaCl (0-1.0 M) at a flow rate of 1ml/min.

Extraction of proteinase from *L. casei* NCDO151: Cells were grown over night at 30 °C in MRS-broth containing 20 mM $CaCl_2$ and 50 mM β-glycerophosphate. Cells were harvested, centrifuged and washed twice with 50 mM β-glycerophosphate, 20 mM $CaCl_2$, pH 7.0. Extraction of cell wall bound proteinase was performed by resuspending the cells 3 successive times in 1/30 of the culture volume of 50 mM Tris-HCl buffer, pH 7.5 at room temperature for 1 h. The extracts were filtered through 0.22 µm filters. Determination of proteolytic activity with ^{14}C-methylated casein or ^{14}C-methylated haemoglobin was performed as described by EXTERKATE (1975). One unit of proteolytic activity was defined as the amount of enzyme releasing 1 % of initial radioactivity of ^{14}C-labelled casein/haemoglobin after 10 min. incubation.

Purification of proteinase: The method used was as described for lipase except for that the equilibration and elution buffer were 20 mM Tris-HCl, pH 8.4.

RESULTS and DISCUSSION: *L. sake* L45 was selected as starter organism due to its capability to grow fast and produce bacteriocin (MØRTVEDT and NES, 1990). In this respect it will dominate the bacterial population and inhibit growth of closely related bacteria. It is of advantage to eliminate the contribution of various microorganisms when single fermentation traits like the effect of proteinase and lipase are to be evaluated. During the fermentation and ripening period the pH dropped from 5.65 to a final value of 5.0 after 36 days. A corresponding increase in total viable counts from $5 \cdot 10^5$ to $2 \cdot 10^8$ CFU/g sausage were detected during the first 5 days, indicating a normal fermentation process.

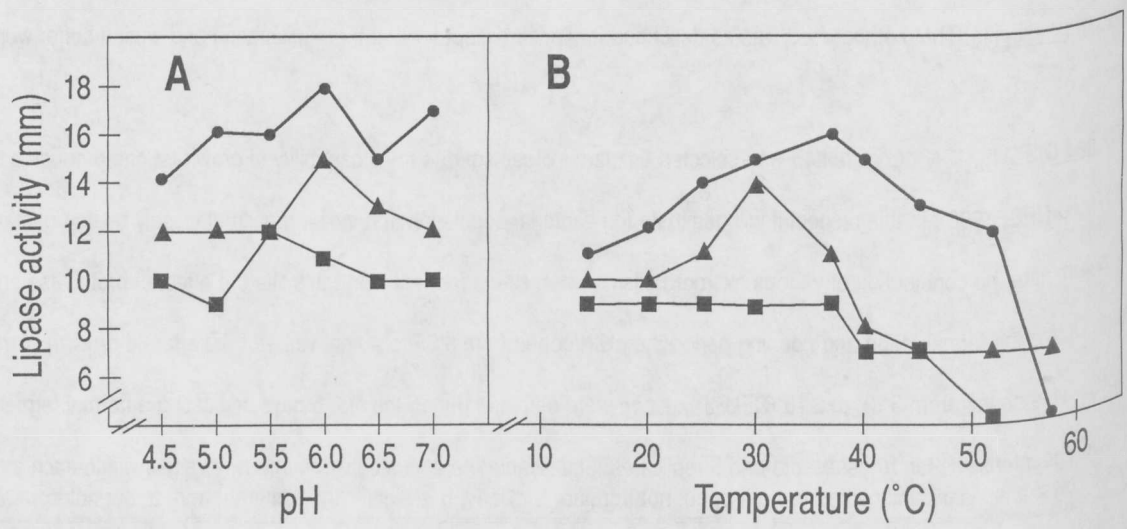
Table 1: Average intensities for 10 assessors and 3 replicates. Significant differences between intensity values within each attribute are indicated as follows: a is significantly different from b, but not from ab. Similarly, b is significantly different from a, but not from ab.

Sensory variables	Control	Lipase (2U/g)	Proteinase (1U/g)	Proteinase (10U/g)	Significance level (%)
off-odour	2.15ab	2.48a	2.24ab	1.95b	3.7
meat whiteness	4.74b	5.46a	5.02b	4.92b	1.0
bitter taste	1.97b	2.62a	1.93b	1.94b	1.0
hardness	4.97ab	4.95ab	4.82b	5.31a	3.1
stickiness	3.61b	4.24a	3.65b	3.54b	2.0

By adding proteinase or lipase to the sausages, differences (< 5% significance level) in off-odour, meat whiteness, bitter taste, hardness and stickiness were observed. The sausage containing lipase had the most bitter tase, showed greatest meat whiteness and was most sticky. Concerning these attributes no differences were observed when comparing the control with those containing proteinase. When comparing the salamis containing the high and low concentration of proteinase, respectively, the latter one was less hard. Proteolytic enzymes degrade proteins into peptides and amino acids and a soft consistency would be expected. However, proteolytic activities early in the fermentation process may result in less water binding capasity. The sausage containing the highest concentration of proteinase may, therefore, has an increased loss of water during the long ripening period. Addition of lipase resulted in increased off-odour compared to the salami containing the highest concentration of proteinase.

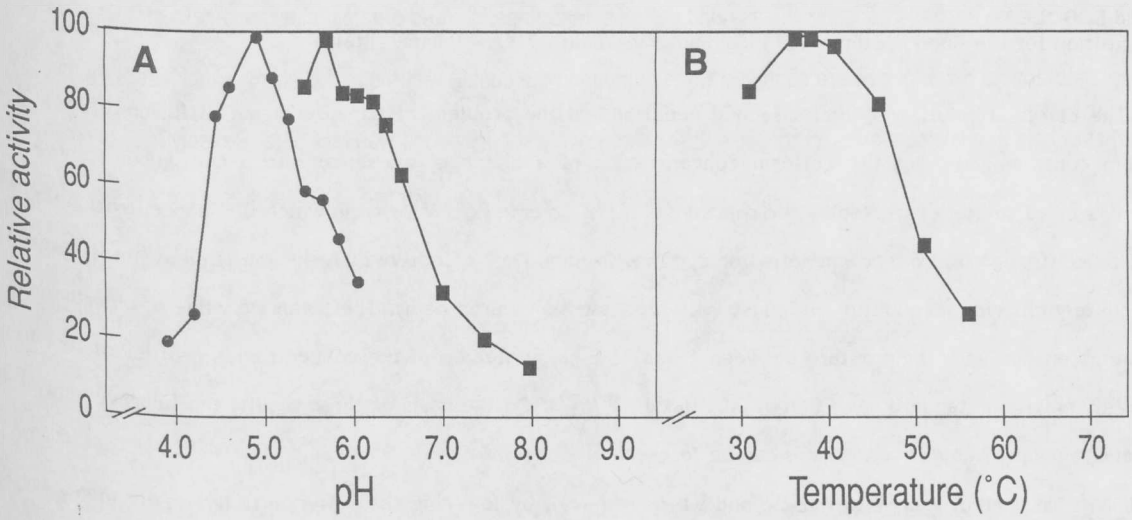
From a total of 78 strains, 11 showed lipolytic activity. Three strains (*Lactobacillus plantarum* MF32, *Pediococcus pentosacceus* SV61 and *Leuconostoc* L1) were selected for further studies due to their broad substrate specificity. They showed lipolytic activity on fat, leaf fat and tributyrin containing Tween-20, -40, -60, or -80. As shown in Fig. 1a, the pH optima for the lipase activities were 5.5 for SV61 and 6.0 for L1 and MF32. SV61 did not show any clear optimal temperature for lipase activity and was constant in a range from 14 to 37 °C (Fig. 1b). The lipase activity of L1 and MF32 had an optimum at 30 °C and 37 °C, respectively. Lipase activity was completely inactivated at 52 °C for SV61 and 57 °C for MF32. Lipase from L1 was still active at these temperatures, indicating a rather temperature stable lipase. It is noteworthy that all strains showed lipolytic activity during actual fermentation conditions and that these enzymes are active against natural fats. Upon purification by anion exchange chromatography on Mono Q, the lipolytic activity peaks eluted between 0.27 M and 0.32 M NaCl for all 3 strains. In addition a lipolytic activity peak was also detected for L1 at 0.18-0.19 M NaCl, indicating the presence of isoenzymes or two different lipases. Due to the high lipase activity of MF32 this strain is suitable for further fermentation studies.

Figure 1: pH and temperature activity curves of lipase from *L. plantarum* MF32 (●), *P. pentosacceus* SV61 (■) and *Lactococcus* L1 (▲)



Anion exchange chromatography of the crude proteinase extract from NCDO151 gave a single peak with proteolytic activity eluting at 0.25 mM NaCl. At this stage a 6-fold increase in specific activity was observed. The effect of pH and temperature on the activity of the NCDO151 proteinase is shown in Fig. 2. The optimum pH was 4.8 when haemoglobin was used as substrate, and with casein the optimal proteolytic activity was at pH 5.6. This fits very well with the conditions during a fermentation.

Figure 2: pH and temperature activity curves of proteinase from *L.casei* NCDO151 on casein (■) and haemoglobin (●).



Hydrolysis of casein was optimal at 35-37 °C and declined sharply above 40 °C.

CONCLUSIONS: It has been shown that addition of lipase and proteinase to sausages give significant differences in sensory characteristics during fermentation. It is of interest to investigate the contribution of lipase and proteinase producing organisms in sausage fermentation. Identification and cloning of genes encoding lipase and proteinase formation are in progress. To evaluate the contribution of single traits, genetically transformed starter strains represent an excellent tool. The same strain is used as an enzyme producer and non enzyme producer, while other growth characteristics are identical. This approach will make it possible to rule out the contribution of the actual enzymes.

REFERENCES:

AMERINE, M.A., PANGBORN, R.M. and ROESSLER, E.B. (1965): Principles of Sensory Evaluation of Food. New York: Academic Press.

EXTERKATE, F.A. (1975): An Introductory Study of the Proteolytic System of *Streptococcus cremoris* strain HP. Neth. Milk Dairy. J. 29: 303-318.

KOUKER, G. and JAEGER, K.-E. (1987): Specific and Sensitive Plate Assay for Bacterial Lipases. Appl. Environ. Microbiol. 53: 211-213.

LIEPE, H.U. (1982): Starter Cultures in Meat Production. In: "Biotechnology" (H.J. REHM and G. REED, eds.) Verlag Chemie, Basel, vol 5: 400-424.

LÜCKE, F.K. (1984): Fermented Sausages. In: "Microbiology of Fermented Foods" (B.J.B. Wood, Ed). Applied Science Publishers, London, vol.2: 41-49.

MØRTVEDT, C.I. and NES, I.F. (1990): Plasmid-associated Bacteriocin Production by a *Lactobacillus sake* strain. J. Gen Microbiol. 136: 1601-1607.

RISVIK, E. (1985): Sensory Analyses. Tecator AB, Höganäs, Sweden.

SELGAS, M.D., SANZ, B. and ORDONEZ, J.A. (1988): Selected Characteristics of Micrococci Isolated from Spanish Dry Fermented Sausages. Food Microbiol. 5: 185-193.

WILKINSON, L. (1990): SYSTAT: The System of Statistics. Systat, Inc. Evanston, IL.