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Partial purification and characterisation of a lipase from *Lactobacillus plantarum* MF32

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A lipase from *Lactobacillus plantarum* MF32 has been partly purified and characterised. The apparent molecular weight of the lipase was estimated to be approximately 75 000. Isoelectric focusing resulted in two separate bands corresponding to pI values of 7·50 and 7·60, respectively. The enzyme has been shown to contribute to sensory quality and reduced production time of fermented dry sausages. Temperature optimum was 37°C with tributyrin as substrate and 41°C with lard as substrate, the overall activity of the lipase being about three times higher with tributyrin as substrate than with lard. Enzyme activity of the lipase was detectable at pH 4·5 and pH 12, with a pH optimum around 9·3 for both substrates. The enzyme activity was only slightly affected by salt concentrations up to 5% NaCl. The temperature dependence of the enzyme as described by the Arrhenius equation with tributyrin as substrate showed a $\Delta H^{\ddagger}_{298}$ (inactivation) of 186 kJ/mol. The activation of the enzyme appears non-linear with increasing temperature, probably due to changes in availability of the substrate with temperature.

INTRODUCTION

Lactic acid bacteria are widely used as starter cultures in the manufacture of fermented meat products (Smith & Palumbo, 1983; Lücke & Hechelmann, 1987), where they are involved not only in suppressing undesirable bacteria, but also in subsequent flavour development (Lücke, 1986). The metabolic activities of these microorganisms are known to play a key role in acid production (Lücke & Hechelmann, 1987), but the role of microorganisms in flavour development in fermented meat products is far from well understood (Sanz *et al.*, 1988; Dainty & Blom, 1994).

Lipolysis appears to be an important event in the ripening of fermented meat products. Recent studies showed that bacterial starter cultures synthesised volatile compounds from free fatty acids. These compounds contributed to the flavour development in fermented meat products (Molina *et al.*, 1991; Berdagué *et al.*, 1993).

The dairy industry has a long history in preparing fermented products with starter cultures containing well-described microorganisms with specific metabolic activities. The purification and characterisation of enzymes, especially proteinases and peptidases (Exterkate & De Veer, 1987; Kiefer-Partsch *et al.*, 1989; Tan & Konings, 1990; Lloyd & Pritchard, 1991; Niven 1991) but also lipases (Chander *et al.*, 1973; Lee & Lee, 1989, 1990; Alhir *et al.*, 1990), have been essential in understanding the general physiology of starter cultures and the improvement of starter culture strains with regard to their technological properties.

In contrast, only a few attempts have been made to purify and characterise different proteolytic and hydrolytic enzymes from microorganisms used as meat starters (Sanz *et al.*, 1988; Papon & Talon, 1989; Næs *et al.*, 1991*a*, 1992, 1993). The successful expression of a lipase gene from *Staphylococcus hyicus* in a meat starter culture (*Lactobacillus curvatus* Lc2-c) (Vogel *et al.*, 1990) and well-developed transformation procedures (Aukrust & Blom, 1992) indicate the possibility of creating tailor-made starter cultures. Thus, it is important that metabolic enzymes from starter cultures

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are purified and characterised with regard to their importance for flavour development in fermented meat.

The present study was undertaken to purify and characterise a lipase from a lactobacillus strain, *Lactobacillus plantarum* MF32, originally isolated from spontaneously fermented meat. This enzyme has been shown to contribute to sensory quality and reduced production time of fermented sausage (Næs *et al.*, 1993).

MATERIALS AND METHODS

Cultural conditions and preparation of crude lipase extract

Lactobacillus plantarum MF32 was cultivated in MRS Broth (Difco Laboratories, MI, USA) at 30°C overnight. The culture was harvested, centrifuged (8000*g*, 10 min) and washed twice (5 mM Tris–HCl, pH 8·0). The pellet was resuspended in 12·5 mM Tris–HCl, pH 8·9 (1% of culture volume) containing 20 mg/ml lysozyme (Sigma, St Louis, MO, USA, 3 × crystallised from chicken egg white, 56500 U/mg) for 30 min at 37°C. Subsequently, the sample was centrifuged (18000*g*, 20 min) and the supernatant filtered through a 0·22 μm filter. This supernatant was then passed through two Sep-Pak cation cartridges (Accell Plus CM, Waters, Milford, MA, USA), which had been pre-washed with 12·5 mM Tris–HCl, pH 8·9. The effluent was then applied to a Sep-Pak anion cartridge (Accell Plus QMA, Waters), pre-washed with 12·5 mM Tris–HCl, pH 8·0. The cartridge was washed with five volumes of 5 mM sodium phosphate buffer pH 7·1 and the bound activity eluted with four volumes of 5 mM sodium phosphate buffer pH 7·1 containing 0·35 M NaCl. The resulting effluent was concentrated three-fold using ultra-filtration with a filter with cut-off value of 10000 (Diaflo, 10 PM 10, Amicon Corp., Lexington, MA, USA) and is in the present report designated crude lipase extract (CLE).

Enzyme assays

Lipolytic activity was assayed at 25°C except in experiments with lard (37°C) and in the determination of optimum temperature. For the determination of optimum pH, optimum temperature and effect of NaCl on the CLE, a pH-stat method was used. The substrate solution contained 10% (w/v) gum arabicum (KEBO, Norway) and 10% (w/v) tributyrin (Sigma) or 10% (w/v) lard, both emulsified by homogenisation (3 × 30 s) using an ultra-turrax macerator mounted with a S25N-18G dispersing tool (Janke and Kunkel, Germany). The pH was adjusted with 0·1 M NaOH. Substrate solution (5 ml) was transferred into a thermostated reaction vessel of the pH-stat (Radiometer Autotitration, Denmark), 0·1 ml CLE was added and the reaction was allowed to proceed. The volume of 0·005 M NaOH consumed, to keep the pH constant, was recorded and the slope of the recorded line was taken as a measure of the reaction rate. For the determination of optimum temperature and effect of NaCl (1, 3 and 5% w/w), the reaction system described was set to pH 9·5.

Enzyme activity in fractions collected after chromatography was measured using a plate assay with TWEEN-80 (Sigma) as substrate. TWEEN-80 (0·25% final concentration) was added to nutrient agar containing Rhodamine B (10 μg/ml, Sigma) and 0·1% pea fibre (Danisco, Denmark). Enzyme activity (in 0·1 ml) was registered by measuring the diameter of the clearing zones (visible upon UV-light illumination) after 18 h incubation at 37°C.

Protein determination

Protein concentration was determined by the Bio-Rad Protein Reagent (BIO-RAD, South Richmond, CA, USA) according to the manufacturer, using lysozyme (Sigma, 3 × crystallised from chicken egg white) as standard.

Ion-exchange chromatography and molecular weight determination

Chromatography was performed using the Pharmacia FPLC system. A flow rate of 1 ml/min was used and fractions of 1 ml were collected. A Mono-Q HR 5/5 ion-exchange column (Pharmacia, Sweden) was equilibrated with Buffer A (5 mM phosphate buffer, pH 7·1). After application of CLE (1 ml), elution was performed in the following manner: 2 min with buffer A, 0–15% buffer B (1 M NaCl in buffer A) for 8 min, 15–30% buffer B for 30 min, 30% buffer B for 5 min and finally 5 min at 100% buffer B. Fractions containing enzyme activity were collected and diluted with one volume of buffer A and subjected to rechromatography on the same column using the same eluting conditions. Fractions containing enzyme activity were collected and designated partly purified enzyme (PPE).

Molecular weight was determined using a Superose 12 10/30 column (Pharmacia). The column was equilibrated and eluted with 0·5 M phosphate buffer, pH 7·2 containing 0·15 M NaCl. An amount of 0.2 ml PPE was applied on the column. Elution was performed with a flow rate of 0·5 ml/min, and 0·25-ml fractions were collected.

Isoelectric focusing

PPE was used for the determination of the isoelectric point of the lipase, employing the PhastSystem of Pharmacia with an IEF 5-8 PhastGel and the standard operational procedures of Pharmacia. Visualisation was obtained through silver staining, according to Pharmacia Development Technique File No. 210.

RESULTS AND DISCUSSION

Purification of the lipase

A summary of the purification procedure is shown in Table 1. Using this procedure, it was possible to achieve

Table 1. Summary of the procedure for partial purification of lipase of *L. plantarum* MF32

Purification step	Total protein (mg)	Total activity[a] (units)	Specific activity[b] (units/mg)	Yield (%)	Fold purification
Lysozyme extract	110	2108	1·9	100	1·0
2'K⁺-cartridge	635	2099	3·3	99·6	1·7
1'A⁻-cartridge	219	1980	9·0	94·0	4·7
Mono-Q 1	8·85	1638	185	77·7	96·4
Mono-Q 2	1·17	408	349	19·4	182

[a]One unit of enzyme is defined as the enzyme necessary to release 1 μmol FFA (free fatty acids) from tributyrin per minute at 25°C.
[b]Specific activity is defined as enzyme units per milligram protein.

a 180-fold of purification, with 20% of the original activity remaining. Cationic and anionic cartridges were used to remove large quantities of protein, including the added lysozyme (cationic cartridge). The highest purification factor was achieved by repeated ion-exchange chromatography. The chromatograms from ion-exchange chromatography are shown in Fig. 1.

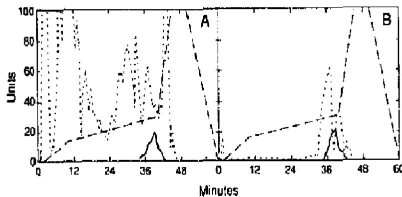

Fig. 1. A) Mono-Q HR 5/5 ion-exchange chromatography of 1-ml CLE and B) Mono-Q HR 5/5 re-chromatography of 20 ml of pooled lipase activity containing fractions from ion-exchange chromatography of CLE. For chromatographic conditions, see text. (....) AU_{280nm}, (--) Gradient and (—) Esterase activity (Tween-80).

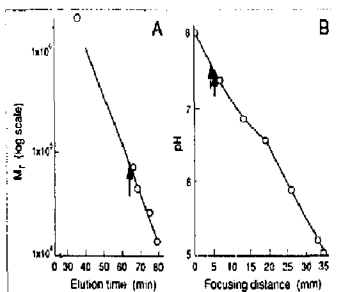

Fig. 2. A) Determination of apparent molecular weight of lipase from molecular sieve chromatography. The arrow indicates the retention time of the lipase. M_r standards: blue dextran (2 000 000), ovalbumin (45 000), chymotrypsinogen (25 000) and ribonuclease A (13 700). B) Isoelectric focusing of lipase-containing fraction after re-chromatography on Mono-Q HR 5/5. The arrows indicate the position of the two proteins appearing after staining of the gel pI standards: soya bean trypsin inhibitor (4·55), β-lactoglobulin A (5·20), bovine carbonic anhydrase B (5·85), human carbonic anhydrase B (6·55), horse myoglobin (acidic) (6·85), horse myoglobin (basic) (7·35), and lentil lectin (8·15).

Enzyme activity profile after repeated ion-exchange chromatography indicates that the lipase elutes in two non-separable fractions.

From molecular sieve chromatography of PPE (Fig. 2A), the apparent molecular weight of the lipase was estimated to be 75 000 on the basis of used standards.

Isoelectric focusing of PPE resulted in two separate bands corresponding to pI values of 7·50 and 7·60 (Fig. 2B).

Characteristics of the lipase

The effects of pH, temperature and NaCl on the activity of CLE with tributyrin and lard as substrates were investigated (Fig. 3). Activity was demonstrated against both tributyrin and lard.

The temperature optimum was found to be 37°C with tributyrin as substrate and around 41°C with lard as substrate (Fig. 3A). However, even at 15°C activity was found with both tributyrin and lard as substrate. This indicates that the lipase will be effective at temperatures normally used during the processing of fermented meat products.

pH optimum was around 9·3 for both substrates. The overall activity of the lipase was about three times higher with tributyrin as substrate than with lard (Fig. 3B). Activity of the lipase has been demonstrated at pH 4·5 using agar diffusion assay (Næs *et al.*, 1991*b*) and at pH 12 (data not shown). These data indicate that the lipase of *Lactobacillus plantarum* MF32 will contribute to lipolysis mainly during the initial ripening of fermented meat products. The amount of lipolysis needed to give rise to detectable amounts of flavour precursors in fermented meat products is not known. The present

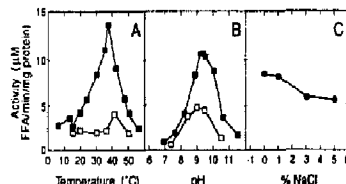

Fig. 3. A) Effect of temperature on lipase activity of CLE and B) effect of pH on lipase activity of CLE using (■) tributyrin and (□) lard as substrates. C) Effect of NaCl on lipase activity of CLE with tributyrin as substrate, pH 9·5 at 25°C.

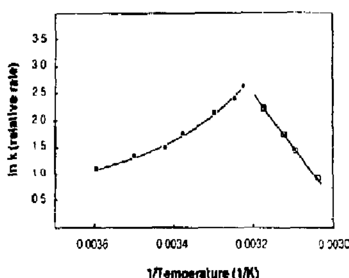

Fig. 4. Arrhenius Plot for CLE with tributyrin as substrate.

lipase shows a distinct alkaline pH optimum in contrast to those of lipases of lactic acid bacteria isolated from dairy sources (pH 6·5–7·5) (Lee & Lee, 1990; El Soda *et al.*, 1986). Crude preparations of lipases from other meat lactobacilli also exhibit alkaline pH optima (unpublished data). The enzyme activity was only slightly affected by salt concentrations up to 5% NaCl (Fig. 3C).

The temperature dependence of the enzyme is described by the Arrhenius equation with tributyrin as substrate (Fig. 4). The inactivation enthalpy ($\Delta H'_{298}$), calculated according to the transition state theory, is 186 kJ/mol, which equals a Q_{10} (inactivation) of 2·3, in the investigated temperature area (42–56°C). The activation of the enzyme appears non-linear with increasing temperature, probably due to changes in the state of the emulsions with temperature.

A partly purified preparation of the *Lactobacillus plantarum* MF32 lipase has been shown to cause accelerated maturation in dry sausage production. When compared to the control sausages (without lipase), significant differences in sensory attributes, such as intensity, maturity, acidity and hardness, were observed ($P < 0{\cdot}05$) (Næs *et al.*, 1993).

Sausages with lipase appeared mature after 14 days of ripening, whereas the control sausages required one more week to reach the same stage of maturity. These findings may be of economic importance to the fermentation industry.

To the knowledge of the present authors, this is the first time a specific lipase from a meat *Lactobacillus*, which has been shown to give rise to sensory changes in a fermented dry sausage (Næs *et al.*, 1993), has been characterised.

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