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Original article

Purification and partial characterization of an esterase from *Lactococcus lactis* ssp *lactis* strain ACA-DC 127

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Summary — An esterase from *Lactococcus lactis* spp *lactis* strain ACA-DC 127, isolated from Greek Feta cheese, was purified on DEAE-cellulose and Sephadex G-100. The enzyme had a molecular weight of 68 000, with an optimum activity on 4-nitrophenyl butyrate at pH 8.0 at 45 °C with K_m = 1.11 mmol.l^{-1}. The esterase was capable of degrading synthetic substrates of low molecular weight. It was strongly inactivated by PMSF. 1,10-phenanthroline and EDTA had no effect on enzyme activity. The bivalent cations Hg^{2+} and Cu^{2+} inhibited the esterase activity.

***Lactococcus lactis* ssp *lactis* / esterase / purification / activity / characterization**

Résumé — Purification et caractérisation partielle d'une estérase intracellulaire de *Lactococcus lactis* ssp *lactis*, souche ACA-DC 127. *Une estérase intracellulaire d'une souche* Lactococcus lactis *ssp* lactis *ACA-DC 127, isolée du fromage grec féta, a été purifiée sur DEAE-cellulose et Sephadex G-100. L'enzyme a un poids moléculaire de 68 000. Son activité est optimale pour le butyrate de 4-nitrophénol à un pH 8,0 et à la température de 45 °C avec une K_m = 1,11 mmol.l^{-1}. L'action hydrolytique de l'estérase est spécifique pour les substrats synthétiques de faible poids moléculaire. L'enzyme est inactivée en présence de PMSF. Le 1,10-phénanthroline et l'EDTA n'ont aucune influence sur l'activité de l'enzyme. Les cations bivalents Hg^{2+} et Cu^{2+} inhibent l'activité de l'estérase.*

Lactococcus lactis *spp* lactis / *estérase / purification / activité / caractérisation*

INTRODUCTION

Cheese ripening is a long and complex process, which proceeds with the breakdown of protein, carbohydrate and fat and results in the characteristic texture and flavour of cheese. Presence in cheese of various compounds is indicative of the complexity of the ripening process.

Lipolysis during cheese maturation is primarily the result of hydrolysis of milk fat catalyzed by various microbial esterolytic

and lipolytic enzymes (Marth, 1963). Esterases are arbitrarily classified as enzymes hydrolyzing substrates in solution (soluble esters of short-chain fatty acids), whereas lipases hydrolyze substrates in emulsions. However, there is no complete distinction between them, as evidence is lacking to indicate that the esterase activity against soluble substrates and lipase activity against insoluble substrates refer to different catalytic mechanisms.

The exact role of esterases in cheese ripening is not completely understood. However, it has been suggested that esterases play an important role in the production of short-chain free fatty acids which contribute to the development of flavour in cheese (Lawrence *et al,* 1976).

Lactic acid bacteria are only weakly lipolytic when compared with similar activity in many other groups of bacteria including *Pseudomonas*, *Aeromonas*, *Acinetobacter* and *Flavobacterium* (Kalogridou-Vassiliadou, 1984).

Stadhouders and Mulder (1958) reported that the lipase activity of lactic acid bacteria is very limited, and suggested that these microorganisms have no significant role in hydrolyzing triglycerides of milk fat during cheese ripening.

Singh *et al* (1973) studied the extracellular and intracellular lipases of lactic streptococci. They found that intracellular lipase from *Streptococcus lactis* hydrolyzed tributyrin, but that hydrolysis of tripalmitin and triolein was very limited.

Piatkiewicz (1987) studied the production of lipase and esterase by mutant strains of lactic streptococci and lactobacilli. The author found that streptococci had greater lipase and esterase activities than did lactobacilli. Esterase activity was relatively higher than lipase activity in all strains.

Harper *et al* (1980) evaluated the esterase activity of cell-free extracts from lactic streptococci in the late logarithmic growth phase. Electrophoresis on polyacrylamide gel, followed by gel staining revealed different pattern for separated esterase enzymes.

In a previous study we reported the post-electrophoretic detection of esterase activities in cell-free extracts from various strains of *Lactococcus lactis* ssp *lactis* (Tsakalidou *et al,* 1992). The present study describes the purification and characterization of an esterase from *Lactococcus lactis* ssp *lactis* strain ACA-DC 127, isolated from Greek Feta cheese.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Lactococcus lactis ssp *lactis* strain ACA-DC 127 was obtained from the laboratory of Dairy Research collection (Agricultural University of Athens). It was isolated from Greek Feta cheese and stored at –30 °C in sterile skim milk. It was subcultured twice in milk and then in M 17 broth (Terzaghi and Sandine, 1975) at 30 °C. Growth was assessed by measurement of absorbance at 600 nm.

Preparation of cell-free extract

Late logarithmic phase cells were collected from the growth medium by centrifugation (12 000 g for 15 min at 4 °C). The pellet obtained was washed twice with 0.9% (w/v) NaCl solution, resuspended in 50 mmol.l^{-1} Tris–HCl buffer, pH 8.5 and then sonicated for 5 min under cooling. The supernatant obtained after centrifugation (12 000 g for 15 min at 4 °C) was designated the cell-free extract.

Purification of enzyme

The crude cell-free extract was first applied to a column of DEAE–cellulose (2 x 8 cm) equilibrat-

ed with 50 mmol.l^{-1} Tris–HCl buffer, pH 8.5. The column was washed with 2 vol equilibration buffer. Elution was performed at a flow rate of 2 ml.min^{-1} with a linear gradient of 0–0.5 mol.l^{-1} NaCl in the same buffer (total vol 360 ml). The enzyme containing fractions were pooled and diluted with 50 mmol.l^{-1} Tris–HCl buffer, pH 7.5. The solution was then applied to the same column as before, this time equilibrated with 50 mmol.l^{-1} Tris–HCl buffer, pH 7.5, and the elution followed under the same conditions. The enzyme containing fractions were pooled, dialyzed against water, lyophilized, dissolved in 0.1 mol. l^{-1} Tris–HCl buffer, pH 7.0 and then filtered on a Sephadex G-100 column (2 x 94 cm) equilibrated in the same buffer, at a flow rate of 12 ml.h^{-1}.

Protein determination

The method of Lowry *et al* (1951) was used with bovine serum albumin as standard.

Measurement of enzyme activity

Enzyme solution (50 µl) and 4-nitrophenyl butyrate (50 µl; 20 mmol.l^{-1} in methanol) were incubated in 400 µl 0.1 mol.l^{-1} Tris–HCl buffer, pH 8.0 at 45 °C (Brandl and Zizer, 1973). Activity was assayed at 400 nm by measuring the degree of substrate hydrolysis. The spontaneous hydrolysis of the substrate was controlled using 50 µl 0.1 mol.l^{-1} Tris–HCl buffer, pH 8.0 was controlled using 50 µl 0.1 mol.l^{-1} Tris–HCl buffer, pH 8.0 instead of the enzyme solution in the assay. A unit of enzyme activity was defined as the amount of enzyme producing 1 µmol 4-nitrophenol (ε_{400} = 15 500 l.mol^{-1}.cm^{-1}) per min. Specific activity was defined as the number of units per mg protein.

Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis according to Laemmli (1970) (12.5% acrylamide gel; 25 mmol.l^{-1} Tris–0.19 mol.l^{-1} glycine buffer, pH 8.3; in the presence of SDS (0.1%, w/v)) was used to control the purification steps of the enzyme and to determine the molecular weight of the purified esterase. Phosphorylase β (92 000), bovine serum albumin (67 000), egg albumin (45 000) and carbonic anhydrase (29 000) were used as marker proteins.

After polyacrylamide gel electrophoresis under the same conditions as before but in the absence of SDS and using 7.5% acrylamide gels, the enzyme was detected directly on the gel according to the method of Harper *et al* (1980) with β-naphthyl butyrate as substrate.

Characterization of the enzyme

The molecular weight of the purified enzyme was estimated by gel filtration on a 2 x 94 cm column of Sephadex G-100 standardized with the following proteins of known molecular weight: Phosphorylase β (92 000), bovine serum albumin (67 000), egg albumin (45 000) and trypsin inhibitor from soyabean (22 000).

pH optimum

Enzyme activity was measured in 0.1 mol.l^{-1} acetate (pH 5.0–5.5), 0.1 mol.l^{-1} phosphate (pH 6.0–7.5) and 0.1 mol.l^{-1} Tris–HCl (pH 8.0–9.0) buffers, at 45 °C, using 4-nitrophenyl butyrate as substrate. The ε_{400} variation of 4-nitrophenol between pH 5–8 was considered and the results respectively corrected (Brandl and Zizer, 1973). The spontaneous hydrolysis of the substrate was controlled using 50 µl of the appropriate buffer instead of the enzyme solution in the assay.

Temperature optimum

Enzyme activity was measured in 0.1 mol.l^{-1} Tris-HCl buffer, pH 8.0, at various temperatures from 15–65 °C using 4-nitrophenyl butyrate as substrate. In all cases the spontaneous hydrolysis of the substrate was controlled using 50 µl 0.1 mol.l^{-1} Tris–HCl buffer, pH 8.0 instead of the enzyme solution in the assay.

Michaelis constant

Enzyme activity measurements were carried out in 0.1 mol.l^{-1} Tris–HCl buffer, pH 8.0, at 45 °C, using various concentrations of 4-nitrophenyl butyrate from 0.1 mmol.l^{-1}–2 mmol.l^{-1}.

Substrate specificity

Enzyme activity measurements were carried out in 0.1 mol.l^{-1} Tris–HCl buffer, pH 8.0, at 45 °C, with the following substrates: 2- and 4-nitrophenyl acetate (C_2), butyrate (C_4), caproate (C_6), caprylate (C_8), laurate (C_{12}) and palmitate (C_{16}). The method described above under measurement of enzyme activity was used. In the case of 2-nitrophenyl derivatives ε_{400} = 4 300 l.mol^{-1}. cm^{-1} for 2-nitrophenol was considered.

Effect of inhibitors and metal ions

N-Ethylmaleimide, iodoacetic acid, diisopropylfluorophosphate (DFP) and EDTA (20 mmol.l^{-1} in 0.1 mol.l^{-1} Tris–HCl buffer, pH 7.0) as well as solutions of the bivalent metal ions Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+} and Hg^{2+} (10 mmol.l^{-1} in 0.1 mol.l^{-1} Tris–HCl buffer, pH 7.0) were incubated with an equal vol of enzyme solution in the same buffer at 25 °C. At various time intervals aliquots of the solution were added to the assay mixture and the remaining activity was measured. Phenylmethylsulfonylfluoride (PMSF) and 1,10-phenanthroline (0.1 mol.l^{-1} in isopropanol) were diluted to 20 mmol.l^{-1} in 0.1 mol.l^{-1} Tris–HCl buffer, pH 7.0 and then incubated with the enzyme as before. The effect of isopropanol on the enzyme activity was also considered.

Effect of sodium chloride

NaCl solutions (4, 8, 12, 16 and 20% in 0.1 mol. l^{-1} Tris–HCl buffer, pH 7.0) were incubated with an equal vol of enzyme solution in the same buffer at 4 °C. At various time intervals aliquots of the solution were added to the assay mixture and the remaining activity was measured.

RESULTS

Purification of enzyme

Separation of the esterase from *Lactococcus lactis* ssp *lactis (Streptococcus lactis)* ACA-DC 127 was performed by ion-exchange chromatography on DEAE–cellulose. The enzyme was eluted at a concentration of 0.17 mol.l^{-1} in the NaCl gradient for both pH 8.5 (fig 1) and 7.5. After the second DEAE column the activity containing fractions were pooled, concentrated and further purified by gel filtration on a Sephadex G-100 column. The elution profile showed that the esterase was eluted as a symmetrical peak. Recovery and degree of purification are summarized in table I.

Polyacrylamide gel electrophoresis in the absence of SDS gave only one band too, with R_f = 0.58; this band reacted with β-naphtyl butyrate as described above (fig 2b).

Characterization of the enzyme

Molecular weight (MW)

A MW of 68 000 was estimated by gel filtration. The symmetry of the single peak of enzyme activity suggested the presence of only one esterase component. When examined by electrophoresis in the presence of SDS and 2-mercaptoethanol (Laemmli, 1970), the purified enzyme was also found to be homogenous since only one band with MW corresponding to 64 000 was observed (fig 2a).

pH optimum

The enzyme was active over a pH range of 5.0–9.0. The optimum pH for the hydrolysis of 4-nitrophenyl butyrate was 8.0 when Tris–HCl buffer was used. Under these conditions the enzyme showed 42% of its maximum activity at pH 9.0 but only 30% at pH 7.0 (fig 3).

Temperature optimum

At pH 8.0 with 4-nitrophenyl butyrate as substrate the optimum temperature of the

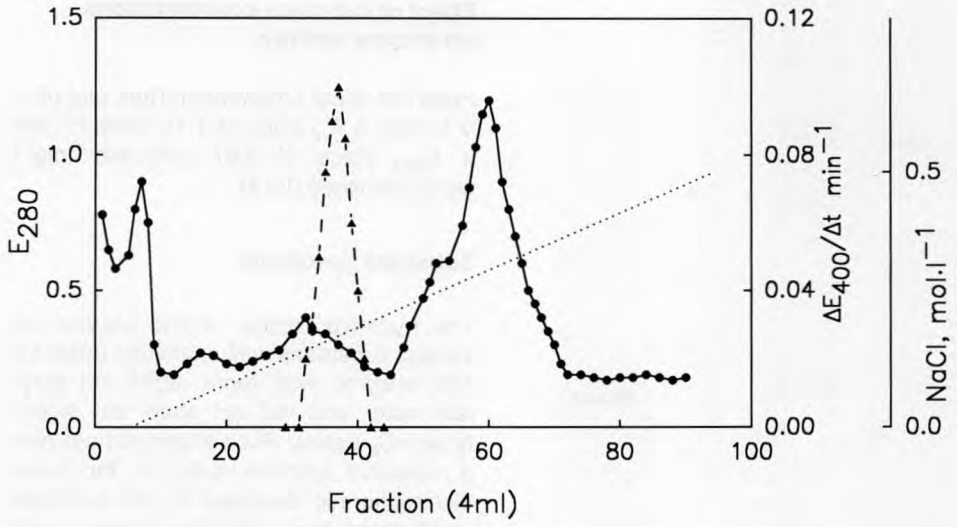

Fig 1. Elution profile of the *Lactococcus lactis* ssp *lactis* ACA-DC 127 crude cell-free extract from DEAE–cellulose equilibrated with 50 mmol.l^{-1} Tris–HCl buffer, pH 8.5, using a linear NaCl gradient, 0–0.5 mol.l^{-1}. Column size: 2 x 8 cm. Flow rate: 2 ml.min^{-1}. Fraction volume: 4 ml.
Profil d'élution du surnageant d'extraction de Lactococcus lactis *ssp* lactis *ACA–DC 127 sur DEAE–cellulose équilibrée en tampon Tris–HCl 50 mmol.l^{-1}, pH 8,5. Élution par un gradient de concentration en NaCl 0–0,5 mol.l^{-1}. Taille de colonne : 2 x 8 cm. Débit : 2 ml.min^{-1}. Volume des fractions : 4 ml.*

Table I. Purification and recovery of the esterase from *Lactococcus lactis* ssp *lactis* ACA-DC 127.
Purification et rendement de l'estérase de Lactococcus lactis *ssp* lactis *ACA-DC 127.*

Purification step	*Total protein (mg)*	*Specific activity (U*/mg)*	*Total activity (U*)*	*Yield (%)*	*Purification (fold)*
Crude extract	290.0	0.02	5.80	100	1
DEAE–cellulose 1	7.0	0.60	4.20	73	30
DEAE–cellulose 2	2.5	1.10	2.75	47	55
Sephadex G-100	0.6	2.40	1.44	25	120

* One unit (U) is defined as the amount of enzyme producing 1 µmol 4-nitrophenol per min from 4-nitrophenyl butyrate.
** Une unité (U) est définie comme étant la quantité d'enzyme produisant 1 µmol de nitro-4-phénol par minute à partir de nitro-4-phényle butyrate.*

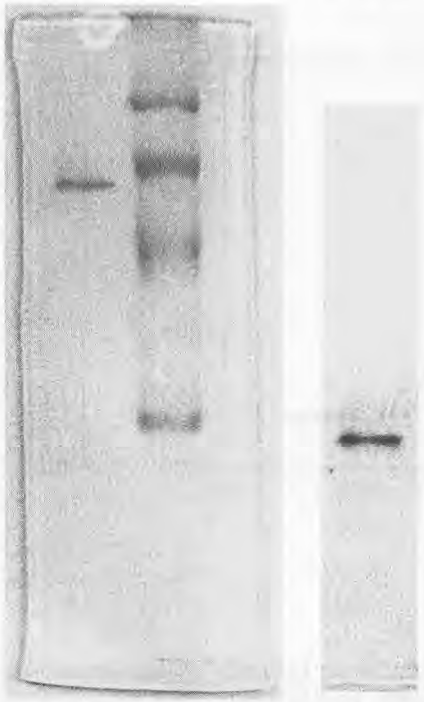

Fig 2. a. SDS–Polyacrylamide gel electrophoresis (12.5% acrylamide; 25 mmol.l^{-1} Tris–0.19 mol.l^{-1} glycine buffer, pH 8.3). A, esterase after Sephadex G-100 column; **B,** marker proteins (92, 67, 45 and 29 kDa from the top to the bottom). **b.** Polyacrylamide gel electrophoresis without SDS (7.5% acrylamide; 25 mmol.l^{-1} Tris–0.19 mol.l^{-1} glycine buffer, pH 8.3). **A,** purified esterase reacted with β-naphthyl butyrate.
***a.** Électrophorèse en gel de polyacrylamide SDS (12,5% acrylamide; solution tampon Tris 25 mmol.l^{-1}–glycine 0,19 mol.l^{-1} pH 8.3). **A,** Estérase après passage sur colonne Sephadex G-100 et **B,** protéines marqueurs (92, 67, 45 and 29 kDa du haut vers le bas). **b.** Électrophorèse en gel de polyacrylamide sans SDS (7,5% acrylamide; solution tampon Tris 25 mmol.l^{-1}–glycine 0,19 mol.l^{-1} pH 8,3). **A,** Estérase purifiée, révélée par réaction avec le β-naphtyl butyrate.*

esterase was 45 °C. Nevertheless, the enzyme showed 73% of its maximum activity at 15 °C and 62% of it even at 65 °C (fig 3).

Effect of substrate concentrations on enzyme activity

From the linear Lineweaver–Burk plot of 1/*V* *v* 1/[S], a K_m value of 1.11 mmol.l^{-1} and a V_{max} value of 5.57 μmol.min^{-1}.mg^{-1} were calculated (fig 4).

Substrate specificity

The hydrolytic action of the enzyme on various substrates was examined (table II). The enzyme was more active on butyrate ester and did not show any activity on >C_8 esters. Such esters did not give a coloured enzyme band in the post-electrophoretic detection of the esterase. In all cases the 4-nitrophenyl esters were more rapidly hydrolysed than the respective 2-nitrophenyl esters.

Effect of inhibitors and metal ions

Reagents reacting with metals (EDTA and 1,10-phenanthroline) had no effect on the enzyme activity even at a final concentration of 10 mmol.l^{-1} and after 60 min incubation at 25 °C. In the case of serine group reagents diisopropylfluorophosphate (DFP) could not inactivate the enzyme; on the contrary, after 60 min incubation of the esterase with phenylmethylsulfonylfluoride (PMSF) (10 mmol.l^{-1}) at 25 °C, only 10% of the total activity remained. As far as sulfhydryl group reagents are concerned very low enzyme inhibition was observed in the case of *N*-ethylmaleimide and iodoacetic acid; nevertheless incubation of the enzyme with Cu^{2+} and Hg^{2+}, which are also known to react with SH groups, resulted in loss of activity after 60 min incubation at 25 °C. Mg^{2+} and Mn^{2+} presented low inhibition effect. Ca^{2+} had no effect on esterase activity (table III).

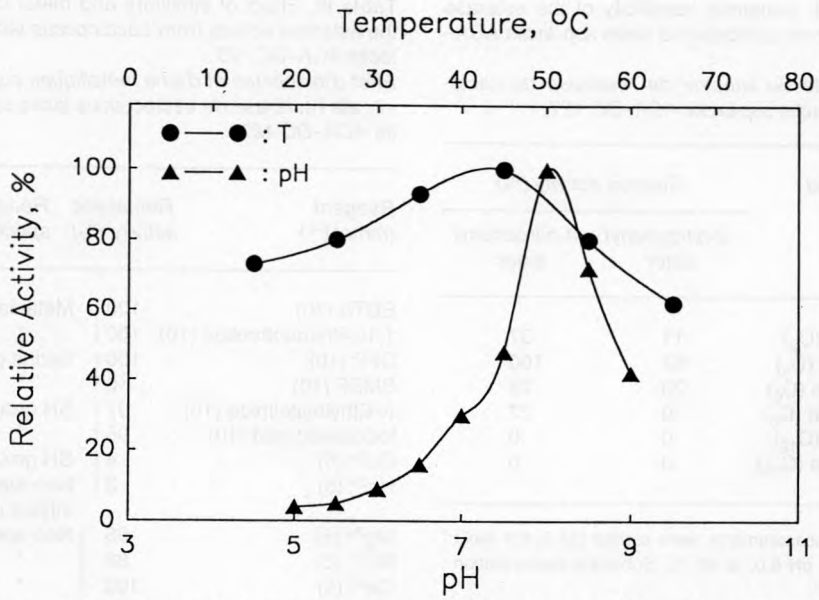

Fig 3. Effect of pH and temperature on the esterase activity from *Lactococcus lactis* ssp *lactis* ACA–DC 127 with 4-nitrophenyl butyrate as substrate.
Effet du pH et de la température sur l'activité de l'estérase de Lactococcus lactis *ssp* lactis *ACA–DC 127 avec, comme substrat, le nitro-4-phényle butyrate.*

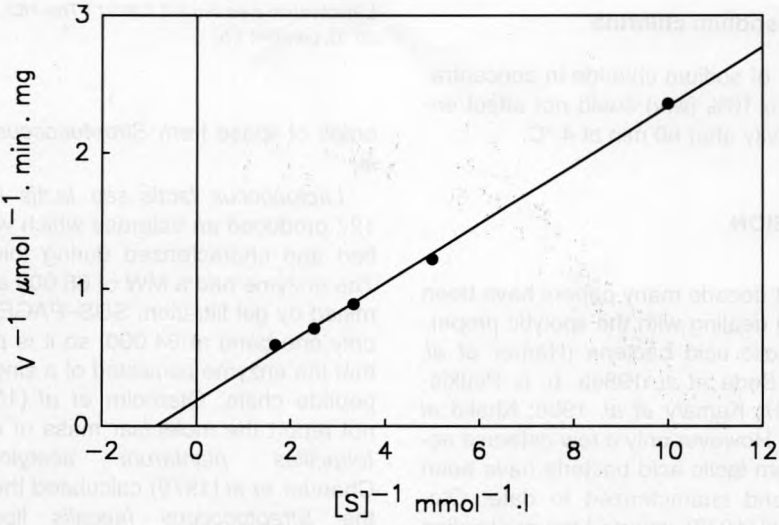

Fig 4. Lineweaver–Burk plot. Effect of 4-nitrophenyl butyrate concentrations on the esterase activity from *Lactococcus lactis* ssp *lactis* ACA–DC 127 at pH 8.0 and 45 °C.
Représentation de Lineweaver-Burk. Effet de la concentration en nitro-4-phényle butyrate sur l'activité de l'estérase de Lactococcus lactis *ssp* lactis *ACA–DC 127 à pH 8,0 et à 45 °C.*

Table II. Substrate specificity of the esterase activity from *Lactococcus lactis* ssp *lactis* ACA–DC 127.
Spécificité du substrat de l'estérase de Lactococcus lactis *ssp* lactis *ACA–DC 127.*

Fatty acid	*Relative activity (%)*	
	2-nitrophenyl ester	*4-nitrophenyl ester*
Acetate (C_2)	11	37
Butyrate (C_4)	62	100
Caproate (C_6)	23	78
Caprylate (C_8)	0	27
Laurate (C_{12})	0	0
Palmitate (C_{16})	0	0

Activity measurements were carried out in 0.1 mol.l^{-1} Tris–HCl, pH 8.0, at 45 °C. Substrate concentration : 2 mmol.l^{-1}.
Mesure de l'activité qui a eu lieu à 0,1 mol.l^{-1} Tris-HCl, pH 8,0, à 45 °C. Concentration de substrat : 2 mmol.l^{-1}.

Table III. Effect of inhibitors and metal ions on the esterase activity from *Lactococcus lactis* ssp *lactis* ACA–DC 127.
Effet d'inhibiteurs et d'ions métalliques sur l'activité de l'estérase de Lactococcus lactis *ssp* lactis *ACA–DC 127.*

Reagent (mmol.l^{-1})	*Remaining activity (%)*	*Reagent specificity*
EDTA (10)	100	Metal ions
1,10-Phenanthroline (10)	100	"
DFP (10)	100	Serine group
PMSF (10)	10	"
N-Ethylmaleimide (10)	97	SH group
Iodoacetic acid (10)	95	"
Cu^{2+} (5)	4	SH group or
Hg^{2+} (5)	3	Non-specific inhibition
Mg^{2+} (5)	95	Non-specific
Mn^{2+} (5)	88	"
Ca^{2+} (5)	100	"

DFP: diisopropylfluorophosphate; PMSF: phenylmethylsulfonylfluoride; EDTA: ethylene diamine tetraacetic acid. Inactivation was carried out in 0.1 mol.l^{-1} Tris–HCl, pH 7.0, at 25 °C for 1 h.
L'inactivation a eu lieu à 0,1 mol.l^{-1} Tris–HCl, pH 7,0, à 25 °C, pendant 1 h.

Effect of sodium chloride

Presence of sodium chloride in concentrations up to 10% (w/v) could not affect enzyme activity after 60 min at 4 °C.

DISCUSSION

In the last decade many papers have been published dealing with the lipolytic properties of lactic acid bacteria (Harper *et al,* 1980; El Soda *et al,* 1986a, b, c; Piatkiewicz, 1987; Kamaly *et al,* 1988; Khalid *et al,* 1990). However only a few esterase activities from lactic acid bacteria have been purified and characterized to date. Oterholm *et al* (1972) reported the purification and properties of an acetyl ester hydrolase from *Lactobacillus plantarum.* Chander *et al* (1979) purified and studied some properties of lipase from *Streptococcus faecalis.*

Lactococcus lactis ssp *lactis* ACA-DC 127 produced an esterase which was purified and characterized during this study. The enzyme had a MW of 68 000 as determined by gel filtration. SDS–PAGE yielded only one band at 64 000, so it is probable that the enzyme consisted of a single polypeptide chain. Oterholm *et al* (1972) did not report the molecular mass of the *Lactobacillus plantarum* acetylesterase. Chander *et al* (1979) calculated the MW of the *Streptococcus faecalis* lipase as 20 900, indicative of an exoenzyme.

The esterase was most active at alkaline pH and lost activity under acidic condi-

tions. It was interesting to note that at pH 5.0 the esterase activity was inhibited (≈ 96%); this may have practical importance, because pH values near 5.0 are encountered in ripening cheese. The pH range from 7.0–8.5 has often been reported as the range in which lipolytic enzymes show maximum activity (Oterholm *et al,* 1970; Chander *et al,* 1979; Bhowmik and Marth, 1990; Kamaly *et al,* 1990).

The esterase was active over a broad temperature range from 15–65 °C, with an optimum at 45 °C. Its high activity in lower temperatures, such as those used during cheese ripening, may partially account for development of cheese flavour. A similar broad temperature profile was also reported for esterases from *Micrococcus* species (Bhowmik and Marth, 1990). *Streptococcus faecalis* lipase (Chander *et al,* 1979) and *Lactobacillus plantarum* acetylesterase (Oterholm *et al,* 1972) presented maximum activity at 40 °C; *Propionibacterium shermanii* lipase at 47 °C (Oterholm *et al,* 1970). Highest specific lipase activity of *Streptococcus lactis* and *Streptococcus cremoris* (Kamaly *et al,* 1990) occurred at 37 °C.

The kinetic studies with the substrate 4-nitrophenyl butyrate, which appeared to be hydrolyzed the best, revealed medium affinity (K_m = 1.11 mmol.l^{-1}) and low maximal hydrolysis rate (V_{max} = 5.57 μmol.min^{-1}.mg^{-1}). Oterholm *et al* (1972) calculated for the *Lactobacillus plantarum* acetylesterase a K_m = 14 mmol.l^{-1} (aqueous triacetin as substrate) and Chander *et al* (1979) for the *Streptococcus faecalis* lipase a K_m = 5 mmol.l^{-1} (emulsified tributyrin as substrate).

Photometrical determination as well as post-electrophoretic detection of enzyme activity with different substrates showed that the esterase presented higher affinity to substrates with low molecular weight, which explains the role of esterases to produce short-chain free fatty acids. In all cases 4-nitrophenyl derivatives were faster hydrolyzed than the respective 2-nitrophenyl ones. The straight configuration of the 4-substituted derivatives makes them more accessible to the enzyme's active site. Similar results have been also reported by other authors (El Soda *et al,* 1986a, b, c; Kamaly *et al,* 1988; Khalid *et al,* 1990).

In order to clarify the mode of action of the isolated esterase, further experiments using aqueous and emulsified mono-, di- and triglycerides as substrates are necessary.

Inhibitors of metalloenzymes had no effect on esterase activity. From the serine group reagents tested PMSF inactivated the enzyme. This could be an indication for essential OH groups in its active site. Nevertheless DFP, which presents similar mode of action as PMSF, could not inactivate the esterase; this might be due to its greater steric demand. Bhowmik and Marth (1990) reported the strong inhibition of *Micrococcus* species esterases by DFP and less by 1,10-phenanthroline. Very low enzyme inhibition by specific sulfhydryl group reagents was observed. Nevertheless, Cu^{2+} and Hg^{2+} resulted in strong inactivation; although this could be a non-specific inhibition, it might suggest the presence of essential SH-groups in the enzyme molecule. The polar character as well as the little steric demand of these metal ions could facilitate their penetration to the enzyme's active site. In the case of Hg^{2+}, similar results have been reported by other authors (Oterholm *et al,* 1970, 1972; Bhowmik and Marth, 1990). Oterholm *et al* (1970) reported that specific sulfhydryl group reagents had little or no inhibitory effect on the *Propionibacterium shermanii* lipase. *Lactobacillus plantarum* acetylesterase was only slightly inhibited by such reagents (Oterholm *et al,* 1972). None of the metal ions tested stimulated esterase activity. Low inhibition was caused by Mg^{2+} and Mn^{2+}.

Sodium chloride yielded no esterase inactivation at concentrations up to 10%. This is important because salt concentrations can occur in cheese during the ripening process. In the case of *Micrococcus* species esterases, Bhowmik and Marth (1990) reported a marked inhibition of esterase activity depending on salt concentration.

The contribution of esterases to the cheese ripening process is not well defined. However, esterases have been linked to flavour development through lipolysis of milk fat (Oterholm *et al,* 1968). The study of an esterolytic enzyme using synthetic substrates is a first step in attempting to determine the mode of catalysis. Further experiments, under conditions resembling these of cheese ripening, could give more information about the role of esterases during this process.

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REFERENCES

Bhowmik T, Marth EH (1990) Esterases of *Micrococcus* species. Identification and partial characterization. *J Dairy Sci* 73, 33-40

Brandl E, Zizer T (1973) Hydrolysis of aromatic esters by microbial preparations with lipolytic and proteolytic activity. *Oesterr Milchwirt Wiss Beil* 28, 15-24

Chander H, Ranganathan B, Singh J (1979) Purification and some properties of lipase from *Streptococcus faecalis. J Food Sci* 44, 1747-1751

El Soda M, Fatallah S, Ezzat N, Desmazeaud MJ, Abou Donia S (1986a) The esterolytic and lipolytic activities of lactobacilli. Detection of the esterase systems of *Lactobacillus plantarum, Lactobacillus brevis* and *Lactobacillus fermentum. Sci Aliments* 6, 545-557

El Soda M, Arb El Wahab H, Ezzat N, Desmazeaud MJ, Ismail A (1986b) The esterolytic and lipolytic activities of lactobacilli. II. Detection of the esterase system of *Lactobacillus helveticus, Lactobacillus bulgaricus, Lactobacillus lactis* and *Lactobacillus acidophilus. Lait* 66, 431-443

El Soda M, Korayem M, Ezzat A (1986c) The esterolytic and lipolytic activities of lactobacilli. III. Detection and characterization of the lipase system. *Milchwissenschaft* 41, 353-355

Harper W, Carmona de Catril A, Chen JL (1980) Esterases in lactic streptococci and their stability in cheese slurry systems. *Milchwissenschaft* 35, 129-132

Kalogridou-Vassiliadou D (1984) Lipolytic activity and heat resistance of extracellular lipases of some Gram-negative bacteria. *Milchwissenschaft* 39, 601-604

Khalid NM, El Soda M, Marth EH (1990) Esterase of *Lactobacillus helveticus* and *Lactobacillus delbrueckii* ssp *bulgaricus. J Dairy Sci* 73, 2711-2719

Kamaly KM, El Soda M, Marth EH (1988) Esterolytic activity of *Streptococcus lactis, Streptococcus cremoris* and their mutants. *Milchwissenschaft* 43, 346-349

Kamaly KM, Takayama K, Marth EH (1990) Acylglycerol acylhydrolase (lipase) activities of *Streptococcus lactis, Streptococcus cremoris* and their mutants. *J Dairy Sci* 73, 280-290

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685

Lawrence RC, Thomas TD, Terzaghi BE (1976) Reviews in the progress of dairy science: cheese starters. *J Dairy Res* 43, 141-193

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* 193, 265-275

Marth EH (1963) Microbiological and chemical aspects of Cheddar cheese ripening. A review. *J Dairy Sci* 46, 869-890

Oterholm A, Ordal ZJ, Witter LD (1968) Glycerol ester hydrolase activity of lactic acid bacteria. *Appl Microbiol* 16, 524-527

Oterholm A, Ordal ZJ, Witter LD (1970) Purification and properties of a glycerol ester hydrolase (lipase) from *Propionibacterium shermanii. Appl Microbiol* 20, 16-22

Oterholm A, Witter LD, Ordal ZJ (1972) Purification and properties of an acetyl ester hydrolase (acetylesterase) from *Lactobacillus plantarum*. *J Dairy Sci* 55, 8-13

Piatkiewicz A (1987) Lipase and esterase formation by mutants of lactic acid streptococci and lactobacilli. *Milchwissenschaft* 42, 561-564

Stadhouders J, Mulder H (1958) Fat hydrolysis and cheese flavour. II. Microorganisms involved in the hydrolysis of fat in the interior of the cheese. *Neth Milk Dairy J* 12, 238-264

Singh A, Srinivasan RA, Dunani AT (1973) Studies on exocellular and endocellular lipases of some of the lipolytic bacteria. *Milchwissenschaft* 28, 164-166

Terzaghi BK, Sandine WE (1975) Improved medium for lactic streptococci and their bacteriophage. *Appl Microbiol* 29, 807-813

Tsakalidou E, Zoidou E, Kalantzopoulos G (1992) Esterase activities of cell-free extracts from strains *Lactococcus lactis* subsp *lactis* isolated from traditional Greek cheese. *J Dairy Res* 59, 111-113