Lait (1993) 73, 345-356

Original article

Esterase activity of dairy *Propionibacterium*

C Dupuis, P Boyaval *

Laboratoire de Recherche de Technologie Laitière, INRA, 65 rue de Saint-Brieuc, 35042 Rennes Cedex, France

(Received 17 March 1993; accepted 2 June 1993)

Summary — This paper reports results on the esterolytic activity of dairy *Propionibacterium*. All the species present an active esterolytic system on naphthyl substrates for fatty acids which contain < 10 carbons. Between 1 to 4 activities of different specificities were revealed on non-denaturing polyacrylamide gel electrophoresis for each species. These activity patterns could be very useful in differentiating between a few *Propionibacterium* species. Ion-exchange chromatography allowed 3 main esterase activities to be separated on a concentrated intracellular extract of *P freudenreichii* subsp *freudenreichii*. These activities have very different substrate specificities which agree closely with those determined by electrophoresis.

esterase / *Propionibacterium* / fatty acid / lipolysis / cheese

Résumé — *Activités estérasiques des bactéries propioniques.* *Les activités estérasiques des bactéries propioniques laitières ont été étudiées. Toutes les espèces possèdent des activités estérasiques significatives sur des dérivés naphtyl d'acides gras contenant jusqu'à 10 atomes de carbone. De une à 4 activités de spécificités différentes ont été mises en évidence pour chacune des espèces. Ces profils d'activités pourraient être un outil de différenciation de certaines espèces de* Propionibacterium. *Une prépurification par chromatographie d'échange d'ions d'extraits intracellulaires de* P freudenreichii *subsp* freudenreichii *a permis de révéler 3 activités distinctes, de spécificités très différentes, en parfait accord avec la mise en évidence d'activité enzymatique sur gel d'électrophorèse.*

estérase* / Propionibacterium / *acide gras* / *lipolyse* / *fromage

* Correspondence and reprints

INTRODUCTION

Propionic acid bacteria play important roles in many industrial processes: production of propionic acid used as a natural preservative in baking products (Boyaval and Corre, 1987); production of vitamin B_{12} and involvement in the development of the characteristic flavor and eye production in Swiss-type cheeses (Hettinga and Reinbold, 1972). Proteolysis and lipolysis are the key mechanisms in Swiss cheese ripening (Biede *et al,* 1979). Fat breakdown is apparently essential to the development of flavor (Ohren and Tuckey, 1965) and it is well-known that ripened Swiss cheese contains more free fatty acids than many other similar hard-ripened cheeses, including Cheddar (Oterholm, 1967). According to Lawrence (1967), it is obvious that large numbers of weakly lipolytic organisms may play an important role in products with a long storage life, such as cheese.

Even if propionic acid bacteria predominate in this type of cheese during ripening, their role in the formation of monocarboxylic acids from triglycerides has not been completely investigated and the results differ widely from one study to another. Propionic acid bacteria seem to be rather deficient in enzymes able to hydrolyze the phospholipid–protein complex of the fat globules in milk (Umanskii *et al*, 1974). Nevertheless, 65% of the strains of *Propionibacterium shermanii* tested by Umanskii and Borovkova (1979) presented a strong lipolytic activity. This strong nonspecific activity had already been noticed by Kaderavek *et al* (1973). Knaut and Mazurek (1974) found low lipolytic activities in 10 strains of propionibacteria. Werner (1967) found no extracellular lipase activity in 8 strains, but Cantoni *et al* (1966) demonstrated that propionic acid bacteria could produce butyric acid from tributyrin and free fatty acid from butterfat. Moreover, a glycerol ester hydrolase activity (EC 3.1.1.3.) was brought to light in *P freudenreichii* subsp *shermanii* (Oterholm *et al*, 1970a). This enzyme preferentially hydrolyzed substrates in emulsion, but also showed some esterase activity (Oterholm *et al*, 1970b).

In this study, the esterolytic activity of the 4 species of propionic acid bacteria involved in hard cheese technology was examined. In line with most published papers, we have chosen to use the term esterase for acylglycerol hydrolases preferentially hydrolyzing esters in true solution (in contrast, lipase preferentially hydrolyzes glycerol esters in emulsion).

MATERIALS AND METHODS

Strains

Five strains, described as the type strain by Cummins and Johnson (1986), of each species or subspecies of dairy *Propionibacterium* were used during this study: *P freudenreichii* subsp *freudenreichii* CIP 103026 (Collection de l'Institut Pasteur, Paris, France), *P freudenreichii* subsp *shermanii* CIP 103027, *P jensenii* CIP 103028, *P thoenii* CIP 103029 and *P acidipropionici* DSM 4900 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany).

Media and growth conditions

Stock cultures were maintained at –70°C in a yeast extract–lactate medium (YEL) (Malik *et al*, 1968) containing 15% (v/v) glycerol (Prolabo, France). All media were sterilized by heat treatment (120°C for 15 min). Two transfers at 1% (v/v) (48 h incubation at 30°C) were performed on YEL before actual use. Cells were grown in a 1-l bottle of YEL at 30°C. Bacterial growth was monitored by optical density (OD) measurements (650 nm) (Beckman spectrophotometer DU7400, USA). Calibration between OD and to-

tal proteins, and OD and population were carried out. Cells from the middle of the exponential growth phase were harvested by centrifugation (Cryofuge M7000, Heraeus, Germany) for 20 min at 7000 *g*, 4°C. They were washed once with cold sterilized distilled water and tested after disruption for esterase activity.

Protein determination

A folin–phenol reagent was used to determine the protein content of samples with bovine serum albumin (Sigma, USA) as standard (Lowry *et al*, 1951).

Preparation of cell-free extracts

Washed cell pellets were frozen at –18°C overnight, and then resuspended in sterilized distilled water at 10^{11} CFU•ml^{-1}. Cells were disrupted using a French Pressure Cell Press (SLM Aminco, USA) for 15 min at 15 600 Psi. This treatment was repeated twice. Preliminary centrifugation on a J2HS (Beckman, USA) for 15 min at 3 840 *g*, 4°C allowed the removal of unbroken cells. Another centrifugation for 20 min at 39 200 *g*, 4°C separated the intracellular fraction (supernatant) from the parietal fraction (containing both cell walls and membranes). The intracellular fraction was kept at –18°C until use. The parietal fraction was washed 3 times in distilled water and then kept in pellet form at –18 °C.

Esterase assay

A survey of esterase activity was made with the API ZYM system (LRA ZYM strips, Bio Mérieux, France). Ten substrates were tested (from 2-naphthyl-butyrate to 2-naphthyl-stearate) on intracellular and parietal fractions and on whole cell suspensions. These suspensions were obtained as follows: cells from the middle of the exponential growth phase were washed twice in sodium phosphate buffer 0.05 mol•l^{-1} pH 7.0, and the OD (650 nm) was adjusted to 1.0 before application on the strips. The strips were then incubated at 30°C and read after 4 h.

The esterase activity was quantified using β- and α-naphthyl (Na) derivatives of acetate, propionate and butyrate (Sigma, USA) as substrates according to the method of Goldberg and Rutenburg (1958) modified as follows. Enzymatic reaction occurred in microplates (Nunc Immuno Plate Maxisorp, Denmark) at 30°C for 10, 30 and 60 min: 50 µl substrate (Na derivatives 0.66 mmol•l^{-1}, dissolved in acetone (5% v/v) in sodium phosphate buffer 0.05 mol•l^{-1}, pH 7.0) were added to 50 µl of the enzyme solution. The reaction was stopped by 50 µl of 'Zym A' [TRIS–hydroxymethyl–aminoethane 25% (w/v), 12.5 N hydrochloric acid 11% (v/v) and sodium lauryl sulfate (SDS) 10% (w/v)]. Then the reaction was revealed with 50 µl of 'Zym B' (Fast Blue BB (Sigma, USA) 0.35% (w/v) in 2-methoxy-ethanol (Prolabo, France)). Absorbance was immediately determined at 540 nm on a microplate autoreader EL309 (Bio-Tek Instruments, USA). A unit (U) of enzyme specific activity was expressed as the change of 0.1 unit absorbance per min for 1 mg of bacterial proteins present in the tested fraction. For myristate, palmitate and stearate fatty acids, the substrate mixture was prepared as described by Gomori (1953): 10 µl of α and βNa derivative stock solutions (52.8 mmol•l^{-1} in acetone) were added to 790 µl 1,2-propanediol (4 N) in distilled water and used as previously described.

Esterase patterns after fractionation of intracellular fractions on polyacrylamide gel electrophoresis (PAGE) were performed on Protean II xi cell (Bio Rad, USA) using an ECPS 3000/150 generator (LKB-Pharmacia, Netherlands). The gel (12 x 17 cm) was made up without SDS using a 5% stacking gel in TRIS buffer (Merck, Germany) 0.062 mol•l^{-1}, pH 6.8 and a 10% running gel in TRIS buffer 0.33 mol•l^{-1}, pH 7.8. The running buffer was composed of TRIS 0.049 mol•l^{-1}, and glycin 0.038 mol•l^{-1}, pH 8.0. Migration was conducted at 150 V, 50 mA at 4°C.

Intracellular fractions were concentrated on Centriflo Membrane Cones CF25 (Amicon, USA), and samples containing 1 mg proteins were loaded using bromophenol blue as tracking dye. Gels were stained according to the method of Harper *et al* (1980): the gel was washed for 5 min in sodium phosphate buffer 0.1 mol•l^{-1}, pH 7.0, then incubated for 30 min at 30°C in 1 ml substrate solution (α and β Na derivative, 1% (w/v) in acetone) and 50 ml revealing solution (Fast Red TR (Serva, Germany) 10% (w/v) in sodium phosphate buffer 0.1

mol•l^{-1}, pH 7.0). Active esterase bands were dark orange and were characterized by their relative mobility (R_f) values. Acetate-βNa, propionate-αNa, butyrate-αNa, valerate-αNa, caproate-αNa, caprylate-αNa, caprate-αNa, laurate-αNa, myristate-αNa, palmitate-αNa, stearate-βNa from Sigma (USA) were used as substrates.

Purification stage

Twenty-five ml of intracellular fraction of *P freudenreichii* subsp *freudenreichii* containing 4.5 mg proteins were applied to a Mono Q HR 5/5 column (Pharmacia, Sweden) equilibrated with TRIS–HCl buffer 50 mmol•l^{-1}, pH 7.5. Elution was performed at a flow rate of 1 ml•min^{-1} with a 4-step linear gradient of NaCl: 0 to 0.3 mol•l^{-1} for 58 min, 0.3 to 1 mol•l^{-1} for 2 min, stationary phase for 10 min and 1 to 0 mol•l^{-1} for 2 min. Detection was performed at 280 nm. Esterase activity was tested on elution fractions (1 ml) as previously described, using acetate-βNa, propionate-αNa and butyrate-αNa as substrates.

RESULTS

Screening of esterase activity

The results of the screening, using API ZYM strips, are shown in table I. Activities were found in all the species of propionibacteria, in whole cells (Cell), intracellular (Intra) and parietal (CW) fractions. A decreased activity with extending chain length substrate was globally exhibited in all strains. As a general rule, activity disappeared or was very slight when chain length of fatty acids exceeded 10 carbons (caprate-βNa). A slight activity (grade of colour intensity: 1) was shown by whole cells of *P freudenreichii* subsp *shermanii* CIP 103027, *P jensenii* CIP 103028 and *P acidipropionici* DSM 4900, and by intracellular fraction of DSM 4900, when stearate-βNa (carbon number: 18) was used as substrate. Most of the highest activity values were shown by intracellular fractions, whereas the difference between parietal fractions and whole cells was less evident. For most of the substrates, parietal fraction activities of *P freudenreichii* subsp *freudenreichii* CIP 103026 were stronger than whole cells activities. *P thoenii* CIP 103029 and *P jensenii* CIP 103028, however, showed the opposite trend and *P acidipropionici* DSM 4900 and *P freudenreichii* subsp *shermanii* CIP 103027 were quite similar.

Quantitative activities of intracellular fractions

With the exception of DSM 4900, all intracellular fractions were active on acetate-βNa, propionate-αNa and butyrate-αNa (fig 1). *P jensenii* CIP 103028 had a singular pattern of activity, showing the highest specific activity value (4.6 units) on acetate-βNa. However, the esterase activity of this strain decreased as chain length extended. The opposite pattern was shown by *P thoenii* CIP 103029. Another pattern was common to *P freudenreichii* subsp *shermanii* CIP 103027, *P freudenreichii* subsp *freudenreichii* CIP 103026 and *P acidipropionici* DSM 4900: activity was at a maximum when using propionate-αNa as substrate.

Esterase electrophoresis patterns

Seven distinct active bands, with R_f values ranging from 0.85 to 0.40, were revealed on the electrophoretic patterns of the 5 strains of *Propionibacterium* (table II). Each strain possessed from 1 to 4 esterase activities. Three trends could be noticed:

– *P freudenreichii* subsp *freudenreichii* CIP 103026 and *P freudenreichii* subsp *sher-*

Table I. Screening on LRA ZYM strips of esterase activity on whole cells, intracellular and parietal fractions, of 5 species of dairy *Propionibacterium.*
Screening des activités estérasiques sur galeries API ZYM, des cellules entières, des fractions intracellulaires et pariétales de 5 espèces de bactéries propioniques laitières.

Strains	*Tested fraction*	*Substrates**									
		C4	*C5*	*C6*	*C8*	*C9*	*C10*	*C12*	*C14*	*C16*	*C18*
P freudenreichii CIP 103026											
	Cell	+++	++	+	+	+	-	-	-	-	-
	Intra	++++	+++	+++	+++	++	+	+	-	-	-
	CW	+++	+++	+++	+++	+	+	-	-	-	-
P shermanii CIP 103027											
	Cell	+++	+++	++	++	++	-	-	-	-	+
	Intra	++++	+++	+++	+++	++	+	-	-	-	-
	CW	+++	+++	++	++	-	-	-	-	-	-
P jensenii CIP 103028											
	Cell	+++	++	++	+	+	+	-	-	-	+
	Intra	++++	+++	+++	+++	++	+	-	-	-	-
	CW	+	+++	+++	+++	+++	+	-	-	-	-
P thoenii CIP 103029											
	Cell	++++	+++	+++	++	++	-	-	-	-	-
	Intra	++++	+++	+++	++	+	-	-	-	-	-
	CW	++	++	+	+	+	-	-	-	-	-
P acidipropionici DSM 4900											
	Cell	+++	++	++	+	+	+	-	-	-	+
	Intra	++++	+++	+++	+++	+++	+	-	-	-	+
	CW	+++	++	++	++	+	+	-	-	-	-

A grade (scale from O to 5) was attributed for activity: –: no activity; from + to +++++: intensity from grade 1 (low) to grade 5 (high). * β - Naphtyl derivatives: C4, butyrate-; C5, valerate-; C6, caproate-; C8, caprylate-; C9 nonanoate; C10, caprate-; C12, laurate-; C14, myristate-; C16, palmitate-; C18 stearate; Cell: whole cells; Intra: intracellular fraction; CW: cell wall fraction

*Une note de 1 à 5 était attribuée aux activités détectées selon l'intensité de la couleur: -: pas d'activité; de + à +++++: intensité faible (note 1) à forte (note 5); * dérivés β - naphtyl: C4, Butyrate-; C5, valérate-; C6, caproate-; C8, caprylate-; C9 nonanoate; C10, caprate-; C12 laurate-; C14, myristate-; C16, palmitate-; C18, stéarate; Cell : cellules entières; Intra : fractions intracellulaires; CW : fractions pariétales.*

manii CIP 103027 had similar patterns: 3 esterase bands, E_1, E_2, E_3, were common to these strains only (R_f 0.85, 0.62 and 0.47). E_1 was only active (high activity) on acetate-βNa and on propionate-αNa. E_2 was active on the 3 substrates, with a noticeable higher activity on butyrate-αNa. In contrast, E_3 was not active on acetate-βNa, showing a strong activity on propionate-αNa and, for CIP 103026 on butyrate-αNa. Moreover, another minor active band (R_f 0.40) was present in CIP 103026.

Table II. Esterase electrophoretic patterns of intracellular fractions of the different species of dairy *Propionibacterium*.
Profils électrophorétiques des activités estérasiques des fractions intracellulaires d'espèces de bactéries propioniques laitières.

Strains	*Band*	R_f	*Intensity of esterasic activity* [a]		
			Substrates		
			Acetate-βNa	*Propionate-αNa*	*Butyrate-αNa*
P freudenreichii CIP 103026					
	E_1	0.85	+++	+++	-
	E_2	0.63	++	++	+++
	E_3	0.47	-	-	++
	E_4	0.40	-	-	+
P shermanii CIP 103027					
	E_1	0.85	+++	+++	-
	E_2	0.62	++	++	+++
	E_3	0.47	-	+	-
P jensenii CIP 103028					
	E_4	0.40	++	++	++
P thoenii CIP 103029					
	E_5	0.50	-	+	+
	E_4	0.40	+++	+++	+++
P acidipropionici DSM 4900					
	E_6	0.78	++	+++	+++
	E_7	0.43	++	+++	+++

[a] Intensity was expressed by: –: no activity; from + to +++: from a thin to a deep-coloured band.
[a] *L'intensité des bandes actives est représentée par : – : pas de bandes actives; de + à +++ : bandes faiblement à fortement colorées.*

– *P jensenii* and *P thoenii* electrophoretic patterns were very similar. CIP 103028 and CIP 103029 had a common esterase activity E_4 with a broad substrate specificity. In addition, another active band (R_f 0.50) on propionate- and butyrate-αNa, was shown by the *P thoenii* strain.

– A singular esterase pattern was shown by *P acidipropionici* DSM 4900: 2 bands (R_f 0.78 and 0.43) were revealed with a broad substrate specificity.

None of the strains presented an active band for longer chain length substrates than butyrate-αNa (valerate-αNa, caproate-αNa, caprylate-αNa, caprate-αNa, laurate-αNa, myristate-αNa, palmitate-αNa, stearate-βNa).

Purification stage

Ion-exchange chromatography of the intracellular fraction of *P freudenreichii* subsp *freudenreichii* CIP 103026 led to the elution of 3 main fractions presenting esterase activities (fig 2). The first fraction, elut-

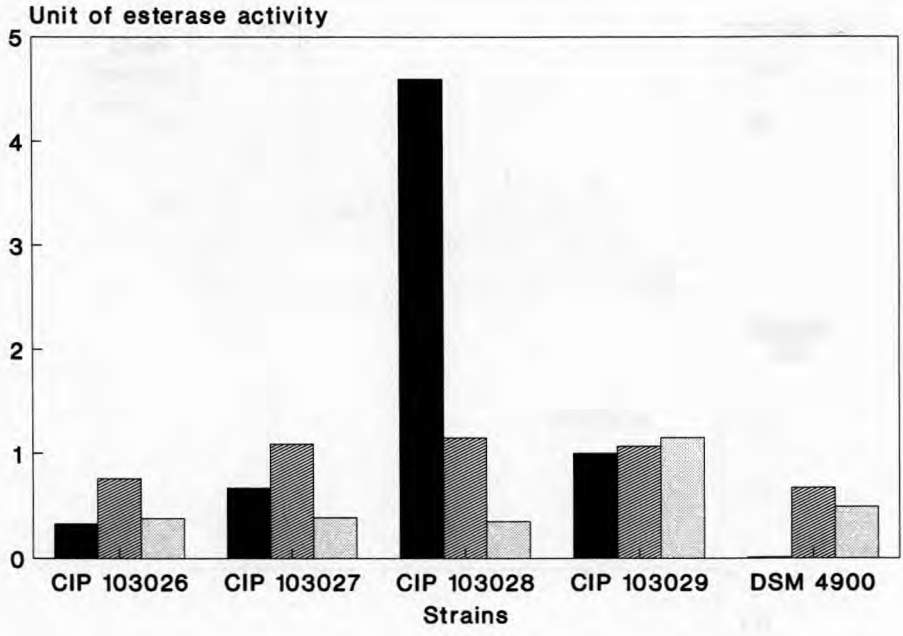

Fig 1. Esterase activity of intracellular fractions of dairy *Propionibacterium* species. A unit of specific esterase activity was defined as the change of 0.1 unit absorbance (540 nm) per min and per mg bacterial proteins. Symbols : ■ acetate-βNa; ▨ propionate-αNa; ⁘ butyrate-αNa.
Activités estérasiques des fractions intracellulaires d'espèces de bactéries propioniques laitières. Une unité d'activité spécifique était définie par la variation de 0,1 unité d'absorbance (540 nm) par min et pour 1 mg de protéines microbiennes. Symboles : ■ acetate-βNa; ▨ propionate-αNa; ⁘ butyrate-αNa.

ed at 0.19 mol•l^{-1} NaCl, was strongly active on acetate-βNa and propionate-αNa, with respective activity values of 0.074 and 0.160 U, whereas only a slight activity of 0.004 U was found on butyrate-αNa. A second fraction which was only active on butyrate-αNa, was eluted at 0.25 mol•l^{-1} NaCl. Finally, the third fraction, which was strongly active on the 3 substrates, appeared at 1 mol•l^{-1} NaCl.

DISCUSSION

In this study, using the API ZYM system Propionibacteria were revealed to be active on many substrates with various specificities depending on substrates. The use of this technique has been recommended by Arora *et al* (1990) for assessment of bacterial enzymatic potential. No evidence of the esterase system localization could be found, even if activities seemed to be stronger in intracellular fractions.

Esterase systems have already been described in many bacterial species (Morichi *et al* 1968; Harper *et al*, 1980; El Soda *et al*, 1986a,b; Kamaly *et al*, 1988; Bhowmik and Marth, 1990; Khalid *et al*, 1990; Tsakalidou and Kalantzopoulos, 1992). Only a few studies on esterase of dairy *Propionibacterium* species can be found in

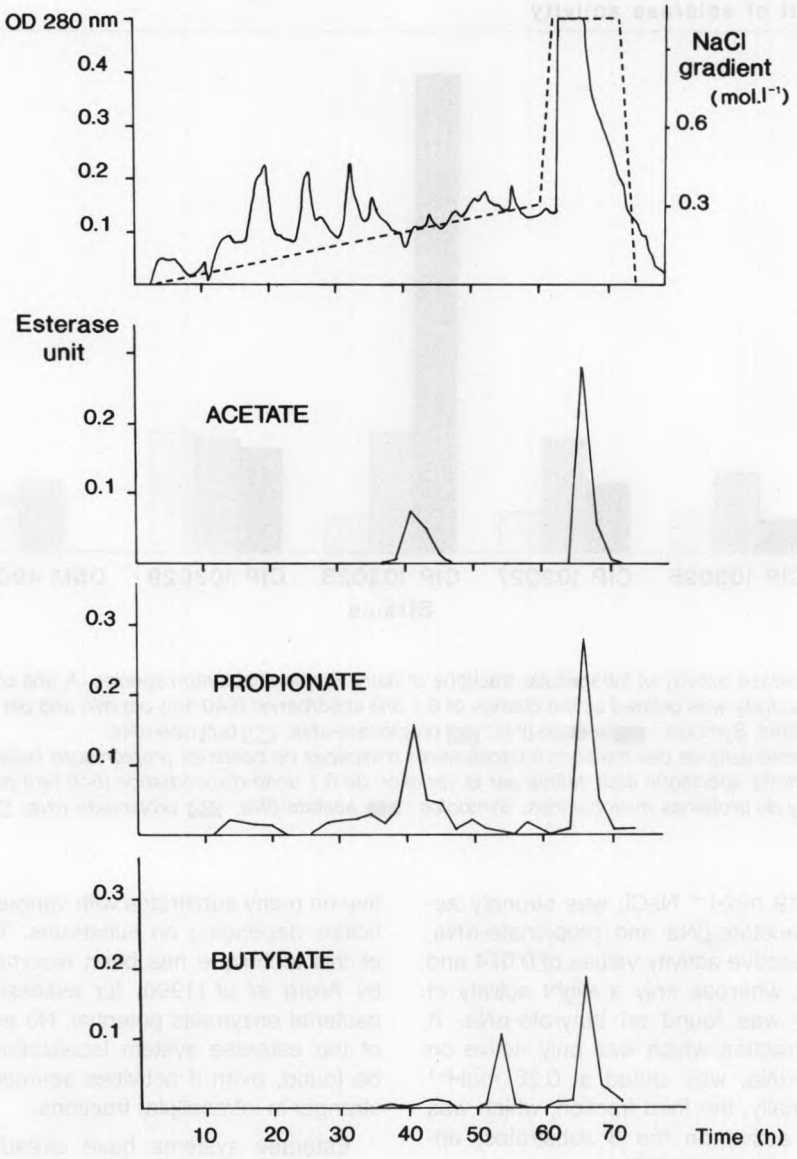

Fig 2. Ion-exchange purification stage of intracellular fraction of *P freudenreichii* subsp *freudenreichii* CIP 103026. Esterase activity was tested using acetate-βNa, propionate-αNa and butyrate-αNa. A unit of esterase activity was defined as the change in 0.1 unit absorbance (540 nm) per min. *Séparation des différentes activités estérasiques, par chromatographie d'échange d'ions, de la fraction intracellulaire de* P freudenreichii *subsp* freudenreichii *CIP 103026. Les activités estérasiques étaient testées sur acétate-βNa, propionate-αNa et butyrate-αNa. Une unité d'activité estérasique était définie comme le changement de 0.1 unité d'absorbance par min.*

the literature. In 2 short congress abstracts, El Soda *et al* (1984) and Ezzat *et al* (1992) mentioned esterase activity respectively in *P freudenreichii* and *P acidipropionici*, but unfortunately without any detail. Oterholm *et al* (1970b) suggested the existence of both an esterase and a lipase in *P freudenreichii* subsp *shermanii*, but finally concluded that these 2 activities were only due to a true lipase. Nevertheless, esterase activity has not been researched systematically.

The intracellular fraction activities of *Propionibacterium* showed various substrate specificities. This has already been described in other bacterial species (El Soda *et al*, 1986a; Bhowmik and Marth, 1990; Khalid *et al*, 1990). El Soda *et al* (1986b), working on strains of *Lactobacillus helveticus*, *L bulgaricus*, *L lactis* and *L acidophilus* found different patterns of activity depending on the chain length substrates, even for strains of the same species. *L helveticus* CNRZ 303 and CNRZ 223 had decreased activity with rising chain length, whereas CNRZ 243 showed the opposite trend; the highest activity was shown by CNRZ 244 on propionate nitrophenyl derivatives. Those 2 trends were found in the 5 strains of *Propionibacterium* of this study. Moreover, El Soda *et al* (1986a) and Bhowmik and Marth (1990) pointed out the importance of the substrate structure: lactobacilli preferentially hydrolyzed *p*-nitrophenyl derivatives rather than *o*-nitrophenyl derivatives. Thus, differences between specific activity values must be interpreted with care. Estimation of the esterase potential of a strain (before industrial use, for example) should at least be made on more than one substrate.

Activities on substrates from acetate to caprate were found when using API ZYM systems. However, these activities could not be confirmed quantitatively in intracellular fractions. Under our operating conditions, substrates such as valerate-βNa were not easily soluble, resulting in opalescent solutions which are not usable in a colorimetric method. Thus, esterase systems of *Propionibacterium* may be more extended. The adsorbed substrate in the API ZYM system is probably more efficient, but esterase activity must normally act on substrate in solution. In order to solubilize longer chain length substrates, some authors enhance the acetone concentration in the medium. But this technique very often leads to a loss of enzyme activity. Gomori (1953) has suggested the use of 1,2-propanediol to better solubilize the long fatty acid derivatives. Using this technique was successful for myristate-αNa but failed for palmitate-αNa and stearate-βNa, which gave unstable opalescent solutions. Unfortunately no activity was revealed with the myristate solution with *P freudenreichii* subsp *freudenreichii* CIP 103026 intracellular fraction. The use of microemulsions to reveal esterolytic (or lipolytic) activity on substrates with a chain length > 5 carbons seems to be a very useful method, even if this technique is still difficult to handle (Haering *et al*, 1987).

The complexity of the esterase system was demonstrated using PAGE separation, but on the 5 tested species, only 3 different patterns were shown. Strains of *P freudenreichii* had different patterns from those of *P thoenii/P jensenii* and *P acidipropionici*. The subspecies *shermanii* and *freudenreichii* could not be distinguished, neither could be strains of *P thoenii* or *P jensenii*. However, esterase electrophoretic patterns remain a useful tool to differentiate *Propionibacterium* species. This has already been pointed out in the literature for other bacterial species (Lund, 1965; Goullet, 1977). Morichi *et al* (1968) were able to distinguish different species of streptococci and other lactic acid bacteria by this mean. El Soda *et al* (1986a, b) and Kamaly *et al* (1988) also highlighted the usefulness of this technique to classify

strains of *Lactobacillus* and *Streptococcus.*

Species of *Propionibacterium* can be differentiated by electrophoresis of their total proteins (Baer, 1987), but this technique is very difficult to handle because of the complexity of the patterns obtained. The revelation of their enzyme activities after electrophoresis could be useful, but not totally discriminant in the identification of propionibacteria after the systematic study of a higher number of strains. Nevertheless, it has been shown that some esterases are active only on a few substrates. This is in accordance with Tsakalidou *et al* (1992): *Lactococcus lactis* subsp *lactis* ACA-DC 128 showed 2 esterases when using acetate-αNa, but 3 when butyrate-αNa was used. Thus, it would be more reliable to check the identity of *Propionibacterium* with acetate, propionate and butyrate naphthyl derivatives. In this study we did not try to show potential variation in activity between α and β naphthyl derivatives. This will be carried out on the purified enzymes.

No activity was found when longer chain length substrates were used. But, as emphasized Morichi *et al* (1968), this does not mean that esterases are inactive on these substrates. Longer substrates may have difficulty in reaching the enzyme in the polyacrylamide gel.

The partial purification of the intracellular fraction of *P freudenreichii* subsp *freudenreichii* CIP 103026 yielded 3 esterases which were differentiated by their elution time and their substrate specificity. These results agree with the esterase electrophoretic pattern: the first eluted esterase was strongly active on acetate-βNa and propionate-αNa, as was the electrophoretic esterase E_1. The second was only active on butyrate-αNa, as was the electrophoretic esterase E_3 and the third was active onto the 3 substrates as was esterase E_2. However, a longer elution gradient must be applied to attempt a possible separation of the last esterase which was eluted at 1 mol•l^{-1} NaCl into different fractions. Nevertheless, the current separation enabled us to independently study these 3 main esterase activities.

The proportion of free fatty acids other than propionic and acetic acids found in Swiss cheese is very similar to that of fatty acids in milk fat (Langler and Day, 1966). This suggests the existence of a lipolytic breakdown of milk fat, mainly of microbial origin, for cheeses made with pasteurized milk in which the natural lipase of the milk is destroyed. Apart from the use of esterases in identification, it seems important to further study their number, their substrate specificity and their activity level. These points appear essential for a better understanding of propionibacteria, one of the main ripening flora of this type of cheese.

ACKNOWLEDGMENTS

The authors are grateful to R Talon for helpful discussions, to JL Maubois and F Girard for their comments on the manuscript. This work was supported by grants from the Région Bretagne. One of us (CD) is greatly indebted to INRA and the Région Bretagne for being awarded a doctoral scholarship.

REFERENCES

Arora G, Lee BH, Lamoureux M (1990) Characterization of enzyme profiles of *Lactobacillus casei* species by a rapid API ZYM system. *J Dairy Sci* 73, 264-273

Baer A (1987) Identification and differentiation of propionibacteria by electrophoresis of their proteins. *Milchwissenschaft* 42, 431-433

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

Biede SL, Paulsen PV, Hammond EG, Glatz BA (1979) The flavor of Swiss cheese. *Dev Ind Microbiol* 20, 203-215

Boyaval P, Corre C (1987) Continuous fermentation of sweet whey permeate for propionic acid production in a CSTR with UF recycle. *Biotechnol Lett* 9, 801-806

Cantoni C, Molnar MR, Renon P (1966) Richerche sull' azione lipolitica dei batteri propionici. *Arch Vet Ital* 17, 335-342

Cummins CS, Johnson JL (1986) *Propionibacterium. In: Bergey's Manual of Systematic Bacteriology* (Sneath PH, Mair NS, Sharpe HE, Holt JG, eds) Williams and Wilkins, Baltimore, vol 2, 1346-1353

El Soda M, Ziada N, Ezzat N, Ismail A (1984) The proteolytic and lipolytic activities of *Propionibacterium freudenreichii. J Dairy Sci* 67 (suppl 1), 86

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

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

Ezzat N, El Soda M, El Shafei H (1992) Cell-wall associated peptide hydrolases and esterases of some cheese related bacteria. *J Dairy Sci* 75 (suppl 1), 95

Goldberg JA, Rutenburg AM (1958) The colorimetric determination of leucine aminopeptidase in urine and serum of normal subjects and patients with cancer and other diseases. *Cancer* 11, 283-291

Gomori G (1953) Human esterases. *J Lab Clin Med* 42, 445-453

Goullet P (1977) Relationships between electrophoretic patterns of esterases from *Salmonella. J Gen Microbiol* 98, 535-542

Haering G, Pessina A, Meussdoeffer F, Hochkoeppler S, Luisi PL (1987) Solubilization of bacterial cells in organic solvents *via* reverse micelles and microemulsions. *Ann NY Acad Sci* 506, 337-343

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

Hettinga DH, Reinbold GW (1972) The propionic-acid bacteria–a review. III. Miscellaneous metabolic activities. *J Milk Food Technol* 35, 436-447

Kaderavek G, Carini S, Saccinto I (1973) Azione delle lipasi microbiche sul grasso del latte. *Riv Ital Sostanze Grasse* 50, 135-136

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

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

Knaut T, Mazurek K (1974) Caractères lipolytiques des bactéries propioniques. *In: XIX Int Dairy Congr* B6, 425

Langler JE, Day EA (1966) Quantitative analysis of the major free fatty acids in Swiss cheese. *J Dairy Sci* 49, 91-93

Lawrence RC (1967) Microbial lipases and related esterases. *Dairy Sci Abstr* 29, 1-8, 59-70

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

Lund BM (1965) A comparison by the use of gel electrophoresis of soluble protein components and esterase of some group D streptococci. *J Gen Microbiol* 40, 413-419

Malik AC, Reinbold GW, Vedamuthu ER (1968) An evaluation of the taxonomy of *Propionibacterium. Can J Microbiol* 14, 1185-1191

Morichi T, Sharpe ME, Reiter B (1968) Esterases and other soluble proteins of some lactic acid bacteria. *J Gen Microbiol* 53, 405-414

Ohren JA, Tuckey SL (1965) Relation of fat hydrolysis to flavor development in Cheddar cheese. *J Dairy Sci* 48, 765

Oterholm A (1967) Glycerol ester hydrolases of some lactic and propionic acid bacteria. Ph D Thesis, Univ Illinois, Urbana, USA

Oterholm A, Ordal ZJ, Witter LD (1970a) Glycerol ester hydrolase activity of *Propionibacterium shermanii. J Dairy Sci* 53, 592-593

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

Tsakalidou E, Kalantzopoulos G (1992) Purification and partial characterization of an esterase from *Lactococcus lactis* ssp *lactis* strain ACA-DC 127. *Lait* 72, 533-543

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

Umanskii MS, Matveeva EK, Borovkova YuA, Ostroumov LA (1974) Étude comparée des méthodes de détermination de l'activité glycérol ester hydrolase des bactéries lactiques et propioniques. *In: XIX Int Dairy Congr* B5, 367-368

Umanskii MS, Borovkova YUA (1979) Lipolytic activity of lactic and propionic bacteria. *Molochn Prom* 6, 20-23,46

Werner H (1967) Untersuchungen über die Lipase und Lecithinase Aktivität von aeroben und anaeroben *Corynebacterium* und von *Propionibacterium* Arten. *Zentralbl Bakteriol Parasitenkd* 204, 127-138