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Lipase and Esterase Activities of *Propionibacterium freudenreichii* subsp. *freudenreichii*

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The lipase and esterase activities of eight strains of dairy *Propionibacterium freudenreichii* subsp. *freudenreichii* were studied. A lipase activity was detected on whole cells and in the culture supernatant. The highest activity was expressed at 45°C and pH 6.8. An esterase activity was also detected in the culture medium. The electrophoresis of the intracellular fractions of the cells revealed from three to six different esterase activities. Two esterases were common to all the strains. The substrate specificity was dependent on each esterase, but no activity was revealed, in our experimental conditions, on ester substrates with a chain length longer than that of butyrate.

Previous studies have shown that the flavor of Swiss-type cheeses depends on organic acids produced by fermentation, fatty acids produced by lipolysis and peptides, and amino acids produced by proteolysis (1, 15, 21). Problems of butyric fermentation and the requirement for constant quality have often led Swiss cheese manufacturers to use technologies such as bactofugation, natural creaming, and now microfiltration of milk to reduce bacteria levels. If the level of *Clostridium butyricum* spores is greatly reduced, the amounts of naturally occurring *Propionibacterium* spp. are also decreased, often resulting in cheese without eyes and considerably reduced value. As a result it is now essential for the professionals to use propionic bacteria with well-known metabolic activities as ripening starters. The lipolytic activity of cheese microflora is considerably less studied than the proteolytic activity, although it is well known that the presence of psychrotrophic microorganisms in milk for extended periods frequently results in production of lipases (6). These heat-resistant enzymes impair the quality of milk and consequently, the quality of cheeses. But the lipolytic activity also has a positive role in cheese ripening, especially in blue cheeses and in Italian cheeses (18). This activity releases free fatty acids in the curd which are the precursors of many aromatic compounds.

Lactic starter bacteria have a very limited activity in hydrolyzing the triglycerides of fat during the ripening of Gouda cheese (34). However, some lipases from lactic acid bacteria have been purified and characterized (3), and a few examples of esterase activity have also been studied (9, 14, 26).

The *Propionibacterium* lipolytic activity has not been explored in detail (20, 36), except for the strains of propionibacteria involved in the hydrolysis of sebum triglycerides in acne vulgaris (13, 16, 37). But Oterholm (27) showed that the lipase activity of *Propionibacterium shermanii* was 100-fold higher than that of lactic bacteria. By comparison with the low level of fatty acids in cheddar cheese, he suggested that propionic acid bacteria must play a major role in fat hydrolysis and flavor development in Swiss and related cheeses.

Moreover, it has been suggested that the esterase patterns of closely related bacteria contribute to their identification (9, 24, 26). As distinction between propionic acid bacteria species is sometimes difficult to achieve, this investigation could be a supplementary tool in naming the strains.

In this paper, the recommendations of the International Union of Biochemistry on the Nomenclature and Classification of Enzymes (17), which has given the name acylglycerol hydrolase (EC 3.1.1.3) as the systematic name for lipase, were followed. Furthermore, in accordance with most of the current published papers, the term esterase will be used for enzymes preferentially hydrolyzing esters in true solution (aryl esterase [EC 3.1.1.2]). We present here a study of the lipase and esterase activities of one of the most frequently used dairy propionibacteria for Swiss-type cheese ripening: *Propionibacterium freudenreichii* subsp. *freudenreichii*.

MATERIALS AND METHODS

Strains. *P. freudenreichii* subsp. *freudenreichii* CIP 103026 (Collection de l'Institut Pasteur, Paris, France) was chosen as the type strain (7). Seven other strains were used for characterization of the esterase activity: CIP 5932, CNRZ 81 (Centre National de Recherche Zootechnique, Jouy en Josas, France), CNRZ 89, CNRZ 435, ORSAY 6207 (generously provided by Paris Sud University, Biochemical Institute, Orsay, France), and strains P103 and P113 (kindly supplied by B. A. Glatz, Iowa State University, Ames).

Lipase activity was also measured on four other type strains (7) of the four species of dairy *Propionibacterium*: *P. freudenreichii* subsp. *shermanii* CIP 103027, *Propionibacterium jensenii* CIP 103028, *Propionibacterium thoenii* CIP 103029, and *Propionibacterium acidipropionici* DSM 4900 (Deutsche Sammlung von Mikroorganismen und Zelkulturen, Braunschweig, Germany).

Media and growth conditions. Stock cultures were maintained at −70°C in a yeast extract-lactate medium (YEL) (25) containing 15% (vol/vol) glycerol (Prolabo, Paris, France). All media were sterilized by heat treatment (120°C for 15 min). The strains were transferred twice (1% [vol/vol]) (48 h of incubation at 30°C) on YEL before actual use. Cells were grown in a 1-liter bottle of YEL at 30°C. Bacterial growth was monitored by optical density (OD) measurements (650 nm) (spectrophotometer DU7400; Beckman, Fullerton, Calif.). Calibrations between OD and total proteins and between OD and bacterial population were carried out (OD of 1 = 1.1×10^9 CFU ml^{-1} = 0.19 mg of bacterial protein).

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Cells from the middle of the exponential growth phase were harvested by centrifugation (Cryofuge M7000; Heraeus, Am Kalkberg, Germany) for 20 min at 7,000 × *g* and at 4°C. They were washed once with cold, sterilized, distilled water and tested as a cell suspension for lipase activity or after disruption for esterase activity.

To study the influence of the growth phase on lipase and esterase activities, cells were grown in a 2-liter glass bioreactor (SET 2M; Setric Genie Industriel, Toulouse, France) containing 1,500 ml of YEL inoculated at 5% (vol/vol) with a 72-h-old culture. Cell growth occurred at 30°C with continuous stirring (50 rpm) but without pH regulation.

Protein determination. The Folin phenol reagent was used to determine the protein content of samples, with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as a standard (23).

Preparation of cell extracts. Washed cells pellets were frozen at −18°C overnight and then resuspended in sterilized distilled water at 10^{11} CFU ml^{-1}. Cells were disrupted twice with a French pressure cell press (SLM Aminco, Urbana, Ill.) for 15 min at 15,600 lb/in^2. The efficiency of breakage ranged from 10 to 50% (estimations were based on spectrophotometry measurements and microscopic observations) depending on the strains considered. A first centrifugation (15 min, 3,840 × *g*, 4°C; Beckman J2HS) allowed the removal of the unbroken cells. Another centrifugation (20 min, 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.

Esterase assay. The esterase activity was quantified by using β- and α-naphthyl (NA) derivatives of acetate, propionate, and butyrate (Sigma) as substrates according to the method of Goldberg and Rutenburg (11), modified as follows. Enzymatic reaction occurred in microplates (Immuno Plate Maxisorp, Nunc, Roskilde, Denmark) at 30°C for 10, 30, and 60 min. A total of 50 μl of substrate (0.66 mM NA derivatives, dissolved in acetone [5% {vol/vol}] in 0.05 M sodium phosphate buffer, pH 7.0) were added to 50 μl of the enzyme solution. The reaction was stopped by 50 μl of Zym A (Tris, 25% [wt/vol]; 12.5 N hydrochloric acid, 11% [vol/vol]; and sodium dodecyl sulfate [SDS], 10% [wt/vol]). The reaction was then quantified with 50 μl of Zym B (fast blue BB [Sigma], 0.35% [wt/vol], in 2-methoxyethanol [Prolabo]). A_{540} was immediately determined on an EL309 microplate autoreader (Bio-Tek Instruments, Winooski, Vt.). A negative control was made as follows: intracellular fraction was inhibited with Zym A and then incubated with esterase substrates. One unit of enzyme specific activity was expressed as a change of 0.1 U in absorbance per min for 1 mg of bacterial protein present in the tested fraction compared with that in the negative control. When medium supernatant was tested during growth, specific activity was expressed as a change of 0.1 U in absorbance per min per mg of bacterial protein.

Esterase patterns of intracellular fractions were analyzed after fractionation by polyacrylamide gel electrophoresis (PAGE) on a Protean II xi cell (Bio-Rad Laboratories, Richmond, Calif.) connected to an ECPS 3000/150 generator (LKB-Pharmacia, Uppsala, Sweden). The gel (12 by 17 cm) was made without SDS by using a 5% stacking gel in 0.062 M Tris buffer (E. Merck AG, Darmstadt, Germany), pH 6.8, and a 10% running gel in 0.33 M Tris buffer, pH 7.8. The running buffer was composed of 0.049 M Tris and 0.038 M glycine (pH 8.0). Migration was conducted at 150 V and 50 mA, during 16 h at 4°C.

Intracellular fractions were concentrated on Centriflo CF25 membrane cones (Amicon, Danvers, Mass.), and samples containing 1 mg of proteins were loaded, using bromophenol blue as a tracking dye. Gels were stained by the method of Harper et al. (14). The gel was washed for 5 min in 0.1 M sodium phosphate buffer, pH 7.0. It was incubated for 30 min at 30°C in 1 ml of substrate solution (α- and βNA derivatives, 1% [wt/vol] in acetone) and 50 ml of coloring solution (10% [wt/vol] fast red TR [Serva, Heidelberg, Germany] in 0.1 M sodium phosphate buffer, pH 7.0). Active esterase bands were dark orange and were characterized by their relative mobility (R_f) values. Acetate-βNA, propionate-αNA, and butyrate-αNA from Sigma were used as substrates.

Lipase activity measurements. Lipase activity was measured on whole cells (1.1×10^9 CFU ml^{-1}) resuspended in 5 mM Tris-HCl buffer–10 mM $CaCl_2$, pH 7.0, or on culture supernatant, by the extraction-titration procedure of Castberg et al. (2) modified as follows. Glyceryl tributyrate emulsion was prepared by sonicating a 10% (wt/vol) aqueous gum arabic solution (Serva) and 10% (vol/vol) glyceryl tributyrate (Merck) in a Vibra Cell (600 W; Bioblock, Illkirch, France) at maximal amplitude for 3 min. The tube was cooled in an ice-water-ethanol bath. One milliliter of cell suspension was added to 1.5 ml of emulsion, 0.5 ml of Tris-HCl buffer (1.0 M, pH 7.0, containing 0.08 M $CaCl_2$), 0.5 ml of 1.0 M NaCl, and 2 ml of distilled water.

After incubation at 37°C for 17 h (31), 1 ml was removed and the reaction was stopped by 1 ml of 0.2 N H_2SO_4–2.0 M NaCl. Each point was repeated three times. Fatty acids were extracted by vigorous shaking for 20 s in 6 ml of hexane-diethyl ether (1:2.75 [vol/vol]). After at least 20 min, 2 ml of the upper phase were removed and titrated with 0.01 M NaOH in propanol-2 (Prolabo), by using phenolphthalein (0.1% [wt/vol]) in propanol-2) as an indicator. Titration was performed with a Dosimat 665 (Metrohm, Herisan, Switzerland) delivering 0.5 μl of titrant, under continuous N_2 bubbling.

Blanks containing buffer and substrate without cells were also tested. A standard curve with butyric acid was plotted to correct for extraction efficiency. Activity was expressed as micromoles of fatty acids liberated per hour per milligram of bacterial protein, compared with that of blanks.

Lipase activity at different pHs (4.8, 6.3, 6.8, 7.9, and 9.3) was studied, using the following buffers: 1.0 M Tris-maleate buffer for pHs ranging from 4.8 to 7.0 and 1.0 M Tris-HCl buffer for pHs ranging from 7.0 to 9.0. Lipase activity was measured at different temperatures (21, 30, 37, 45, and 55°C) at pH 7.0.

RESULTS

Lipase activity. Lipase activity in whole cells varied with *Propionibacterium* species from 0.32 to 0.49 U. Although the differences are not very marked, we subsequently used *P. freudenreichii* subsp. *freudenreichii* CIP 103026, which always exhibited the highest values, and other strains of this species.

(i) Lipase and growth phase. Lipase activity was detected on whole cells at the beginning of the growth phase (Fig. 1). Activity appeared after 3 h of growth and reached a maximum of 1.28 U after 7.5 h. Activity decreased rapidly (in less than 10 h) and stabilized at a level of 0.05 to 0.11 U when cells reached the end of the exponential growth phase. A similar activity was detected in the culture supernatant. This activity exhibited the same time course as previously de-

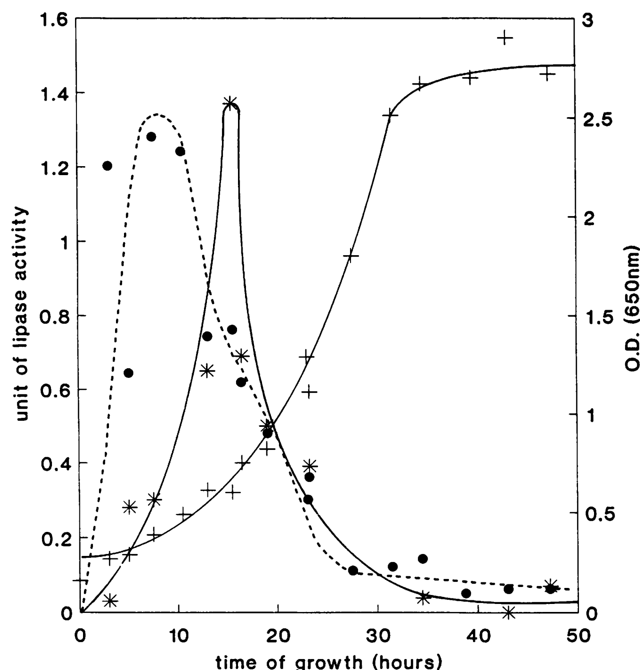

FIG. 1. Cell-associated and extracellular lipase activities during growth of *P. freudenreichii* subsp. *freudenreichii* CIP 103026. Experiments were performed twice (each point is the average of three measurements). Symbols: +, OD_{650} of the cell culture; ●, whole cell lipase activity; *, lipase activity measured on growth medium. One unit of lipase specific activity was expressed as the production of 1 μmol of butyric acid per h per mg of bacterial protein, compared with that of blanks.

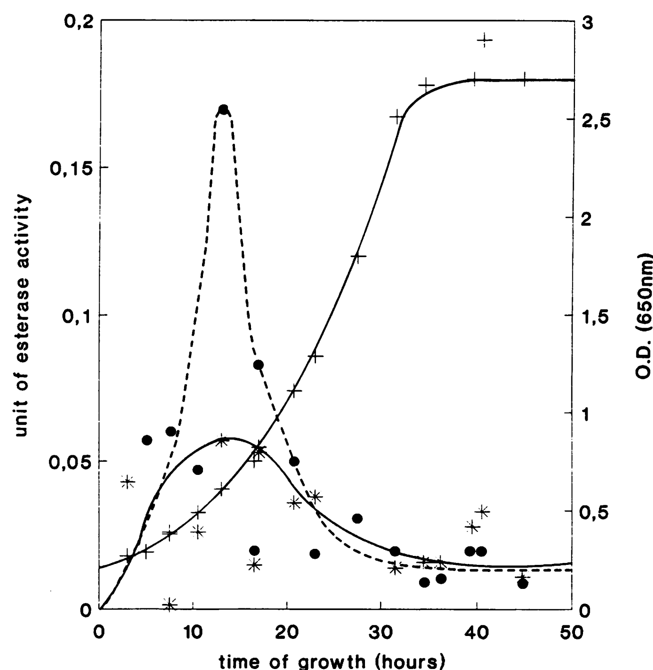

FIG. 2. Extracellular esterase activity during growth of *P. freudenreichii* subsp. *freudenreichii* CIP 103026. Experiments were performed twice. One unit of esterase activity was expressed as the change of 0.1 U of OD_{540} per min per mg of bacterial protein. Symbols: +, OD_{650} of the cell culture; ●, esterase activity measured by using propionate-αNA as a substrate; *, esterase activity measured by using butyrate-αNA as a substrate.

scribed (Fig. 1) with a slight lag in time: a peak of maximum activity (1.37 U) was reached after 15.5 h of growth and was very sharp (activity was 50% less in a range from 13 to 19 h). The same results were obtained when the pH value was adjusted to 7.0 before the cells were separated from the growth medium.

(ii) Lipase characterization. The lipase activity rose until the temperature reached 45°C and then rapidly decreased. Specific activity values were close to 0 for a temperature of 55°C. In the same way, pH was tested in a range from 4.8 to 9.3, and cells showed an optimum activity at pH 6.8.

Esterase activity. (i) Esterase and growth phase. In the culture supernatant of strain CIP 103026, an esterase activity was revealed by using propionate- and butyrate-αNA, whereas no activity was measured on acetate-βNA. The evolution of this enzyme activity is reported in Fig. 2. Activity was higher on propionate-αNA than on butyrate-αNA. Production of this activity followed the same time course as lipase production: a maximum value was reached (after 13 h of growth, 0.057 U on butyrate-αNA and 0.17 U on propionate-αNA) and then decreased rapidly. Nevertheless, the maximum was more marked for propionate-αNA than for butyrate-αNA.

(ii) Activity of crude intracellular fractions. Eight strains of *P. freudenreichii* subsp. *freudenreichii* were studied. The intracellular fractions from all strains were active on acetate-, propionate-, and butyrate-NA derivatives (Fig. 3). Butyrate-αNA was preferentially hydrolyzed by six of the eight strains, especially by *P. freudenreichii* subsp. *freudenreichii* CNRZ 85 and P103.

(iii) Esterase patterns on PAGE. Eight distinct active bands with R_f values ranging from 0.42 to 0.95 were revealed in eight strains. From three to six esterase activities were shown in each strain (Table 1).

Two esterase bands, E_1 (R_f, 0.95) and E_2 (R_f, 0.79), were common to all strains. E_1 was only active on acetate-βNA and propionate-αNA, showing a narrow specificity, and seemed to be a main esterase for all strains (strong color intensity). Even if E_2 was expressed by all the extracts, in four strains E_2 was active on the three substrates (slightly less on acetate-βNA), whereas in the strains ORSAY 6207, CNRZ 435, CIP 103026, and CNRZ 81, E_2 was less active and did not react on acetate-βNA. Another esterase band, E_3 (R_f, 0.75) was shown in seven of eight strains (except in CIP 5932). ORSAY 6207, CNRZ 435, CIP 103026, and CNRZ 81 had the same broad pattern of specificity, whereas other strains had a narrow one (only active on propionate-αNA for CNRZ 89 and P113 or on acetate-βNA for P103).

In addition, five other bands of minor intensity were revealed, active only on propionate-αNA and/or butyrate-αNA. An esterase band, E_4, at an R_f value of 0.57 was detected in ORSAY 6207, CNRZ 435, and CNRZ 81. Except for CIP 103026, the other strains showed an activity (E_5) at R_f of 0.65. Finally, two strains had single patterns: CNRZ 89 with an E_8 band at R_f 0.71 and CNRZ 81 with two other activities, E_6 and E_7, at R_f 0.42 and 0.61.

Activity of the intracellular fraction of CIP 103026 after fractionation on PAGE was also tested on longer-chain-length substrates (valerate-, caproate-, caprylate-, caprate-, laurate-, myristate-, and palmitate-αNA and stearate-βNA), but no active band was revealed under our experimental conditions.

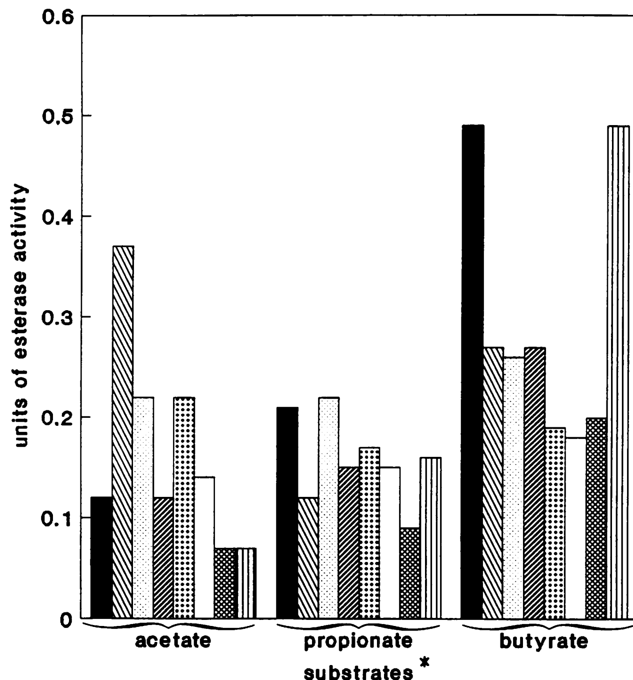

FIG. 3. Esterase activity of intracellular fractions of eight strains of *P. freudenreichii* subsp. *freudenreichii*. Each point is the average of three measurements. One unit of esterase activity was defined as the change of 0.1 U of A_{540} per min per mg of bacterial proteins. Symbols: ■, P103; ▧, P113; ▨, CNRZ 81; ▤, CNRZ 435; ▥, CNRZ 89; ■, ORSAY 6207; ▩, CIP 5932; □, CIP 103026. *, NA derivatives: acetate-βNA, propionate-αNA, and butyrate-αNA.

DISCUSSION

The lipase activity evidenced in this work (1.28 U) was significantly higher than the activities of dairy lactic acid bacteria which ranged from 0.02 to 0.12 U (28, 33).

No extracellular lipase was found in *P. shermanii* (29); in *P. jensenii*, *P. freudenreichii*, and other nondairy strains of *Propionibacterium* (20); and in unknown strains of propionibacteria (33). In this study, the existence both of a cell-associated and of an extracellular lipase was clearly shown for the first time to our knowledge. According to Lawrence et al. (22), the appearance of an extracellular lipase activity could be due to death or lysis of a fraction of cells. The existence of a real extracellular lipase is supported by the slight delay between expression of activity on whole cells and activity in the growth medium and by the fact that lipase activity did not increase when growth was stopped. Both the excretion of the intracellular lipase and the coexistence of two distinct lipases are possible. The sharp decrease in the activity could be explained by the presence of some proteinases or by modifications of the physicochemical properties of the surrounding medium. However, the existence of a pH-dependent balance between the cell-associated and the extracellular lipase has been rejected, as activities were the same even if the pH value was controlled before examination of the enzymatic activities.

According to Fox and Stepaniak (10) and Papon and Talon (31), the greatest cell-associated lipase activity was obtained during the logarithm growth phase or the beginning of the stationary phase, whereas *P. freudenreichii* subsp. *freudenreichii* CIP 103026 expressed an activity associated to whole cells from the very beginning of the exponential growth phase. This activity decreased rapidly during growth, as observed by Papon and Talon (31) on *Lactobacillus curvatus*, relating this trend to the influence of the growth medium pH.

The lipase activity of *P. freudenreichii* subsp. *freudenreichii* was the highest at 45°C in accord with the findings of Oterholm et al. (30). Knaut and Mazurek (20), however, mentioned an optimum temperature of 30°C for 10 strains of *Propionibacterium*. Lipases from other species are most active at temperatures ranging from 30 to 40°C (3–5, 10, 32). Optimum pH of 6.8 was close to neutrality, and on this point

TABLE 1. Esterase electrophoretic patterns of intracellular fractions of the different strains of *P. freudenreichii* subsp. *freudenreichii*

Strains and esterase band	R_f	Intensity of esterase activity[a] on the following substrate:		
		Acetate-βNA	Propionate-αNA	Butyrate-αNA
ORSAY 6207				
E_1	0.95	+++	+++	−
E_2	0.79	−	++	+
E_3	0.76	++	++	+
E_4	0.59	−	−	+
CNRZ 435				
E_1	0.95	+++	+++	−
E_2	0.78	−	+	+
E_3	0.74	++	++	+
E_4	0.56	−	++	+
CNRZ 81				
E_1	0.95	+++	+++	−
E_2	0.79	−	+	+
E_3	0.75	++	++	+
E_6	0.61	−	−	++
E_4	0.57	−	+	−
E_7	0.42	−	+	−
CNRZ 89				
E_1	0.95	+++	+++	−
E_2	0.79	++	+++	+++
E_3	0.74	−	+	−
E_8	0.71	−	−	+
E_5	0.65	−	+	−
P103				
E_1	0.95	+++	+++	−
E_2	0.79	++	+++	+++
E_3	0.74	+	−	−
E_5	0.64	−	+	−
P113				
E_1	0.95	+++	+++	−
E_2	0.79	++	+++	+++
E_3	0.74	−	++	−
E_5	0.65	−	++	+
CIP 103026				
E_1	0.95	++	+++	−
E_2	0.80	−	++	++
E_3	0.75	++	+	++
CIP 5932				
E_1	0.95	+++	+++	−
E_2	0.79	++	+++	+++
E_5	0.65	−	+	−

[a] −, no activity; + to +++, thin band to deeply colored band.

lipase activity seems to exhibit properties similar to those of many other microbial lipases (3–5, 20, 30, 32).

El Soda et al. (9) and Khalid et al. (19), working on intracellular extracts of *Lactobacillus* species, showed that esterase activity was present from the beginning of the exponential growth phase and that it increased and reached a maximum value during the early stationary phase. Nevertheless, lipases are also known to hydrolyze substrates in solution such as acetate-, propionate-, and butyrate-NA derivatives (22), even if their true substrate must be in emulsion. Therefore, further experiments are required to prove the existence of a true extracellular esterase: this activity may be attributed to one of the lipase activities detected (27), even if the highest activities were not shown at the same time.

As observed in this study, there are no general rules for hydrolysis of esterase substrates related to the length of the carbon chain. Khalid et al. (19), working on *Lactobacillus helveticus*, and Tsakalidou and Kalantzopoulos (35), working on *Lactococcus lactis* subsp. *lactis*, observed an increased activity with the chain length substrate, but this trend was reversed once the substrate contained more than four carbons. As described by El Soda et al. (9), strains of the same species had different esterase systems, with a variation in substrate specificity. Thus, care must be taken when only one substrate is used to compare the esterase activities of different strains.

The electrophoretic patterns revealed the complexity of the esterase system of *P. freudenreichii* subsp. *freudenreichii* as it was frequently observed for many other species (12, 14, 26). Patterns were closely related and distinct from those obtained with other propionibacteria species (8). Esterase may be inactive on longer chain length than butyrate substrates, but, as was highlighted by Morichi et al. (26), care must be taken because it is more difficult for a substrate of low solubility, such as laurate-αNA, to diffuse in the polyacrylamide gel.

P. freudenreichii subsp. *freudenreichii* has both esterase and lipase activities. Even if the role of these enzymes in the cheese-ripening process is not well defined, Oterholm et al. (28) pointed out their importance in the development of flavors in cheeses. Thus, it would be interesting to elucidate the involvement of lipolysis enzymes of *Propionibacterium* spp. in Swiss-type cheese ripening.

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