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Original article

Purification and characterization of an intracellular esterase from *Propionibacterium freudenreichii* ssp. *freudenreichii* ITG 14

Evangellia KAKARIARI, Marina D. GEORGALAKI, George KALANTZOPOULOS, Effie TSAKALIDOU*

Laboratory of Dairy Research, Department of Food Science and Technology, Agricultural University of Athens, Iera Odos 75, 118 55 Athens, Greece

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Abstract —An intracellular esterase from *Propionibacterium freudenreichii* ssp. *freudenreichii* ITG 14 was purified by anion exchange and gel filtration chromatography. The enzyme had a molecular weight of 47 400 g·mol^{-1} as determined by gel filtration chromatography, with an optimum activity on α-naphthyl-acetate at pH 6.0 and at 65 °C, with K_M = 1.2 mmol·L^{-1}. The esterase hydrolyzed synthetic substrates of low molecular weight (C2-C4), and among triglycerides only triacetin. Sulfhydryl group reagents and metal chelators had limited or no effect on enzyme activity; highest inhibition was observed with phenylmethylsulfonylfluoride (PMSF).

***Propionibacterium freudenreichii* ssp. *freudenreichii* / esterase / purification / characterization**

Résumé — Purification et caractérisation d'une estérase intracellulaire de *Propionibacterium freudenreichii* ssp. *freudenreichii* ITG 14. Une estérase intracellulaire extraite de *Propionibacterium freudenreichii* ssp. *freudenreichii* ITG 14 a été purifiée par chromatographie échangeuse d'anions et chromatographie d'exclusion de taille. L'enzyme avait une masse moléculaire de 47 400 g·mol^{-1}, comme déterminé par chromatographie d'exclusion de taille, et elle avait une activité maximale sur le substrat α-naphthyl-acétate à pH 6,0 et 65 °C, avec K_M = 1,2 mmol·L^{-1}. L'estérase dégradait des substrats synthétiques de faible poids moléculaire (C2–C4), et parmi les triglycérides seulement la triacétine. Des groupements sulfydryles et des chélateurs de métaux n'avaient aucun effet ou un effet limité sur l'activité enzymatique ; la plus forte inhibition a été observée lors de l'utilisation du PMSF.

***Propionibacterium freudenreichii* ssp. *freudenreichii* / estérase / purification / caractérisation**

* Correspondence and reprints
Tel.: (30) 1 529 4676; fax: (30) 1 529 4672; e-mail: et@aua.gr

1. INTRODUCTION

The classical or dairy propionic acid bacteria have a long history of use in the fermentation and dairy industries. They could be used for the industrial production of vitamin B12, and they could serve as alternate sources of natural food preservatives such as propionic and acetic acid. Moreover, propionic acid bacteria play an important role in the production of Swiss-type cheeses [9–11].

During cheese ripening, propionic acid bacteria convert lactate produced by the starter lactic acid bacteria to propionate, acetate and carbon dioxide. The carbon dioxide assimilates in weak pockets in the softening cheese curd and thus leads to eye formation, while propionic and acetic acid contribute strongly to the final cheese flavor. However, proteolysis and lipolysis are also considered as key mechanisms in Swiss-type cheese ripening. Previous studies have shown that fatty acids produced by lipolysis, and peptides and amino acids produced by proteolysis are responsible for the characteristic sweet and nutty flavor of these cheeses [15].

Over recent years, several attempts have been undertaken in order to elucidate the lipolytic system of propionic acid bacteria [4, 5, 19–21]. Lipolysis during cheese maturation is primarily the result of hydrolysis of milk fat catalyzed either by endogenous milk enzymes or by various microbial esterolytic and lipolytic enzymes. Esterases are arbitrarily classified as enzymes hydrolyzing substrates in solution, whereas lipases hydrolyze substrates in emulsion. However, there is no complete distinction between them. Knowledge of the properties of these enzyme activities is vital to understanding their functions both in bacterial nutrition and in cheese maturation.

The present study describes the purification and characterization of an intracellular esterase from *Propionibacterium freudenreichii* ssp. *freudenreichii* strain ITG 14.

2. MATERIALS AND METHODS

2.1. Strain and growth conditions

Propionibacterium freudenreichii ssp. *freudenreichii* ITG 14 was obtained from the ITG collection (Institut Technique Français du Fromage, France) and stored at –70 °C in sterile YEL agar [17]. It was subcultured twice in YEL broth. Final growth was performed in YEL broth at 30 °C (1% inoculum). Growth was monitored by optical density measurement at 650 nm.

2.2. Fractionation of the cell extract

Samples (10 mL) were taken at 8, 16, 24, 32, 40, 48, 60 and 72 h during growth. The growth medium was collected by centrifugation (12 500 *g*, 10 min, 4 °C) and cells were washed twice with 0.9% (w/v) NaCl, containing 20 mmol·L^{-1} $CaCl_2$. Growth medium and supernatants obtained during cell washing were combined and designated as the Growth Medium Fraction (GMF). Part of the intact cell suspension was kept apart as such, and designated as the Cell Fraction (CF). The rest was incubated in a 50 mmol·L^{-1} phosphate buffer, pH 7.0 for 2 h at 30 °C, in order to extract the cell wall associated proteins. The supernatant obtained after centrifugation (12 500 *g*, 10 min, 4 °C) was designated as the Cell Wall Extract 1 (CWE1). Subsequently, cells were resuspended in the 50 mmol·L^{-1} phosphate buffer, pH 7.0, containing 20% (w/v) saccharose and 2 mg·L^{-1} lysozyme, and incubated for 2 h at 37 °C. The supernatant obtained after centrifugation (12 500 *g*, 10 min, 4 °C) was designated as the Cell Wall Extract 2 (CWE2). Finally, cells were resuspended again in 50 mmol·L^{-1} phosphate buffer, pH 7.0 and sonicated for 30 s under cooling. The supernatant obtained after centrifugation (12 500 *g*, 10 min, 4 °C) was designated as the Intracellular Extract (IE). In all fractions obtained during growth (GMF, CF, CWE1, CWE2 and IE), enzyme

activity was measured using derivatives of α-naphthol with fatty acids from C2 to C18.

2.3. Preparation of cell-free extract

For the enzyme purification, cells were collected from the growth medium (2 L) by centrifugation (10 000 *g*, 15 min, 4 °C). The pellet obtained was washed twice with 0.9% (w/v) NaCl. Subsequently, it was resuspended in a 50 mmol·L^{-1} Tris·HCl buffer, pH 8.5, sonicated for 5 min, and then incubated in the presence of 5 mg·mL^{-1} lysozyme for 2 h at 37 °C. The supernatant obtained after centrifugation (10 000 *g*, 15 min, 4 °C) was designated as the cell-free extract.

2.4. Enzyme purification

The cell-free extract was firstly applied on a DEAE-cellulose column (2.5 × 11 cm), equilibrated with 50 mmol·L^{-1} Tris·HCl, pH 8.5. The column was washed with 2 volumes of equilibration buffer. Elution was performed at a flow rate of 1.9 mL·min^{-1} with a linear gradient of 0–0.5 mol·L^{-1} NaCl in the same buffer. The enzyme containing fractions were pooled and applied on a Mono Q HR 5/5 column equilibrated with 50 mmol·L^{-1} Tris·HCl, pH 8.5. Elution was performed at a flow rate of 1 mL·min^{-1} with a linear gradient of 0–0.5 mol·L^{-1} NaCl in the same buffer. The enzyme containing fractions were pooled and applied on the same column, this time equilibrated with 50 mmol·L^{-1} Tris·HCl, pH 7.0. Elution was performed at a flow rate of 1 mL·min^{-1} with a linear gradient of 0–0.5 mol·L^{-1} NaCl in the same buffer. The enzyme containing fractions were pooled, lyophilized, dissolved in H_2O and filtrated on a Sephacryl S-300 16/60 column equilibrated with 50 mmol·L^{-1} Tris·HCl buffer, pH 7.0, containing 0.1 mol·L^{-1} KCl. Elution was performed at a flow rate of 0.5 mL·min^{-1}. The enzyme containing fractions were pooled, stored at –30 °C and further used for the enzyme characterization. In all chromatographic steps described above, protein elution was monitored by measuring the absorbance at 280 nm.

2.5. Esterase assay

Enzyme solution (50 µL) and α-naphthyl-acetate (50 µL; 20 mmol.L^{-1} in acetone) were incubated in 350 µL of 50 mmol·L^{-1} phosphate buffer, pH 7.0, at 37 °C in the presence of Fast Red TR (50 µL; 10 mmol·L^{-1} in 50 mmol·L^{-1} phosphate buffer, pH 7.0). Activity was assayed at 560 nm by measuring the degree of substrate hydrolysis. Spontaneous, non-enzymatic substrate hydrolysis was also measured. A unit of enzyme activity was defined as the amount of the enzyme giving ΔE_{560} = 0.01 per min. Specific activity was defined as the number of units per mg protein.

2.6. Protein determination

The method of Lowry et al. [16] was used with bovine serum albumin as a standard.

2.7. Polyacrylamide gel electrophoresis

SDS-Polyacrylamide gel electrophoresis (10% acrylamide gel, in the presence of SDS and β-mercaptoethanol) was used to control the purification steps of the enzyme and to determine the molecular mass of the purified enzyme [14]. Myosin (205 000 g·mol^{-1}), β-galactosidase (116 000 g·mol^{-1}), phosphorylase *b* (97 400 g·mol^{-1}), bovine serum albumin (66 000 g·mol^{-1}), egg albumin (45 000 g·mol^{-1}) and carbonic anhydrase (29 000 g·mol^{-1}) were used as marker proteins.

The same electrophoretic system (10% acrylamide gel), but in the absence of SDS and β-mercaptoethanol, was used for the post electrophoretic detection of the enzyme, using α-naphthyl-acetate (C2), -propionate (C3), -butyrate (C4), -caproate (C6),

-caprylate (C8), -caprate (C10), -laurate (C12), -myristate (C14), -palmitate (C16) and -stearate (C18) as substrates [8].

2.8. Molecular mass determination

The molecular mass of the enzyme was estimated by gel filtration on a Sephacryl S-300 16/60 column standardized with the following proteins of known molecular mass, namely β-amylase (200 000 g·mol^{-1}), bovine serum albumin (66 000 g·mol^{-1}), carbonic anhydrase (29 000 g·mol^{-1}) and cytochrome *c* (12 400 g·mol^{-1}).

2.9. pH optimum

Enzyme activity measurements were carried out in 50 mmol·L^{-1} acetate (pH 4.0–5.0), 50 mmol·L^{-1} phosphate (pH 6.0–7.0) and 50 mmol·L^{-1} Tris·HCl (pH 8.0– 9.0) buffers, at 37 °C, using α-naphthyl-acetate as substrate.

2.10. Temperature optimum

Measurements of enzyme activity were carried out in 50 mmol·L^{-1} phosphate buffer, pH 6.0 at various temperatures from 15 to 75 °C, using α-naphthyl-acetate as substrate.

2.11. Heat stability

Enzyme solution was pre-incubated either in the presence or in the absence of substrate (α-naphthyl-acetate) for 2 min at various temperatures from 45 to 85 °C. The measurement of the remaining enzyme activity was carried out in 50 mmol·L^{-1} phosphate buffer, pH 6, at 65 °C, using α-naphthyl-acetate as substrate.

2.12. Michaelis constant

Enzyme activity measurements were carried out in 50 mmol·L^{-1} phosphate buffer, pH 6.0 at 65 °C, using various concentrations of α-naphthyl-acetate from 0.125 to 2 mmol·L^{-1}.

2.13. Substrate specificity

Enzyme activity measurements were carried out in 50 mmol·L^{-1} phosphate buffer, pH 6.0 at 65 °C with various substrates: α-naphthyl-acetate (C2), -propionate (C3), -butyrate (C4), -caproate (C6), -caprylate (C8), -caprate (C10), -laurate (C12), -myristate (C14), -palmitate (C16) and -stearate (C18). The method described under measurement of enzyme activity was used.

When triglycerides were used as substrates, Petri dishes were plated with bacteriological agar (1.5% w/v), containing 1% (v/v) substrate (triglycerides of various fatty acids from C2–C18) and 1% (w/v) arabic gum, after sonication for 1 min and sterilization for 10 min at 121 °C. Enzyme solution (40 µL) was added in wells, and after incubation at 37 °C for 48 h, the dishes were observed for halo formation around the wells.

2.14. Effect of inhibitors

Enzyme solution was incubated in 50 mmol·L^{-1} Tris·HCl buffer, pH 7.0 at 30 °C for 60 min, in the presence of diisopropylfluorophosphate (DFP), phenylmethylsulfonylfluoride (PMSF), *N*-ethylmaleimide, iodoacetamide, EDTA and 1,10-phenanthroline (final concentration 5 mmol·L^{-1}). Then, aliquots of the solution were added to the assay mixture and the remaining activity was measured.

2.15. Effect of metal ions

Enzyme solution was incubated in 50 mmol·L^{-1} Tris·HCl buffer, pH 7.0 at 30 °C for 60 min, in the presence of $MnSO_4 \times H_2O$, $MgSO_4 \times 7H_2O$, $CuSO_4 \times 5H_2O$,

$BaCl_2 \times 2H_2O$, $CaCl_2 \times 2H_2O$, $ZnSO_4 \times 7H_2O$, $MgCl_2 \times 4H_2O$, LiCl, $HgCl_2$, $FeSO_4 \times 7H_2O$, $AgNO_3$ and $(CH_3COO)_2Pb \times 3H_2O$ (final concentration 5 mmol·L^{-1}). Then, aliquots of the solution were added to the assay mixture and the remaining activity was measured.

3. RESULTS AND DISCUSSION

The *P. freudenreichii* ssp. *freudenreichii* ITG 14 esterase activity was assayed in the Growth Medium Fraction (GMF), the whole Cell Fraction (CF), the Cell Wall Extract 1 (CWE1), the Cell Wall Extract 2 (CWE2) and the Intracellular Extract (IE) obtained at certain time intervals during strain growth. No activity was detected in the GMF, CWE1 and CWE2 fractions with any of the α-naphthol derivatives used. The activity, against α-naphthyl-acetate, -propionate and -butyrate, was present only in the Cell Fraction (CF) and Intracellular Extract (IE). This is an indication that the enzyme is either cytoplasmic or cell membrane associated, meaning that substrates have first to be transferred through the cell wall before being hydrolyzed. Similarly, Oterholm et al. [19, 20] reported that *P. shermanii* produced an intracellular glycerol ester hydrolase activity, which was not excreted in the growth medium. On the contrary, Dupuis and Boyaval [4], and Dupuis et al. [5] reported extracellular or cell wall bound lipase and esterase activities for several *Propionibacterium* strains. Extracellular lipase has been also reported for *P. acidipropionici* [21], while the existence of extracellular lipases in the case of cutaneous propionic acid bacteria has been clearly confirmed [12, 18].

The production of the esterase, as assayed in the intracellular extract during growth, reached a maximum in the middle of the logarithmic phase and then declined rapidly (Fig. 1). This time course was rather different from that described for the extracellular lipase and esterase by Dupuis et al. [5], where a maximum value was reached at the beginning of the logarithmic phase and then decreased rapidly too. However, the decrease of the activity of the intracellular enzyme of the present study can not be easily attributed to the presence of proteinases or to the physicochemical modifications of the medium as suggested for the extracellular enzyme by Dupuis et al. [5]. In our case, it could be speculated that at the beginning of growth some lipid character components of the yeast extract in the growth

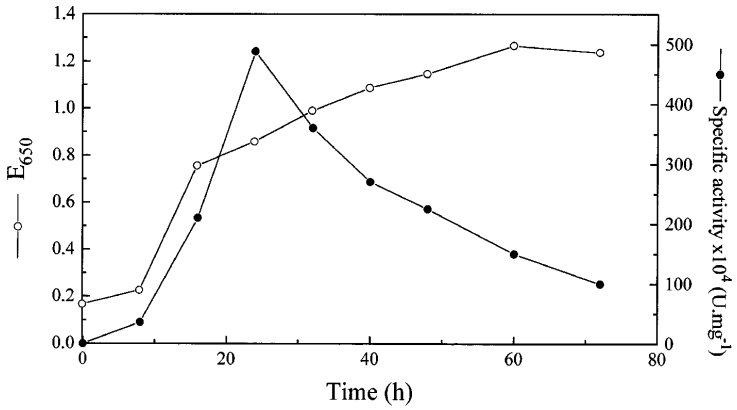

Figure 1. Esterase production during growth of *P. freudenreichii* ssp. *freudenreichii* ITG 14.
Figure 1. Production d'estérase pendant la croissance de *P. freudenreichii* ssp. *freudenreichii* ITG 14.

medium induce the synthesis of the intracellular enzyme, while enzyme synthesis diminishes as soon as these components are exhausted. To test this hypothesis, further experiments with modified YEL medium by adding various glycerides/ lipids, are necessary.

Purification of the esterase was performed in three steps of anion exchange chromatography and finally by gel filtration chromatography. The enzyme was eluted at a concentration of 0.15 mol·L^{-1} and 0.19 mol·L^{-1} in the NaCl gradient, from the Mono Q column pH 8.5 and pH 7.0 (Fig. 2), respectively. Separation on Sephacryl S-300 gave a symmetrical single peak. No activity losses were observed during lyophilization. Recovery and degree of purification are summarized in Table I. Post electrophoretic detection of the purified enzyme with α-naphthyl-acetate, -propionate and -butyrate revealed only one colored band with R_f = 0.48 (Fig. 3b). This band was also the one only obtained with the crude cell-free extract, indicating a simple enzyme system, significantly different from the interesting, complex esterase system of the *P. freudenreichii* ssp. *freudenreichii* strains described by Dupuis and Boyaval [4], and Dupuis et al. [5].

A molecular mass of 47 400 g·mol^{-1} was estimated by gel filtration on Sephacryl S-300 column. A similar result was obtained after electrophoresis in the presence of SDS and β-mercaptoethanol (Fig. 3a). The purified enzyme gave only one band with molecular mass corresponding to 52 000 g·mol^{-1}, indicating that the enzyme is a monomer protein. There are very limited data concerning the size of the lipolytic enzymes of dairy propionic acid bacteria. Oterholm et al. [20] reported a relatively large molecule of approximately 50 000 g·mol^{-1} for the intracellular *P. shermanii* lipase, as the enzyme

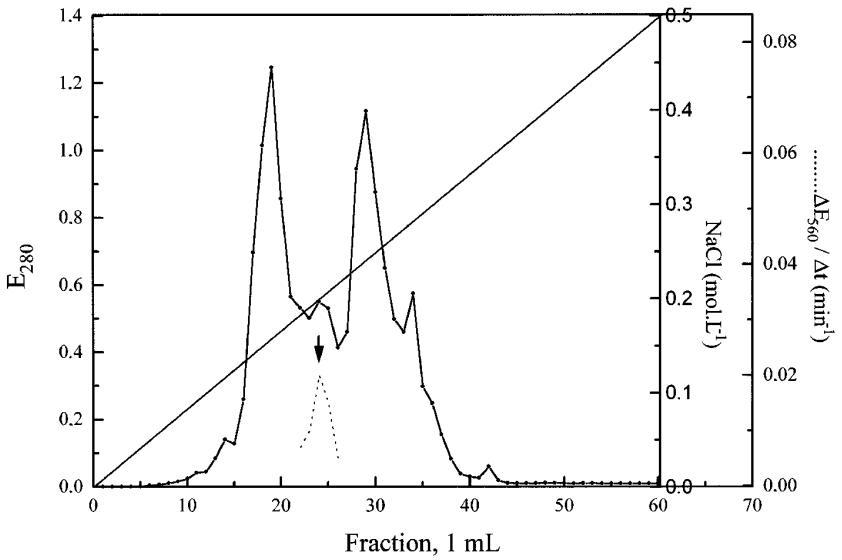

Figure 2. Elution profile of the esterase from the Mono Q HR 5/5 column with a linear gradient of 0–0.5 mol·L^{-1} NaCl in 50 mmol·L^{-1} Tris·HCl buffer, pH 7.0. Flow rate 1 mL·min^{-1}. Fraction volume 1 mL.

Figure 2. Profil d'élution d'estérase par la colonne Mono Q HR 5/5, en utilisant un gradient linéaire de 0–0,5 mol·L^{-1} NaCl dans 50 mmol·L^{-1} Tris·HCl, pH 7,0. Vitesse d'élution : 1 mL·min^{-1}. Volume des fractions : 1 mL.

was eluted with the void volume on a Sephadex G-75 column, while a small molecular mass (6 000–8 000 g·mol^{-1}) extracellular lipase has been reported for *P. acidipropionici* [21]. In the case of *P. acnes*, Ingham et al. [12] detected a major

Table I. Purification and recovery of the esterase from *P. freudenreichii* ssp. *freudenreichii* ITG 14.
Tableau I. Purification et rendement de l'estérase de *P. freudenreichii* ssp. *freudenreichii* ITG 14.

Purification step	Protein (mg)	Specific activity (U·mg^{-1})	Total activity (U)	Yield (%)	Purification fold
Crude extract	264	0.05	13.20	100	1
DEAE, pH 8.5	102	0.11	11.22	85	2.2
Mono Q, pH 8.5	17	0.21	3.57	27	4.2
Mono Q, pH 7.0	1.5	0.60	0.90	6.8	12
Sephacryl, pH 7.0	0.5	0.70	0.35	2.7	14

One unit (U) is defined as the amount of enzyme giving ΔE_{560} = 0.01 per min.
Une unité (U) est définie comme la quantité d'enzyme qui donne ΔE_{560} = 0,01 par min.

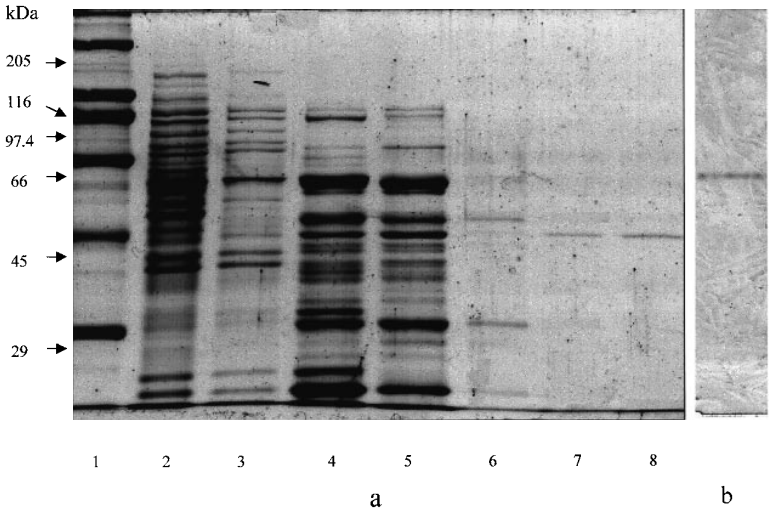

Figure 3. (**a)** SDS-Polyacrylamide gel electrophoresis (10% acrylamide; 0.025 mol·L^{-1} Tris–0.19 mol·L^{-1} glycine buffer, pH 8.3). Lanes: 1, marker proteins; 2, crude cell free extract; 3, after DEAE-cellulose; 4, after Mono Q at pH 8.5; 5, after Mono Q at pH 7.0; 6, 7 and 8, Sephacryl S-300 active fractions 37, 38 and 39, respectively. (**b)** Polyacrylamide gel electrophoresis without SDS and β-mercaptoethanol (10% acrylamide; 0.025 mol·L^{-1} Tris–0.19 mol·L^{-1} glycine buffer, pH 8.3): purified esterase reacted with α-naphtyl-acetate and Fast Red TR.

Figure 3. (a) Electrophorèse en gel de polyacrylamide et SDS (10 % acrylamide ; tampon 0,025 mol·L^{-1} Tris–0,19 mol·L^{-1} de glycine, pH 8,3). Puits : 1, standards de masse moléculaire ; 2, extrait acellulaire ; 3, après DEAE-cellulose ; 4, après MonoQ à pH 8,5 ; 5, après MonoQ à pH 7,0 ; 6, 7 et 8, Sephacryl S-300 fraction active 37, 38 et 39, respectivement. **(b)** Electrophorèse en gel de polyacrylamide sans SDS et β-mercaptoethanol (10 % acrylamide ; tampon 0,025 mol·L^{-1} Tris–0,19 mol·L^{-1} glycine, pH 8,3) : estérase purifiée, revélée par réaction avec le α-naphthyl-acétate et Fast Red TR.

extracellular lipase of 46 770 g·mol^{-1}, together with some minor protein components with lipolytic activity and of higher molecular mass, which were attributed to the formation of aggregates of the lower molecular weight under certain ionic strength conditions.

The *P. freudenreichii* ssp. *freudenreichii* ITG 14 esterase was active over a pH range of 4.0–9.0, using α-naphthyl-acetate as substrate (Fig. 4). With optimum pH of 6.0, close to neutrality, it exhibited similar behavior to other propionic acid bacteria lipolytic enzymes [5, 12, 20, 21]. Although it showed only 16% of its maximum activity at pH 4.0, it was considerably more active at alkaline pH, with 72 and 66% of its maximum activity at pH 8.0 and 9.0, respectively. Similar behavior was reported for the *P. acidipropionici* extracellular lipase [21], while fairly rapid decline was observed in the case of *P. acnes* [12] and the *P. shermanii* lipase [20].

The *Propionibacterium freudenreichii* ssp. *freudenreichii* ITG 14 lipolytic enzyme was active over a temperature range from 15 to 75 °C, with optimum activity at 65 °C (Fig. 4); it showed 11% of its maximum activity at 15 °C and 50% at 75 °C. Since the highest optimum temperature reported for lipolytic enzymes of propionic acid bacteria is around 45 °C [5, 20], it was considered necessary to study the heat stability of the enzyme.

In the absence of substrate, the esterase was stable after 2 min up to 75 °C, while 50% of the initial activity was retained after 2 min at 85 °C. Surprisingly, the enzyme was less stable in the presence of substrate; it retained full activity when heated for 2 min up to 65 °C, only 46% of the initial activity remained at 75 °C, and it was completely inactivated at 85 °C (Fig. 5). When the stability of the enzyme was tested only at 85 °C, at various time intervals, it was observed that in the absence of substrate 10% of the initial activity was retained after 3 min pre-incubation, while esterase was completely inactivated after 4 min. Ingham et al. [12] reported complete inactivation of the extracellular lipase of *P. acnes* after 30 min at 60 °C, while glycerol ester hydrolase from *Corynebacterium acnes* retained 26 and 15% of activity against *p*-nitrophenyl-acetate and tributyrin, respectively, after 15 min at 85 °C [7].

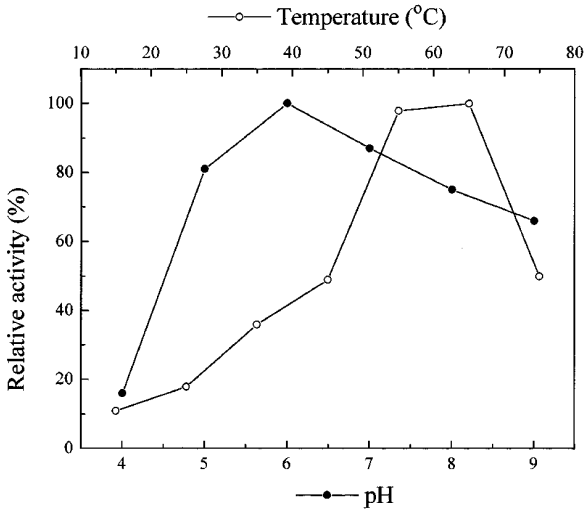

Figure 4. Effect of the pH (●) and the temperature (○) on the esterase from *P. freudenreichii* ssp. *freudenreichii* ITG 14, with α-naphthyl-acetate as substrate.

Figure 4. Effet du pH (●) et de la température (○) sur l'estérase de *P. freudenreichii* ssp. *freudenreichii* ITG 14 en utilisant le substrat α-naphthyl-acétate.

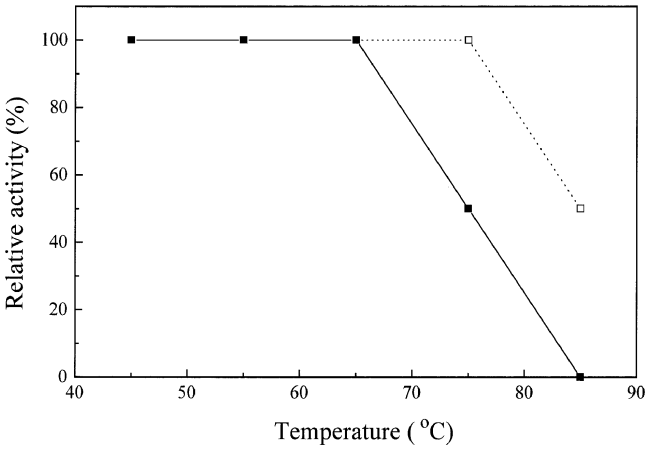

Figure 5. Thermal inactivation of the esterase of *P. freudenreichii* ssp. *freudenreichii* ITG 14, in the absence (□) and in the presence (■) of α-naphthyl-acetate as substrate.

Figure 5. Inactivation thermique de l'estérase de *P. freudenreichii* ssp. *freudenreichii* ITG 14, en absence (□) et en présence (■) du substrat α-naphthyl-acétate.

From the linear Lineweaver-Burk plot of $1/V$ vs. $1/[S]$ a relatively high K_M value of 1.2 mmol·L^{-1} and a rather low V_{max} value of 0.16 U·mg^{-1} were calculated using α-naphthyl-acetate as substrate (Fig. 6). Concerning other propionic acid bacteria, Oterholm et al. [20] determined for the *P. shermanii* lipase a K_M = 2 mmol·L^{-1} (emulsified tributyrin as substrate), while Sarada and Joseph [21] determined for the *P. acidipropionici* lipase a K_M = 4.16 mmol·L^{-1} with emulsified tributyrin as substrate, and a K_M = 0.045 mmol·L^{-1} with *p*-nitrophenyl-acetate as substrate.

By both photometric and post electrophoretic methods used in this study, it was shown that the enzyme hydrolyzed only α-naphthyl-acetate (C2), -propionate (C3) and butyrate (C4) (Tab. II). These findings were in agreement with the general findings that the esterolytic system of microbial enzymes is specific against short chain fatty acids [1, 6, 13]. Dupuis et al. [5] have detected electrophoretically esterolytic activities of *P. freudenreichii* ssp. *freudenreichii* strains only up to C4; however, when photometric determination was performed, esterase activities up to C10 could be

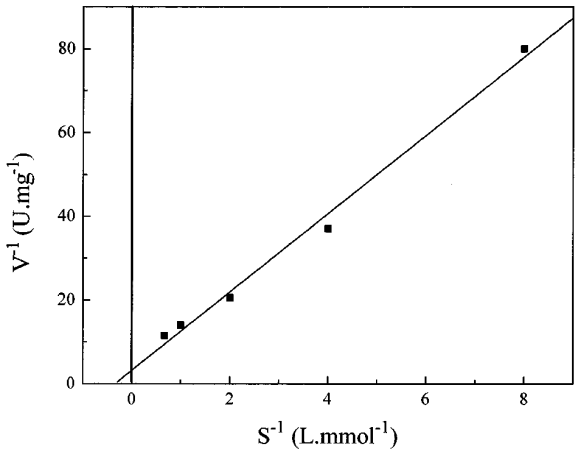

Figure 6. Lineweaver-Burk plot. Effect of α-naphthyl-acetate concentrations on the esterase of *P. freudenreichii* ssp. *freudenreichii* ITG 14, at pH 6.0 and 65 °C.

Figure 6. Tracé de Lineweaver-Burk. Effet de la concentration en α-naphthyl-acétate sur l'estérase de *P. freudenreichii* ssp. *freudenreichii* ITG 14, à pH 6,0 et à 65 °C.

Table II. Substrate specificity of the esterase from *P. freudenreichii* ssp. *freudenreichii* ITG 14.
Tableau II. Spécificité de substrat de l'estérase de *P. freudenreichii* ssp. *freudenreichii* ITG 14.

Substrate	Photometrically (relative activity, %)	Electrophoretically (R_f)
α-naphthyl-acetate (C2)	100	0.48
α-naphthyl-propionate (C3)	89	0.48
α-naphthyl-butyrate (C4)	10	0.48

detected for some of the strains [4], meaning that the determination method plays an important role in the characterization of esterolytic enzymes. Concerning triglycerides, the enzyme studied in the present work was capable of degrading only triacetin; no hydrolysis was observed when triacylglycerols with butyric up to stearic acid were used as substrates. Short chain triacylglycerols are slightly soluble in aqueous solutions and because of this are accessible as ester substrates. We have therefore designated the enzyme of the present study as esterase.

Reagents reacting with serine and sulfhydryl groups as well as metal chelators had no or low inactivation effect on the esterase activity (Tab. III). Highest inhibition was observed with PMSF; this is an indication that the serine group(s) might be essential for enzyme activity, even though DFP, another OH-group reagent, did not affect considerably the enzyme activity. No data exist about inhibition of esterases and lipases of propionic acid bacteria by serine group specific reagents. However, serine esterases have been reported for other bacteria [2, 7, 22]. Concerning the effect of metal ions, the enzyme was completely inhibited by Cu^{2+}, Hg^{2+}, Pb^{2+}, Fe^{2+} and partially by Zn^{2+}. Divalent ions are known to inhibit numerous enzymes non-specifically and thus inhibition of esterases especially by Hg^{2+} has been reported elsewhere [2, 20, 22].

Milk fat hydrolysis during cheese manufacture is due to the endogenous milk lipase, the lipolytic enzymes of starter and non-starter bacteria, lipases from psychotrophic bacteria, and depending on cheese variety, exogenous enzyme preparations. Fatty acids produced can be further converted to methylketones and thioesters, which are also implicated as cheese flavor compounds. Propionic acid bacteria are only weakly

Table III. Effect of inhibitors and metal ions on the esterase from *P. freudenreichii* ssp. *freudenreichii* ITG 14.
Tableau III. Effet des inhibiteurs et des ions métalliques sur l'estérase de *P. freudenreichii* ssp. *freudenreichii* ITG 14.

Reagent (5 mmol·L^{-1})	Remaining activity (%)	Reagent specificity
EDTA	100	Metal ions
1,10 phenanthroline	90.6	Metal ions
DFP	99.8	Serine group
PMSF	77.5	Serine group
N-ethylmaleimide	100	Sulfhydryl group
Iodoacetamide	100	Sulfhydryl group
Mn^{2+}	100	Non-specific
Mg^{2+}	100	Non-specific
Cu^{2+}	0	Non-specific
Ba^{2+}	100	Non-specific
Ca^{2+}	100	Non-specific
Zn^{2+}	50	Non-specific
Mg^{2+}	100	Non-specific
Li^{+}	100	Non-specific
Hg^{2+}	0	Non-specific
Ag^{+}	100	Non-specific
Pb^{2+}	0	Non-specific
Fe^{2+}	0	Non-specific

lipolytic, when compared with other groups of microorganisms. However, due to the low taste threshold of some fatty acids, a large number of weakly lipolytic microorganisms may play an important role in products which are stored for a long period, such as ripened cheeses [3].

Strain *P. freudenreichii* ssp. *freudenreichii* ITG 14 produces an intracellular esterase, which is active only against low molecular weight substrates in aqueous solution. However, the enzyme is active in a broad pH and temperature range, and it shows considerable stability at high temperatures. It could thus contribute to milk fat hydrolysis during cheese maturation, which is usually a long period process.

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