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Autolysis of dairy propionibacteria: isolation and renaturing gel electrophoresis of the autolysins of *Propionibacterium freudenreichii* CNRZ 725

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Summary — Dairy propionibacteria contribute to proteolysis and lipolysis of the curd in Swiss-type cheese technology by their autolysis and the concomitant release of intracellular enzymes such as esterases and peptidases. The spontaneous autolysis of *P freudenreichii* CNRZ 725 just after maximal growth in sodium lactate broth was shown to occur at pH 6.0–6.2, when the main carbon source (lactate) was depleted. This spontaneous autolysis was also observed with 6 other strains of *P freudenreichii,* and its extent as well as its rate were clearly strain-dependent. The cell walls of *P freudenreichii* CNRZ 725 were isolated by mechanical disruption and suspended in 0.05 mol/l potassium chloride at 40°C. As they contained active autolysins, rapid cell wall lysis was observed in these conditions. Concomitantly, 15% of the cell wall proteins were released in the supernatant, as well as a high amount of neutral polysaccharides. The presence of autolysins among the released proteins was demonstrated by the lytic activity of the supernatant, and by renaturing gel electrophoresis. This method, applied here for the first time to analyse the autolysins of propionibacteria, revealed the complexity of their autolytic system. In *P freudenreichii* CNRZ 725, at least 8 lytic activities of various intensities and apparent molecular masses were observed: the most intense at 121 kDa, 6 very close activities between 81 and 118 kDa and a rather weak and not completely translucent one at 34 kDa. Interestingly, all these activities were observed when the gels were incubated in Emmental juice instead of the optimal renaturation buffer (0.1 mol/l potassium phosphate buffer pH 5.8, 1% w/v of Triton X-100). Attempts made to separate and tentatively purify by chromatography (gel filtration, anion exchange) these 8 lytic activities from the supernatant of cell wall lysis were all unsuccessful. Finally, a method for extracting directly autolysins from whole cells was developed, using 1 mol/l LiCl solution. This LiCl treatment led to a crude extract of autolysins with a low content of contaminating neutral polysaccharides; however, the 34 kDa lytic activity was seemingly missing in this extract.

propionibacteria / autolysis / autolysin / renaturing SDS-PAGE / cell wall

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Résumé — Autolyse des bactéries propioniques laitières : isolement et analyse sur gel d'acrylamide des autolysines de *Propionibacterium freudenreichii* CNRZ 725. *Les bactéries propioniques laitières interviendraient dans la protéolyse et la lipolyse des fromages à pâte pressée cuite après libération de leurs enzymes intracellulaires, par autolyse bactérienne. Dans un milieu de culture YEL, la phase d'autolyse spontanée de* P freudenreichii *CNRZ 725 débutant juste après la phase de croissance maximale, a lieu lorsque le pH du milieu de culture atteint 6,0-6,2 et que la principale source carbonée (lactate) est épuisée. L'ampleur de cette autolyse spontanée, également observée au cours de la croissance de 6 autres souches de* P freudenreichii, *est un caractère souche-dépendant. Des parois de* P freudenreichii *CNRZ 725 préparées par rupture mécanique des cellules à la presse de French, sont placées dans une solution de chlorure de potassium à 0,05 mol/l, à 40°C. Dans ces conditions, les autolysines associées à ces parois et encore dotées de leur activité, provoquent une hydrolyse pariétale rapide. Lors de cette hydrolyse 15% des protéines pariétales sont libérées dans le surnageant parallèlement à d'importantes quantités de polysaccharides neutres pariétaux. Parmi ces protéines libérées, la présence d'autolysines a été mise en évidence par mesure de l'activité lytique du surnageant et par analyse sur gel de polyacrylamide (SDS-PAGE) après renaturation des enzymes en présence de Triton X-100. Cette méthode, utilisée pour la première fois pour l'analyse des autolysines des bactéries propioniques, révèle la complexité de leur système autolytique. Chez* P freudenreichii *CNRZ 725, 8 activités lytiques d'intensité et de masses moléculaires apparentes variables ont été mises en évidence : une activité très intense de 121 kDa ; 6 activités regroupées entre 81 et 118 kDa, et une activité plus diffuse et jamais totalement translucide de 34 kDa. De manière très intéressante, toutes ces activités sont observées lorsque le gel de polyacrylamide est directement incubé dans du jus d'emmental au lieu d'utiliser la solution de renaturation optimale (tampon phosphate de potassium à 0,1 mol/l, pH 5,8, à 40°C, additionné de 1% (p/v) de Triton X-100). Les tentatives de séparation et de purification par chromatographie (filtration sur gel et échangeur d'anions) de ces 8 activités lytiques isolées du surnageant d'hydrolyse pariétale sont restées vaines. Néanmoins, une méthode d'extraction des autolysines directement des cellules entières, a été développée par l'utilisation d'une solution de LiCl à 1 mol/l. Ce traitement permet l'obtention d'un extrait brut d'autolysines pauvre en polysaccharides neutres. Mais l'activité de 34 kDa est absente de cet extrait.*

bactérie propionique / autolyse / autolysine / SDS-PAGE suivie d'une renaturation / paroi

INTRODUCTION

Autolysis is a spontaneous cell breakdown phenomenon. This process is carried out by endogenous cell wall located autolysins hydrolysing covalent bonds of the peptidoglycan, which is the main protective cell wall component (Rogers *et al,* 1980; Shockman and Höltje, 1994). Several autolysins of distinct specificity can be found at the same time in the cell wall and constitute the "autolytic system" of the bacteria.

The dairy propionibacteria (mainly *Propionibacterium freudenreichii)* are essential for the ripening of Swiss-type cheeses such as Emmental (Langsrud and Reinbold, 1973a, b; Bergère and Accolas, 1986; Steffen *et al,* 1993), where they transform lactate into propionate and acetate with the concomitant production of CO_2 which is essential for the formation of the expected opening of the cheese. Moreover, they contribute to the proteolysis and lipolysis of the curd by releasing intracellular enzymes after their autolysis. Indeed, intracellular esterases and peptidases have been shown in this genus (Langsrud, 1974; El Soda *et al,* 1991, 1992; Dupuis and Boyaval, 1993; Dupuis *et al,* 1993; Dupuis, 1994). Thus, the ability of a strain to autolyse could be a new characteristic to take into consideration when selecting starters for rapid and convenient ripening of cheese.

In 1974, Langsrud was the first to observe large differences between the

induced autolyses of 6 strains of *Propionibacterium freudenreichii* (4), *P acidipropionici* (1) and *P jensenii* (1). More recently, we have compared the autolysis of 57 strains of dairy propionibacteria we have compared in 0.1 mol/l potassium phosphate buffer (pH 6.2), at 37°C (36 strains of *P freudenreichii;* 5 strains of *P thoenii;* 7 strains of *P acidipropionici* and 9 strains of *P jensenii;* the strains were from international collections and from industrial origin) (Lemée *et al,* 1994a). This study obviously confirmed the strain-dependent character of the autolysis phenomenon: 7 strains were shown to be highly prone to lysis (this represented 14% of the strains tested) and they belonged to the species *P freudenreichii,* which is the principal species encountered in Swiss-type cheeses (Baer and Ryba, 1992). One of them, *P freudenreichii* subsp *shermanii* CNRZ 725, was chosen as a model strain to further study autolysis (Lemée *et al,* 1994b). In sodium lactate broth (YEL), the maximal growth of *P freudenreichii* CNRZ 725 was immediately followed by a spontaneous autolysis phase which was quantified by viability and cell dry weight measurements at different times, as well as visualized by transmission electron microscopy. During this spontaneous autolysis, cell walls were extensively damaged. On the other hand, the autolysis was induced *in vitro* by suspending harvested whole cells in appropriate buffers or salt solutions and the optimal conditions leading to extensive lysis were determined: 0.1–1 mol/l potassium phosphate buffer, pH 5.8 at 40°C. Nevertheless, it must be stressed that, even reduced, the autolysis can be observed in a wide range of pH and temperature values (from 4 to 40°C, and from pH 4.5 to 8.5) (Lemée *et al,* 1994b). These results, confirming previous observations of Langsrud (1974) using *P freudenreichii* subsp *shermanii* P59, showed that autolysis of propionibacteria can occur at pH values and ionic strengths found in Swiss cheese.

When the cell walls of strain CNRZ 725 were isolated by mechanical disruption (French press) of exponential cells, their endogenous autolytic activity was at least partially preserved. Thus, a high level of cell wall lysis was observed when suspending them in the optimal conditions described earlier and the involvement of a major *N*-acetylglucosaminidase activity was clearly demonstrated (Lemée *et al,* 1994b).

In this work, growth and subsequent spontaneous autolysis were followed up for several strains of dairy propionibacteria, and some experiments related to the effect of the temperature of growth and the role of lactate starvation on the induction of the phenomena are proposed. Nevertheless, this presentation is mainly concerned by the release of autolysin(s) of *P freudenreichii* CNRZ 725 during the lysis of isolated cell walls, and their preliminary characterization; in particular, by using the efficient method recently described in the literature to visualize lytic enzymes after SDS-PAGE (Potvin *et al,* 1988; Leclerc and Asselin, 1989; Foster, 1991).

MATERIALS AND METHODS

Bacterial strains and growth conditions

Most of the strains were obtained from the CNRZ collection (INRA, Jouy-en-Josas) (CNRZ 725, CNRZ 277, CNRZ 727) or from our collection (INRA, Rennes) (TL 12). Some strains (referred to as letters M, N, P and T) were of commercial origin (Lemée *et al,* 1994a). The strain *P freudenreichii* subsp *shermanii* CNRZ 725 appeared to be the most appropriate for studying autolysis. It showed the highest extent of autolysis in a potassium phosphate buffer (0.1 mol/l, pH 6.2, 37°C) compared with 56 other strains of dairy propionibacteria from our collection. Strains were stored at –80°C in YEL (Malik *et al,* 1968) containing 15% (v/v) glycerol. They were grown statically in YEL broth at 30°C for 2 d from a 1% (v/v) inoculum of 2-d culture. Growth was monitored by mea-

suring the optical density at 650 nm (OD_{650}) using a spectrophotometer (Beckman DU 7400). For OD_{650} greater than 1, samples were diluted with distilled water. In order to maintain constant culture conditions (without stirring), a large volume of the culture freshly inoculated was distributed in tubes (10 ml) which were used to measure OD_{650} and viability at different times of the growth. Viability was determined on YEL agar plates after incubation for 5 d at 30°C under anaerobic conditions (Anaerocult A from Merck).

Lactic, propionic and acetic acid concentrations

Lactic, propionic and acetic acid concentrations were estimated using a high-performance liquid chromatography (HPLC) system (Beckman, USA) equipped with an UV detector (214 nm). Separation took place in a 7.5 x 300 mm (Aminex A6, Biorad) stainless steel column, operated at ambiant temperature with H_2SO_4 0.01 mol/l (1 ml/min) as an eluent (Bio-Rad, 1987).

Protein and neutral polysaccharide quantification

The protein content was estimated by the Bradford (1976) procedure (Biorad microprocedure, Hercules, CA, USA) using serum albumin (Sigma) as standard. Neutral polysaccharides were quantified using the phenol-sulfuric acid method of Dubois *et al* (1956).

Preparation of cell walls, the crude extract (CE) and the heat-treated cell walls

The cell walls were obtained by mechanical disruption (French pressure cell) as follows: All operations were carried out at 4°C. Cells were harvested at the beginning of the exponential growth phase by centrifugation at 5 500 *g* for 15 min, washed twice in distilled water and disrupted by 2 runs (15 and 5 min) at 138 MPa in a refrigerated French pressure cell (French and Milner, 1955). Undisrupted cells were removed by centrifugation at 5 500 *g* for 15 min. Cell walls were pelleted by centrifugation at 30 000 *g* for 15 min, and washed at least 3 times with distilled water (Lemée *et al,* 1994b). In order to characterize the time course of cell wall lysis in buffer, we considered the extent of lysis expressed as the percent of decrease in OD_{650} after 3 h of incubation. For the preparation of the crude extract, the cell walls were suspended in 0.05 mol/l KCl, at 40°C (2.4 mg dry weight/ml). After 90 min of incubation, the supernatant (70 ml) was separated by centrifugation (35 000 *g*, 15 min, 4°C), dialysed against distilled water and concentrated to 10 ml using an ultrafiltration cell (Amicon model 8200, diaflo ultrafiltration membranes YM 10, cutoff Mr 10 000, 40 PSI at 4°C). The retentate was further concentrated to 1.5 ml using centrifugal concentrators (Centricon 10, cutoff Mr 10 000) at 5 000 *g*, and at 4°C. This final concentrated retentate was centrifuged at 100 000 *g* for 30 min at 4°C and has been called *crude extract* (CE).

In order to be used as substrates for the detection of the lytic activity in CEs, samples of cell walls were heated at 100°C for 10 min to completely denature their endogenous autolytic activity (Lemée *et al,* 1994b).

Purification of the autolysins

Ammonium sulfate precipitation

The precipitation of the CE by ammonium sulfate was carried out according to the table of Dawson *et al* (1969). Precipitation was performed at 4°C, during 15 h. The precipitated proteins were sedimented by centrifugation (30 000 *g*, 15 min at 4°C).

Chromatography

All the purification steps were performed on a Pharmacia (Upsala, Sweden) FPLC system. Samples were applied to a Superose 12 HR 10/30 column (1 x 30 cm) previously equilibrated with 50 mmol/l potassium phosphate buffer, pH 5.8, containing 0.5 mol/l KCl. The column was eluted with 1 bed volume of the same buffer at a flow rate of 0.5 ml/min. The CE or enzyme fraction was applied to a prepacked Mono Q HR 5/5 column (1 x 5 cm), a strong anion-exchanger, previously equilibrated with 10 mmol/l potassium phosphate buffer, pH 5.8 or 8.0. Elution was at a flow rate of 1 ml/min

using a linear KCl gradient (0 to 1 mol/l over 25 min).

The column was monitored on the basis of $A_{280\ nm}$ and $A_{260\ nm}$ and 1.0 ml fractions were collected. Portions of each fraction, or pooled fractions, were concentrated through a Centricon-10 (cutoff Mr 10 000) or Centricon-3 (cutoff Mr 3 000) and tested for lytic activity.

Assay for lytic activity

The standard assay was done as follows: Autoclaved (121°C, 15 min) whole cells of CNRZ 725 where suspended in 0.1 mol/l potassium phosphate buffer, at pH 5.8 (0.21 mg cellular dry weight/ml equivalent to an OD_{650} of 0.9). One hundred µl of autolysins containing sample were added to 1.9 ml of this cell suspension.

One unit of lytic activity (UA) was defined as the quantity of enzyme which reduced the turbidity of this whole cell suspension by 0.001 OD_{650}/min during the first 15 min incubation at 40°C. The specific activity was defined as the lytic activity per mg of proteins (UA/mg proteins).

Extraction of autolysins from whole cells

Using LiCl

Exponential cells of CNRZ 725 were washed twice with distilled water and suspended in a precooled 1 mol/l LiCl solution (7 mg cellular dry weight/ml). The suspension was gently stirred for 3 h at 4°C and centrifuged at 5 500 *g* for 15 min at 4°C. The supernatant was then dialysed at 4°C against 2 l of distilled water for 72 h (using Spectrapor molecular porous membrane tubing, cutoff Mr 6 000–8 000). The enzymatic extract was then concentrated using an ultrafiltration device (Centricon 30, cutoff Mr 30 000).

Using SDS

Exponential growth phase cells were washed twice with distilled water and suspended at room temperature in 1% SDS solution (1 mg cellular dry weight/ml). The suspension was gently stirred for 16 h at room temperature and centrifuged at 5 500 *g* for 15 min at 4°C. The supernatant was then dialysed at 4°C against 2 l of distilled water for 72 h (using Spectrapor molecular porous membrane tubing, cutoff Mr 6 000–8 000). The enzymatic extract was then concentrated by evaporation using a speed vacuum apparatus (Savant Instrument), and stored at –20°C.

Renaturing SDS-PAGE, SDS-PAGE and sample preparation

The renaturing polyacrylamide gel electrophoresis (renaturing SDS-PAGE) was similar to that described by Potvin *et al* (1988), Leclerc and Asselin (1989) and Foster (1991), with some modifications which will be described elsewhere. Denaturing 0.1% (w/v) sodium dodecyl sulfate (SDS)-polyacrylamide separating gels (10% acrylamide in 370 mmol/l Tris-HCl buffer, pH 8.8) containing 0.2% (w/v) whole cells of *P freudenreichii* CNRZ 725 previously autoclaved at 121°C for 15 min in distilled water, were used to detect lytic activity.

Gels (minigels) were subjected to electrophoresis at 150 V of constant voltage for approximately 2 h, under a continuous SDS-Tris-glycine buffer system (0.1% [w/v] SDS, 25 mmol/l Tris, 192 mmol/l glycine, pH 8.3). After electrophoresis, gels were soaked for 30 min in distilled water at room temperature with gentle shaking. The gels were then transferred in 0.1 mol/l potassium phosphate buffer, pH 5.8, containing 1% (w/v) Triton X-100 (the renaturation solution, except if stated otherwise). The gels were gently shaken for 16 h at 40°C to allow renaturation. Following incubation, the gels were rinsed with distilled water, stained in 0.1% (w/v) methylene blue in 0.01% KOH (according to Jayaswal *et al*, 1990) for at least 3 h, and destained in distilled water. The lytic activities were visualized as translucide bands in the blue background.

For SDS-PAGE or renaturing SDS-PAGE, samples were prepared identically as follows: The samples were mixed volume to volume with the Laemmli buffer (62.5 mmol/l Tris-HCl pH 6.8, containing 10% [v/v] glycerol and 2% [w/v] SDS, 5% [v/v] β-mercaptoethanol, 0.025% [w/v] bromophenol blue). Samples were boiled for 2 min and loaded onto the gels. As all electrophoreses were run in denaturing conditions, the apparent Mr of the proteins as well as of the lytic bands observed could be estimated. The calibration proteins used ranged from 14 400 to 212 000 (Low Molecular Weight Calibration Kit; Pharmacia:

phosphorylase B [94 kDa], bovine serum albumin [67 kDa], ovalbumin [43 kDa], carbonic anhydrase [30 kDa], soybean trypsin inhibitor [20.1 kDa], α-lactalbumin [14.4 kDa] and High Molecular Weight Calibration Kit; Pharmacia: myosin [212 kDa], α2-macroglobulin [170 kDa], β-galactosidase [116 kDa], transferrin [76 kDa] and glutamic dehydrogenase [53 kDa]).

In renaturing SDS-PAGE, the lane containing the standard was cut and stained by Coomassie Blue R250. For the protein analysis by SDS-PAGE (Laemmli, 1970), gels were stained with Coomassie Blue R 250 and silver nitrate according to Tunon and Johansson (1984). All observations were confirmed in at least 2 independent experiments.

RESULTS

Spontaneous autolysis after maximal growth

Previous work (Lemée *et al*, 1994a) showed that the extent of autolysis observed by suspending exponential phase cells in phosphate buffer was highly strain-dependent. Two clusters were defined: cluster A containing 7 strains highly prone to lysis (with an average decrease of OD_{650} about 31 ± 10% in 2 h and 86 ± 5% in 24 h) and cluster B containing the 50 other strains. In addition, the complete growth curve of 1 of the strains belonging to cluster A (*P freudenreichii* CNRZ 725) was studied and showed a spontaneous large autolysis just after maximal growth. In order to see whether the autolysis in phosphate buffer could be correlated with a more or less extensive spontaneous autolysis in broth, the growth of 5 cluster A strains and 3 cluster B strains was followed under the same conditions (figs 1a and b, respectively). All cultures presented a decrease of OD_{650} just after the maximal growth. The absence of any stationary phase, or the presence of only a very limited one, has already been observed in propionibacteria (Langsrud, 1974; de Carvalho, 1994; Lemée, 1994), and could thus be a general feature of their growth in YEL medium. Nevertheless, the comparison of these 2 graphs revealed that the rate and final extent of the spontaneous autolytic phase were obviously more important for the strains belonging to cluster A compared to those of cluster B, while their exponential growth phases were virtually identical. It could therefore be concluded that the screening in phosphate buffer gives a valuable indication about the ability of a given strain to autolyse extensively after its maximal growth. Nevertheless, this assessment should be confirmed by analyzing a larger number of cluster B-type strains.

Using our model strain, *P freudenreichii* CNRZ 725, the influence of the temperature of incubation (15, 24 and 30°C) on its growth and on the subsequent spontaneous autolysis phase was studied (fig 2). At these 3 temperatures, the maximal OD_{650} was about 3 (corresponding to $5\ 10^9$ cfu/ml; data not shown) and spontaneous autolysis occurred more rapidly the higher the temperature was. The rates of the OD_{650} decrease (expressed in uOD/min) were $0.27\ 10^{-4}$, $2.8\ 10^{-4}$ and $3.8\ 10^{-4}$ at 15, 24 and 30°C, respectively. At 37°C, which is even closer to the optimal temperature of the autolytic system of the strain (40°C), no significant growth was observed. Interestingly, if samples of the cultures at 30°C where transferred to 43°C during the exponential phase (t = 41 h; OD_{650} = 2.2), spontaneous autolysis occurred immediately at a rate of $1.8\ 10^{-4}$ uOD/min (data not shown).

Because spontaneous autolysis in the growth medium is often related to a nutrient starvation, the contents of lactate, propionate and acetate, as well as the pH, were followed during the growth of CNRZ 725 on YEL at 30°C (fig 3). The acidification of growth medium was concomitant with the organic acid productions. The starting point of the spontaneous autolysis corresponded effectively to a consumption of 95% of the

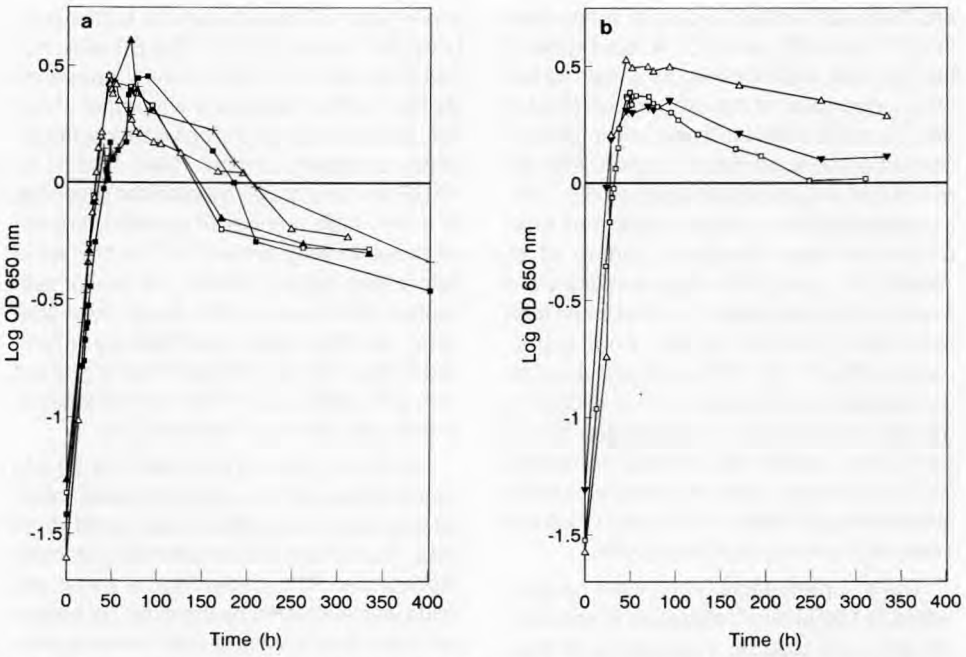


Fig 1. Comparison of the growth curves (log OD_{650}) in YEL medium at 30°C, of (**a**) dairy propionibacteria strains highly prone to autolysis in potassium phosphate buffer (cluster A, strains: CNRZ 725, ■; CNRZ 727, +; TL 12, Δ; P, ❑; T, ▲) and (**b**) strains more resistant to autolysis (cluster B, strains: CNRZ 277, ❑; M, Δ; N, ▼).

*Comparaison des courbes de croissance (Log DO_{650}) en milieu de culture YEL à 30°C (**a**) de souches de bactéries propioniques laitières présentant une forte autolyse en solution tamponnée de phosphate de potassium (groupe A, souches : CNRZ 725, ■ ; CNRZ 727, +; TL 12, Δ ; P, ❑ ; T, ▲) et (**b**) de souches plus résistantes à cette autolyse induite (groupe B, souches : CNRZ 277, ❑ ; M, Δ ; N, ▼).*

initial lactate content and occurred at pH 6.2. From the beginning of spontaneous autolysis, the metabolism of lactate as well as the production of propionic acid (maximum concentration of 5 g/l) or acetic acid (maximum concentration of 2.6 g/l) were stopped. If the depletion of lactate is the principal inducing factor of cell autolysis, providing lactate during the lytic phase should arrest the phenomena. Effectively, the addition of lactate alone (to a final concentration identical to that of YEL medium, 10 g/l)) at the end of the exponential growth phase, as well as during the spontaneous autolysis, induced immediately a new growth of strain CNRZ 725 (data not shown) and this was verified for another strain (CNRZ 277) belonging to the cluster B (data not shown).

The autolytic system of P freudenreichii *CNRZ 725*

Release of the autolysin(s) during the lysis of isolated cell walls; obtention of a CE of autolysin(s)

The cell walls of *P freudenreichii* CNRZ 725 were isolated as described in the Materials

and Methods section, and were suspended in 0.05 mol/l KCl at 40°C. A rapid lysis of the cell walls was obtained as shown by the OD_{650} decrease of the suspension (fig 4a) and paralleled the increase of the protein content of the supernatant fraction. After 90 min of cell wall lysis (at this stage 55 ± 7.7% of peptidoglycan amino acids and hexosamines were liberated; Lemée *et al*, 1994b), 57 μg eq BSA of proteins/ml were found in the supernatant, and this value then remained constant (fig 4a). Four supernatants (S0, S1, S2, S3) were recovered by centrifugation (35 000 *g*, 15 min, 4°C) at 0, 40, 90 and 150 min, respectively. It was noted that sample S0 already contained 21.7 μg of protein per ml, released just by suspending cell walls in KCl at 40°C without seemingly yet any significant lysis.

The 4 supernatants (2 ml) were concentrated to 100 μl by ultrafiltration (Centricon-30) and were added to a suspension of heat-treated cell walls (10 min at 100°C) in 0.1 mol/l potassium phosphate (pH 5.8 at 40°C, 0.6 mg wall dry weight/ml). The 4 concentrated supernatants were able to hydrolyse the heated cell walls (fig 4b), indicating that they all contained some autolysin(s). The supernatant S2 was chosen for further purifying the autolysin(s). The S3 was not retained even if its lytic activity appeared slightly higher because it was much more contaminated by peptidoglycan fragments (data not shown; Lemée, 1994). The SDS-PAGE analysis of S2 revealed the presence of a very high number of proteins, but not all those initially present in the cell walls before lysis (fig 4c). Indeed, the total protein content (estimated by the Bradford procedure) was 380 μg/mg of cell wall dry weight, and it could thus be estimated that only about 15% (57 μg/380 μg) of this content was liberated after 90 min of cell wall lysis.

For the purpose of purification, a 30-fold concentration of the volume of supernatant S2 was obtained by ultrafiltration (cutoff Mr 10 000). The activity recovered in the retentate represented 92% of the total activity; this value was estimated by the extent of heated cell walls lysis obtained after concentration (percent decrease of OD_{650} after 3 h of incubation) (data not shown). The retentate was named *crude extract* (CE) for all subsequent purification and characterization steps.

Further biochemical analysis of CE revealed a very high content of neutral

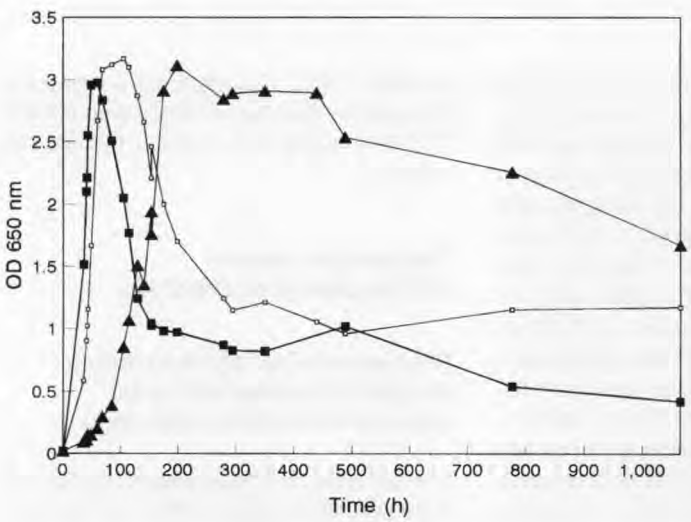


Fig 2. Growth of *P freudenreichii* CNRZ 725 at different temperatures in YEL medium. Growth was followed by OD_{650} for 1 100 h of incubation at 15°C (▲), 24°C (❑) and 30°C (■). *Croissance de* P freudenreichii *CNRZ 725 en milieu YEL à différentes températures d'incubation. La croissance a été suivie par la mesure de la DO_{650} sur une période de 1 100 h, pour 3 températures d'incubation : 15°C (▲), 24°C (❑) et 30°C (■).*

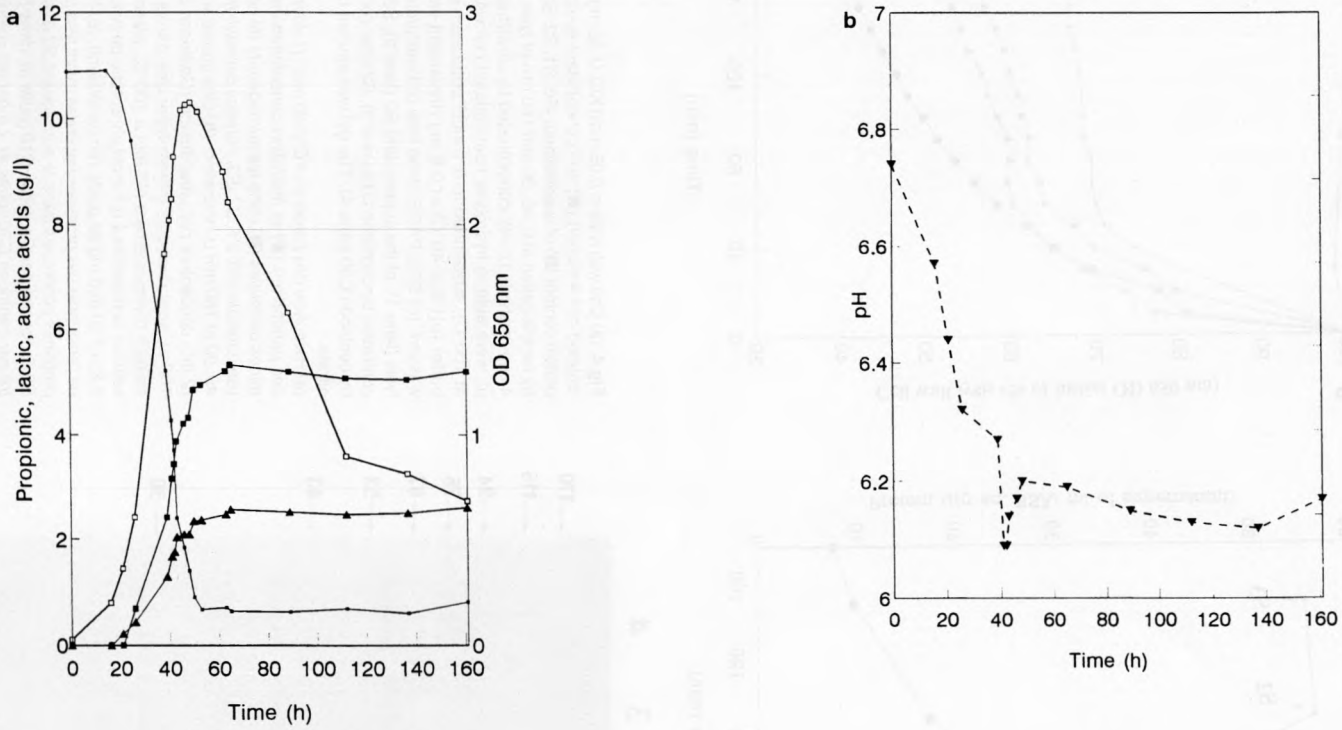
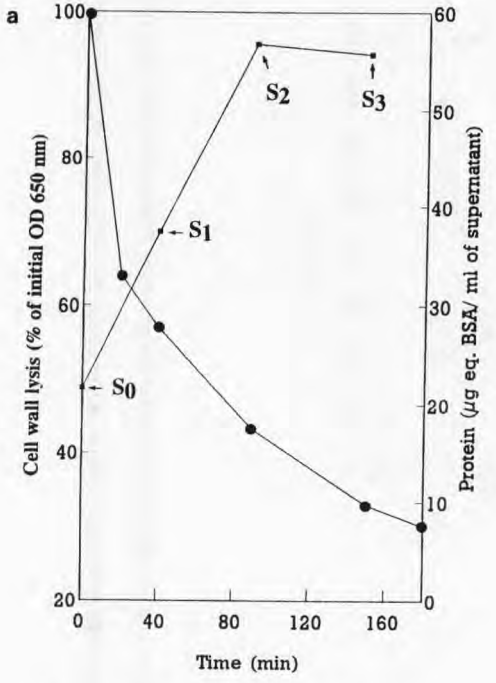
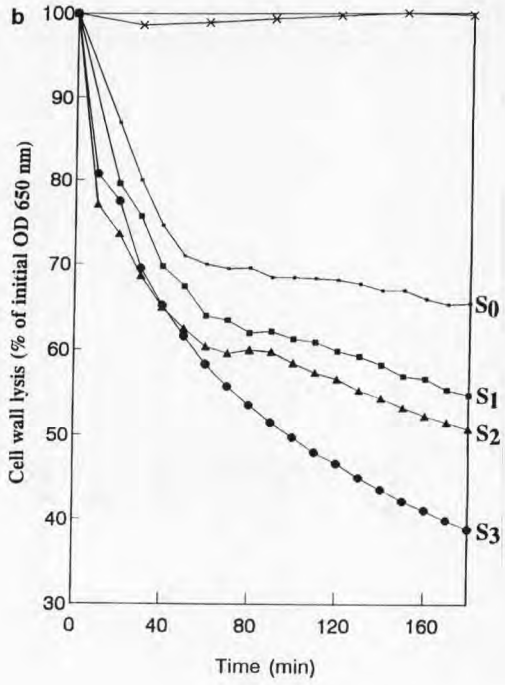


Fig 3. Lactate depletion (▪), propionic acid (■) and acetic acid (▲) productions; and pH decrease (▼) during the growth of *P freudenreichii* CNRZ 725 in YEL medium at 30°C (❑).
Consommation du lactate (▪), parallèlement à la production d'acide propionique (■) et d'acide acétique (▲), et baisse du pH du milieu (▼) par P freudenreichii *CNRZ 725 au cours de sa croissance (□) sur YEL à 30°C.*





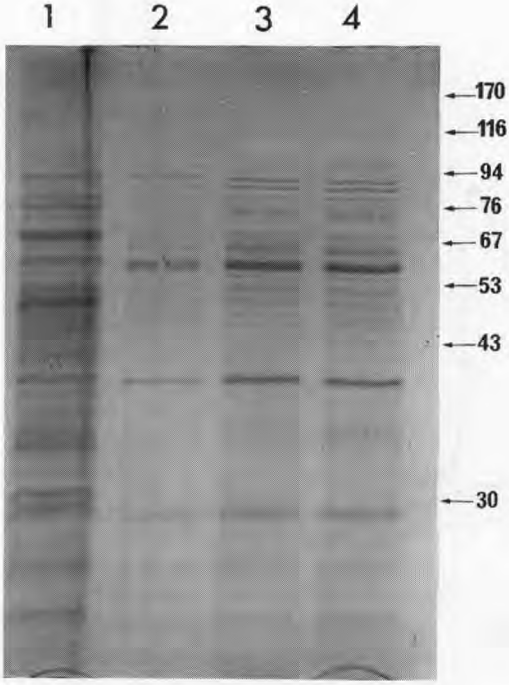


Fig 4. (**a**) Cell wall lysis in 0.05 mol/l KCl (1.46 mg dry weight isolated cell walls/ml) (●) and concomitant evolution of the protein content (■) of supernatants (S0, S1, S2, S3) obtained by centrifugation at 0, 40, 90 and 160 min of lysis. (**b**) These 4 supernatants (2 ml), concentrated by ultrafiltration to 100 µl, were able to hydrolyse heat-isolated cell walls (10 min at 100°C), suspended in 0.1 mol/l potassium phosphate buffer (pH 5.8; 40°C) at 0.6 mg dry weight isolated cell walls/ml. (**c**) SDS-PAGE of the total cell wall proteins before lysis (lane 1), of the supernatant S2 (lane 2), S2 after concentration by centricon C10 (lane 3), S2 after concentration by centricon C30 (lane 4). The gel was stained using silver nitrate.

*(a) Hydrolyse des parois en KCl 0,05 mol/l (1,46 mg de poids sec pariétal/ml) (●) et libération concommittante des protéines pariétales (■) dans les surnageants de cette hydrolyse pariétale (S0, S1, S2, S3) obtenus par centrifugation à 0, 40, 90 et 160 min d'incubation. (**b**) Ces quatre surnageants (2 ml), concentrés par ultrafiltration (Centricon-30) jusqu'à 100 µl, sont capables d'hydrolyser des parois isolées et traitées thermiquement (10 min à 100°C), placées dans la solution tamponnée à 0,1 mol/l phosphate de potassium (pH 5,8; 40°C) (0,6 mg de poids sec pariétal/ml). (**c**) SDS-PAGE de l'ensemble des proteines pariétales avant lyse (puits 1) des protéines contenues dans le surnageant S2 (puits 2), dans S2 concentré par Centricon C10 (puits 3), dans S2 concentré par centricon C30 (puits 4). Le gel est coloré au nitrate d'argent.*

polysaccharides, since the neutral polysaccharide/protein (w/w) ratio was as high as 12 (table I). This ratio dramatically increased with cell walls from late exponential phase cells, even if the extent of cell wall lysis was comparable (table I). However, the lytic activity in the CE (per mg of protein) decreased when the neutral polysaccharide content increased (data not shown). According to table I, and in order to minimize the contamination by neutral polysaccharides, the CE was always prepared by the lysis of cell walls from cells harvested at an OD_{650} below 0.5.

Preparation of heated whole cells as substrate for the quantification of the lytic activities

Ideally, the detection and quantification of lytic activities should be made on whole cells devoid of endogenous autolytic activity. Whole cells of strain CNRZ 725 were harvested in the early exponential growth phase (OD_{650} < 0.5) and submitted to several heating conditions or to a drastic formic acid treatment. Similarly, lyophilized whole cells of *Micrococcus luteus* were submitted to some of these treatments. Thereafter, they were suspended in 0.1 mol/l potassium phosphate buffer (pH 5.8) at 40°C, in order to verify, by the stability of the OD_{650} during a 3-h incubation, the inactivation of their own autolysins, which was the first condition for being a convenient substrate. The second condition was the susceptibility to the lytic activity of the CE (table II). The *M luteus* cells were not stable at all in the conditions of incubation used, except after the formic acid treatment, but the cells obtained in this case were not lysed by the CE. In contrast, 3 of the 4 heat treatments applied to whole cells of strain CNRZ 725 as well as the formic acid treatment led to stable cellular suspensions, which could be all lysed by the addition of some CE (table II). The treatment at 121°C for 15 min was retained since it could be made easily by autoclaving the cells and thus ensured, because of the sterility, a good preservation of the cells after treatment. In conclusion, autoclaved whole cells were used as substrate in the conditions described in detail in the Materials and Methods section for the quantification of the lytic activity of any crude

Table I. Obtention of cell walls from cells harvested at different growth phases: extent of cell wall lysis and neutral polysaccharide to protein ratio (w/w) (NP/P) of the crude extract (CE) obtained in each case.
Préparation de parois à partir de cellules récoltées à différents stades de culture ; hydrolyse des parois isolées et rapport entre les polysaccharides neutres et les protéines (p/p) (NP/P) contenus dans l'extrait brut (CE), obtenu dans chaque cas.

Whole cells	*Isolated cell walls*	*CE*	
OD_{650}nm of harvesting	*Extent of lysis * (%)*	*Protein content (µg eq BSA/ml)*	*NP/P ratio (w/w)*
0.3	71	488	12
0.53	76	780	11
1	66	1 295	41

* Percent decrease of OD_{650} after 3 h of incubation.
* *Pourcentage de diminution de la DO_{650} après 3 h d'incubation.*

Table II. Assays of several substrates for quantifying by spectrophotometry the lytic activity of the crude extract (CE)*.
Recherche d'un substrat pour la quantification de l'activité lytique de l'extrait brut (CE) par spectrophotométrie.

Substrate	*Treatment*	*Loss of OD_{650} nm after 3 h incubation (%) without CE*	*Sensibility to CE* [a]
Whole cells of CNRZ 725	15 min at 100°C	10	+
	30 min at 100°C	3	+
	60 min at 100°C	2.5	+
	15 min at 121°C	1–3	+
	formic acid (1 N, 30 min, 4°C)	1	+
Whole cells of *Micrococcus luteus*	without treatment	40	ND
	30 min at 100°C	45–50	ND
	formic acid (1 N, 30 min, 4°C)	4	–

* The substrates were suspended in 0.1 mol/l potassium phosphate buffer (pH 5.8, 40°C).
[a] +, loss of OD_{650} nm > 35% after a 3 h incubation at 40°C; – : no significant loss of OD_{650}; ND: not determined.
** Les substrats étaient placés dans une solution de 0,1 mol/l phosphate de potassium (pH 5,8; 40°C).*
[a] +, perte de DO_{650} nm > 35% après une incubation de 3 h à 40°C; – : pas de perte significative de DO_{650}; ND : non déterminé.

or partially purified extract. The main parameter used was the initial rate of the OD_{650} decrease (over a 15 min period) induced by a 100 µl sample of extract. One UA was defined as the quantity of enzymatic activity leading to a decrease of 0.001 uOD/min. As shown, for example, in table III, 100 µl of CE contained 11.7 UA.

Nevertheless, it was not possible by this way to know whether the activity was due to one or several autolysins. For this reason, we tried to apply to dairy propionibacteria a method described by Potvin *et al* (1988) and Leclerc and Asselin (1989) for visualizing lytic activities in the polyacrylamide gel after denaturing electrophoresis and this is discussed in the next section.

Qualitative analysis of the autolysins by renaturing electrophoresis

In the method described by Potvin *et al* (1988), whole cells or cell walls of *M luteus*, or of the species considered, are included in the polyacrylamide network, and lytic activities are observed as clear bands in the turbid gel after incubation in a 1% Triton X-100 containing buffer. In the present work, autoclaved whole cells of strain CNRZ 725 were included in the acrylamide network. A CE sample was mixed with an equivalent volume of Laemmli buffer, heated for 2 min at 100°C and 20 µl aliquote was loaded on the gel. After electrophoresis, the gel was soaked in distilled water for 30 min and then incubated under stirring in 0.1 mol/l phosphate buffer, pH 5.8, at 40°C (optimal conditions previously defined for this autolytic system) and containing 1% (w/v) Triton X-100. After 3 h, at least 8 lytic activities were observed (fig 5, lane 1). The most intense had an apparent molecular mass of 121 kDa; 6 other activities were visualized between 80 and 118 kDa (81, 87, 92, 100, 109 and 118 kDa); the last activity at an apparent molecular mass of 34 kDa was

Table III. Protein and neutral polysaccharide contents, and lytic activity of the crude extract (CE) of autolysins and of the different fractions obtained after an ammonium sulfate precipitation.
Teneur en protéines et polysaccharides neutres, et activité lytique de l'extrait brut d'autolysines (CE), et des différentes fractions obtenues après une précipitation au sulfate d'ammonium.

Fractions	*Proteins (mg eq BSA/ml)*	*Neutral polysaccharides (mg eq glucose/ml)*	*NP/P ratio* (w/w)*	*UA (0–15 min) (100 µl)*	*Specific activity*
CE	1.92	21	11	11.7	61
P50	1.60	2.4	1.5	12.1	76
S50	0.80	20.6	26	11.8	148
P70	0.41	1.5	3.7	10.5	256
S70	0.21	13.5	64	1.3	62

* NP/P ratio: neutral polysaccharide/protein ratio. NB: the isolated cell walls of CNRZ 725 used for the obtention of CE, contained 380 µg eq BSA of proteins/mg walls dry weight and 560 µg eq glucose of neutral polysaccharides/mg walls dry weight; then, the NP/P ratio was about 1.5.
* *NP/P ratio : rapport polysaccharides neutres/protéines. NB: les parois isolées de CNRZ 725 utilisées pour l'obtention de CE, contiennent 380 µg eq SAB de protéines/mg de poids sec pariétal et 560 µg eq glucose de polysaccharides neutres/mg de poids sec de parois ; ainsi, le rapport NP/P est de 1,5 pour les parois isolées.*

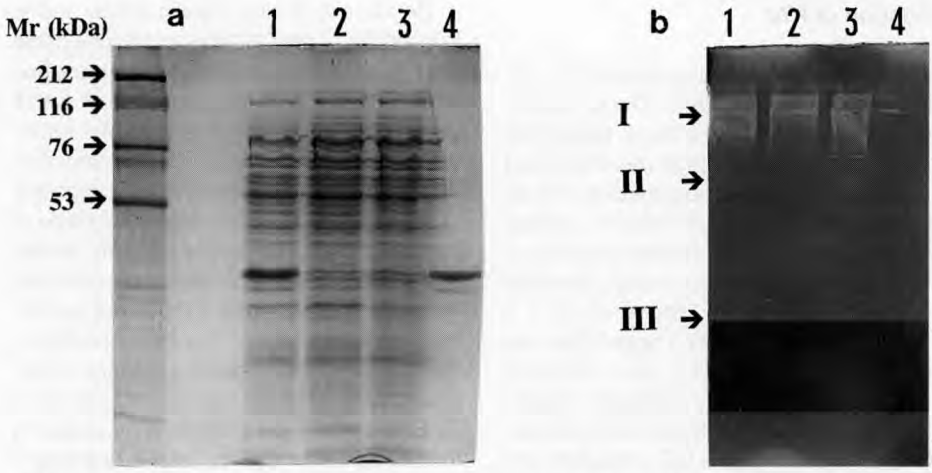


Fig 5. (**a**) Protein profiles and (**b**) profiles after renaturing SDS-PAGE of the following extracts: lane 1: CE; lane 2: pellet sulfate ammonium precipitation of CE at 70% (P70); lane 3: supernatant of CE sulfate ammonium precipitation at 70% (S70); lane 4: pellet sulfate ammonium precipitation of CE at 50% (P50).(Renaturation buffer: 0.1 mol/l potassium phosphate, pH 5.8 with 1 % (w/v) Triton X-100; 40°C). (Lytic activities: I: 120 kDa; II: 81 to 118 kDa; III: 34 kDa).
*Profils protéiques (**a**) et profils d'activité (**b**) après renaturation des autolysines en tampon de 0,1 mol/l de phosphate de potassium, pH 5,8, additionné de 1% de Triton X-100, à 40°C. Les extraits enzymatiques sont les suivants : puits 1 : extrait brut (CE) ; puits 2 : culot après précipitation du CE à 70% de sulfate d'ammonium (P70) ; puits 3 : surnageant après précipitation du CE à 70% de sulfate d'ammonium (S70) ; puits 4 : culot après précipitation du CE à 50% de sulfate d'ammonium (P50) ; (activités lytiques : I: 120 kDa ; II: 81-118 kDa ; III: 34 kDa).*

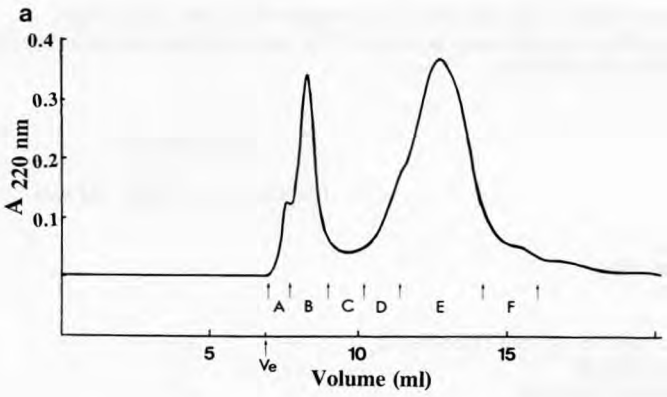
weak, and not completely translucent. A quantity as low as 4 µg protein (corresponding to 1 UA) in the CE sample loaded was sufficient to observe all these lytic activities, indicating that the method is sensitive.

It could be noted that other substrates have been tentatively included in the gel as *i)* whole cells of autoclaved *M luteus*, but no lytic bands at all were obtained; *ii)* heat-treated cell walls of CNRZ 725, and the profile obtained was exactly the same as with autoclaved whole cells (data not shown). In order to determine which lytic activity detected on the gel corresponded to the *N*-acetylglucosaminidase activity mainly involved in the autolysis of strain CNRZ 725 (Lemée *et al,* 1994b), the purification was undertaken.

Ammonium sulfate precipitation of autolysin(s) from the CE: obtention of P70

The CE was precipitated by ammonium sulfate successively at 50 and 70% of saturation (w/v) leading to 4 fractions: pellet P50 and supernatant S50, which was then used to obtain pellet P70 and supernatant S70. As summarized in table III, all fractions, except S70, contained significant amounts of lytic activity (about 10 UA). Surprisingly, the total activity of P50 and S50 together (12.1 + 11.8 = 23.9 UA) was 2-fold higher than the initial activity of CE (11.7 UA). A similar observation has been made by Foster (1993) in the course of the ammonium sulfate precipitation from a CE containing the endolysin of a bacteriophage infecting *B subtilis*. On the other hand, the sum of the S70 (1.3 UA) and P70 (10.5 UA) activities was coherent with the S50 (11.8 UA) activity. It was also noted that the neutral polysaccharides initially present in the CE remained mainly soluble in the final supernatant S70, and the ammonium sulfate precipitation was thus an easy way for eliminating them, at least partially. P70 was the sample showing the highest specific activity, with a 4-fold increase compared to the initial CE (256/61). Fractions CE, P50, P70 and S70 were analyzed by SDS-PAGE (fig 5a) and by renaturing gel electrophoresis (fig 5b). The protein profiles of CE, P50 and P70 were very similar, indicating that almost all the proteins precipitated by the addition of ammonium sulfate at the concentrations used. The only exception was seemingly a predominant protein of about 30 kDa, which clearly remained in S70 (fig 5a). In terms of lytic activities, the 8 lytic bands found in CE were also observed in the P50 and the P70 profiles (fig 5b). In the S70 sample, no lytic bands could be observed except a residual weak band at 121 kDa, and this observation was coherent with its low total activity (1.3 UA). The low protein content of S70 (0.21 mg/ml) and its protein profile (fig 5a, lane 4) were also in agreement.

Because of its high specific activity and its low content of neutral polysaccharides (table III), P70 was chosen for the following steps of purification (next paragraph). It was also used to assay several media for the renaturation of the 8 lytic activities detected after denaturing gel electrophoresis, especially in order to find potential inhibitors of some of these activities. The results are summarized in table IV. The first observation was that the presence of Triton X-100 was necessary for renaturation. The best conditions were to incubate the gel in *i)* 0.1 mol/l phosphate buffer pH 5.8 or *ii)* KCl 100 mmol/l or *iii)* Emmental juice (obtained by pressing the cheese after the end of the ripening in warm room), all containing 1% Triton X-100 (w/v). This was not surprising with media *i)* and *ii)* since they were previously described as inducing efficiently the autolytic system of the strain (Lemée *et al,* 1994b). The fact that the renaturation and the lytic activities of all the bands occurred in Emmental juice was an exciting observation, of practical interest. Finally, the divalent cations Mg, Cu and Zn were able to inhibit the renaturation



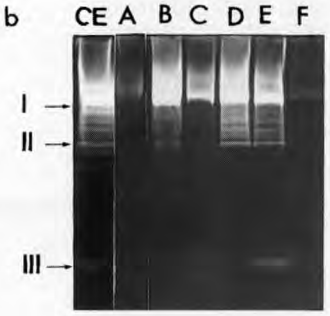


Fig 6. Elution profile (**a**) of P70 extract on gel permeation chromatography (Superose 12) (50 mmol/l potassium phosphate buffer, pH 5.8 containing 50 mmol/l KCl) and (**b**) renaturing SDS-PAGE profiles of the fractions obtained: lanes 1: CE; 2: A; 3: B; 4: C; 5: D; 6: E; 7: F (Renaturation buffer: 0.1 mol/l potassium phosphate buffer pH 5.8 - Triton X-100); (Lytic activities: I: 120 kDa; II: 81 to 118 kDa; III: 34 kDa).
*Profil d'élution de l'extrait P70 (**a**) en chromatographie de gel filtration (Superose 12), en tampon de phosphate de potassium (50 mmol/l, pH 5,8) à 50 mmol/l de KCl et (**b**) profils d'activité des différentes fractions obtenues : 1) CE ; 2) A ; 3) B ; 4) C ; 5) D ; 6) E ; 7) F. (Renaturation de l'activité dans 0,1 mol/l phosphate de potassium à pH 5,8 - Triton X-100) ; (activités lytiques : I: 120 kDa ; II: 81-118 kDa ; III: 34 kDa).*

and/or the enzymatic activity of all or some of the lytic activities of P70, depending on the concentrations used.

Gel permeation and anion exchange chromatography of P70

P70 was loaded on a gel permeation column. Figure 6 shows the elution profile obtained as well as the analysis by renaturing gel electrophoresis of the 6 fractions collected (A,B,C,D,E,F). The fractions were concentrated to an identical volume of 3 ml by using Centricon-3 (cutoff 3 000) before analysis. The 8 lytic activities were not really separated by this gel permeation chromatography. Several conditions of elution were tested unsuccessfully for improving the separation: molarity of the phosphate buffer (10 to 50 mmol/l), pH (5.8, 6.0, 6.5), addition of 0.15 to 2 mol/l KCl in the phosphate buffer and reduction of the elution rate from 0.5 to 0.3 ml/min. Nevertheless, fractions C and E were interesting because: *i)* the fraction C contained seemingly only the 121 kDa activity; *ii)* the fraction E contained all the lytic activities, and moreover the lytic activity at 34 kDa was more intense than in P70.

Fraction C (40 µg eq BSA) was added to 1.5 mg of heated cell walls (10 min, 100°C), and induced a decrease of about 21% of the OD_{650} of the cell wall suspension after 6 h in 0.05 mol/l KCl at 40°C. The concomitant apparition of glucosaminitol was

Table IV. Renaturation in various media of the lytic activities of fraction P70, after SDS-PAGE. *Renaturation des activités lytiques contenues dans la fraction C70, dans diverses solutions, après une électrophorèse en conditions dénaturantes.*

Solution of renaturation	*Lytic activities*		
	121 kDa	*81–118 kDa*	*34 kDa*
Distilled water without Triton X-100	–	–	–
Distilled water	–	–	–
Potassium phosphate 0.1 mol/l pH 5.8, without Triton X-100	+/–	+/–	–
Potassium phosphate 0.1 mol/l pH 5.8	++	++	+
Potassium phophate 25 mmol/l pH 5.8 (KPB)	+	+++	+
KPB + $MgSO_4$ 10 mmol/l	++	++	+
KPB + $MgSO_4$ 100 mmol/l	++	++	+
KPB + $MgCl_2$ 10 mmol/l	++	++	+/–
KPB + $MgCl_2$ 100 mmol/l	++	++	–
KPB + $CuSO_4$ 10 mmol/l	+/–	–	–
KPB + $CuSO_4$ 100 mmol/l	–	–	–
KPB + $CuCl_2$ 10 mmol/l	+	–	–
KPB + $CuCl_2$ 100 mmol/l	–	–	–
KPB + $ZnCl_2$ 10 mmol/l	+	+/–	–
KPB + $ZnCl_2$ 100 mmol/l	+	–	–
Potassium phoshate 25 mmol/l pH 8.0	++	++	–
Tris-HCl 0.1 mol/l pH 8.0	++	+	–
KCl 100 mmol/l	+++	+++	++
Emmental juice (pH 5.7)*	+++	+++	++

All the solutions contained 1% (w/v) of Triton X-100 (except if indicated otherwise in the table). The substrate included in the gel was autoclaved whole cells of *P freudenreichii* CNRZ 725. * The Emmental juice obtained from a 32-d ripened Emmental according to the Morris *et al* (1988) method, contained 30 g/l lactate, 0.2 mol/l Ca^{2+}, 0.3 mol/l Na^+, 0.2 mol/l Cl^-, 0.06 mol/l K^+, 0.03 mol/l Mg^{2+} (Salvat-Brunaud *et al*, 1995).

Tous les milieux d'incubation contiennent 1% (p/v) de Triton X-100 (sauf indication contraire dans le tableau). Substrat inclus dans le gel : cellules entières de P freudenreichii *CNRZ 725 autoclavées. * le jus d'emmental obtenu à partir d'un emmental affiné de 32 jours selon la méthode de Morris* et al *(1988), renferme entre autres 30 g/l de lactate, 0,2 mol/l Ca^{2+}, 0,3 mol/l Na^+, 0,2 mol/l Cl^-, 0,06 mol/l K^+, 0,03 mol/l Mg^{2+} (Salvat-Brunaud* et al, *1995).*

detected (data not shown), indicating that this 121 kDa activity might be the N-acetyl-glucosaminidase mainly involved in the autolysis of strain CNRZ 725 (Lemée *et al,* 1994b). Furthermore, fraction E (270 µg eq BSA) was loaded on an anion exchange column (Mono Q) and again the lytic activities were not separated, and were mainly excluded all together (fraction 1) (fig 7).

Extraction of autolysins from whole cells

The preliminary attempts of purification described earlier revealed the complexity of separating the lytic activities present in the CE. In addition, these assays suffered from the fact that the CE was a starting material tedious to obtain, thus limiting the number of chromatography steps which

could be realized. For this reason, we have tried to find a direct way to extract autolysins from whole cells of strain CNRZ 725. In the literature, autolysins have been extracted directly from whole cells by anionic detergent (Sugai *et al,* 1990; Foster, 1992; Valence and Lortal, 1995), 5 mol/l NaCl (Brown, 1972) or LiCl (0.5 to 5 mol/l) (Pooley *et al,* 1970; Valence and Lortal, 1995).

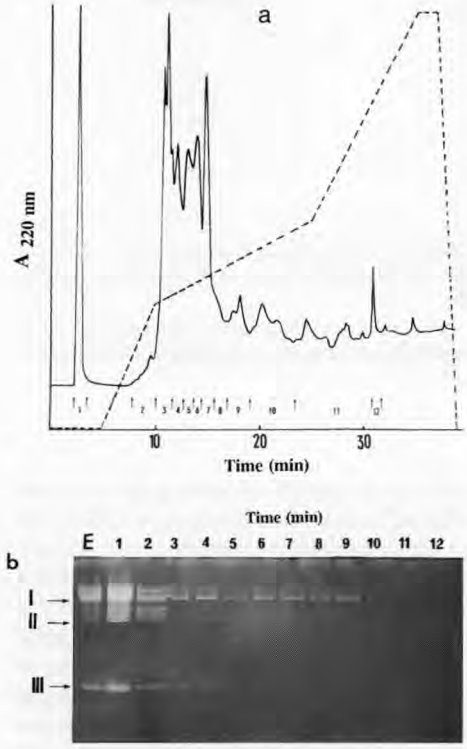


Fig 7. Elution profile of the fraction E, obtained in figure 6 by gel permeation, on (**a**) anion exchanger chromatography (Mono Q) and (**b**) renaturing SDS-PAGE profiles of the fractions obtained: lane 1: E fraction; lanes 2 to 13: the 12 pooled fractions obtained by chromatography, called 1 to 12. (Renaturation buffer: 0.1 mol/l potassium phosphate buffer pH 5.8 - Triton X-100); (Lytic activities: I: 120 kDa; II: 81 to 118 kDa; III: 34 kDa).

*Profil d'élution de l'extrait E (obtenu par gel filtration, fig 6) en chromatographie échangeuse d'anions (**a**) et profils d'activité des différentes fractions obtenues (b) : puits 1 : fraction E ; puits 2 à 13 : 12 fractions obtenues par chromatographie, numérotées de 1 à 12. (Renaturation de l'activité dans 0,1 mol/l phosphate de potassium à pH 5,8 - Triton X-100); (activités lytiques : I: 120 kDa ; II: 81 - 118 kDa ; III: 34 kDa).*

As the autolysins of strain CNRZ 725 can be observed (8 lytic activities) after denaturing gel electrophoresis, their resistance toward SDS can be presumed. Washed exponential-phase cells of this strain (5 mg dry weight) were suspended either in 1% SDS or in 1 mol/l LiCl (as described in detail in the Materials and Methods section). After extraction and centrifugation, the soluble lytic activity was qualitatively analyzed by renaturing SDS-PAGE and quantified by the spectrophotometric assay on autoclaved whole cells of strain CNRZ 725. The results (table V) indicated that the 1 mol/l LiCl treatment was an efficient way to extract autolysin from whole cells. Moreover, the contamination of the LiCl extract by neutral polysaccharides was very low (8-fold lower than in CE).

DISCUSSION

The autolytic activity of the dairy propionibacteria is dependent on the strain and even seemingly on the species (the strains most prone to autolysis were all of the species *P freudenreichii)* (Langsrud, 1974; Lemée *et al,* 1994a). In order to see if the screening previously developed to detect "autolytic strains" by suspending them in phosphate buffer (Lemée *et al,* 1994a) could be correlated with their spontaneous behavior in culture medium, the growth of 7 strains was followed in this work in lactate broth at 30°C during 1 100 h. The strains most prone to autolysis in buffer were again those showing the highest rate and extent of spontaneous autolysis in the growth medium. Even if the growth of a greater number of strains should be followed up before any definitive conclusion, the present work supported the idea

Table V. Extraction of autolysins from whole cells by 1% SDS and 1 mol/l LiCl: lytic activity, protein and neutral polysaccharide contents of the autolysin extracts.
Extraction des autolysines à partir de cellules entières par du SDS 1% et du LiCl 1 mol/l : activité lytique, teneur en protéines et polysaccharides neutres de ces extraits d'autolysines.

	SDS extract	*LiCl extract*
Extent of lysis (%)	14.5	30
UA (500 µl)	2.2	6.3
Protein (µg eq BSA/ml)	ND[a]	341
Specific activity		37
NP/P ratio [b] (w/w)		1.4

The extent of lysis was expressed as the percent decrease of OD_{650} after 3 h of incubation. [a] ND: not determined, because of the interference of the SDS with the Bradford procedure; [b] NP/P ratio: neutral polysaccharide/protein ratio (w/w).
Le taux de lyse est exprimé comme étant le pourcentage de diminution de la DO_{650} après 3 h d'incubation. [a] ND, non déterminé, du fait de la présence de SDS qui est incompatible avec la méthode de Bradford ; [b] NP/P ratio : rapport polysaccharides neutres/protéines (p/p).

of the validity of the screening in buffer applied by Lemée *et al* (1994a) over 57 strains. This obvious strain dependence of autolysis in dairy propionibacteria is of practical value for the choice of cheese starters, but also leads to an interesting fundamental question. It suggests the presence of different and specific autolytic systems for each strain and/or different levels of regulation. According to the suggestions of Jolliffe *et al* (1981) and Kemper and Doyle (1993), the apparent resistance of the cells to autolysis during normal growth reflects the strong control of autolysins by energized membrane. Effectively, the cell life principally depended on the intracellular pH (buffering capacity and ionic force of the cytoplasm), and on the turgor pressure which are directly linked to the pH and content of the environmental medium (Ingraham, 1987). In this work, the spontaneous autolysis of *P freudenreichii* CNRZ 725 after maximal growth in YEL medium occurred at pH 6.2 when the lactate was depleted and at a compatible temperature (15–24–30°C). These conditions (in terms of absence of nutrient, pH and temperature) were close to those that led to extensive autolysis of CNRZ 725 in buffered solution (Lemée *et al*, 1994b). The hypothesis from which the pH of the growth medium had an effect upon the induction of the whole cell autolysis of CNRZ 725 was in agreement with the results of Langsrud (1974). He showed that the initial pH of the growth medium influenced the release of the intracellular proline iminopeptidase by spontaneous autolysis of *P freudenreichii* P59 cells. In experimental Swiss-type cheese, Kurtz *et al* (1959) observed relationships between the initial pH off the press and the growth curve (and in particular the loss of viability after maximal growth) of dairy propionibacteria (*Propionibacterium freudenreichii* subsp *shermanii)*. A pH off the press of about 5.25 accelerated the starting point of the loss of viability in the curd, compared to an initial pH of about 5.37 and 5.82.

Nevertheless, if pH decrease and nutrient depletion are undoubtedly key factors of autolysis induction, it must be emphasized that autolyses have been observed even in

the presence of lactate (as, *eg,* when the temperature of growth is suddenly increased from 30 to 40°C during the exponential phase; or when exponential whole cells are suspended in Emmental juice obtained by pressing the cheese before the warm room) (data not shown). Further experiments must be undertaken to understand precisely the molecular mechanism of this induction. Nevertheless, it could be underlined that the conditions leading to autolysis of CNRZ 725 cells were similar in terms of pH and salinity (Lemée *et al,* 1994b) to those of the aqueous phase of Emmental during ripening (Salvat-Brunaud *et al,* 1995). Moreover, this work showed that the Emmental juice allowed the expression of the autolysins of strain CNRZ 725. In spite of the high optimal temperature (40°C) of this autolytic system, we showed that CNRZ 725 cells were able to autolyse in growth medium at 15 or 24°C (temperature of Swiss-type cheese ripening). Thus, this work demonstrated that the aqueous phase of Swiss-type cheeses would be adequate to promote the autolysis of propionibacteria at the ripening temperature.

The autolysins from a dairy propionibacteria strain were isolated here for the first time and analyzed by renaturing gel electrophoresis. This method has revealed the complexity of the autolytic system for the strain CNRZ 725, as at least 8 lytic bands were obtained in the profile: *i)* 1 at 121 kDa, very intense and which could correspond from the data presented here to the *N*-acetylglucosaminidase activity mainly involved in the autolysis (Lemée *et al,* 1994b); *ii)* 6 lytic activities between 81 and 118 kDa and *iii)* 1 relatively weak activity at 34 kDa. At this stage, it was not possible to assume that all these lytic activities corresponded to distinct autolysins. Some of them could correspond to proteolytically processed forms of the same autolysin, in particular the lytic activities with close molecular mass (between 81 and 118 kDa). Since the development of this renaturing gel electrophoresis method, autolytic systems which were supposed to be relatively well known, were, in fact, shown to contain many lytic activities: 8 lytic activities for *S aureus* (Sugai *et al,* 1990); 11 for *Klebsiella pneumoniae* and 17 for *E coli* (Bernadsky *et al,* 1994). The correlation between a lytic band and a specific autolysin is still an open question.

The attempts presented here to separate these 8 lytic activities and purify them by using classical methods such as ammonium sulfate precipitation, gel filtration and ion-exchange chromatography have not resulted in extensive purification. Several difficulties were encountered, in particular the fact that the enzymes were always associated with cell wall neutral polysaccharides in high amount. For this reason, a method of direct extraction from whole cells was successfully developed, based on a LiCl treatment which had been previously described in the literature as an extracting agent for autolysins in several species (*Bacillus subtilis* [Ortiz *et al,* 1972; Rogers *et al,* 1984; Foster, 1992]; *Streptococcus faecalis* [Pooley *et al,* 1970]; *Clostridium perfringens* [Williamson and Ward, 1979] and *Lactobacillus helveticus* [Valence and Lortal, 1995]).

Further work implies *i)* the demonstration of the impact of propionibacteria autolysis on cheese ripening using strains highly prone to autolysis; *ii)* the purification, or at least, the separation of the 8 lytic activities constituting the autolytic system of *P freudenreichii* CNRZ 725 in order to determine whether they are all distinct autolysins, and the definitive determination of which one corresponds to the *N*-acetylglucosaminidase activity mainly involved in the autolysis of this strain (Lemée *et al,* 1994b).

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