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Strain variability of the cell-free proteolytic activity of dairy propionibacteria towards β-casein peptides

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Abstract — Strain selection of dairy propionibacteria is based on CO_2, acetate and propionate production. However, another criterion such as proteolytic activity could be of industrial interest for selection of flavor ripening agents. Given that the propionibacteria are variously able to autolyse and that proteolytic activity is mainly intracellularly located, the proteolytic potential was evaluated on the cell-free extracts of 37 dairy propionibacteria strains. The substrate used to detect proteolytic activity was a tryptic/chymotryptic hydrolysate of β-casein. The evaluation was performed at a temperature of 30 °C and pH of 5.7. The increase in the free NH_2 groups over a 24 h period was monitored as well as the evolution of the reversed-phase chromatographic profiles. In addition, for 14 strains, the free amino acids released from the β-casein peptides were determined. All the strains were able to hydrolyze β-casein peptides. Difference of proteolytic activity was observed among and within the four dairy propionibacteria species. This variability was expressed more in terms of nature of the products released (peptides and free amino acids) than in terms of degree of proteolytic activity. From the results, a strain selection system may be developed using as substrate the tryptic/chymotryptic β-casein hydrolysate and as criteria the degree of hydrolysis at 24 h and the amount and the nature of free amino acids released by cell-free extracts. Such a selection will give useful data for cheesemakers, especially if characterization of autolytic ability in the cheese environment is also determined. © Inra/Elsevier, Paris.

propionibacteria / proteolysis / peptide / free amino acid / selection

Résumé — **Souche dépendance de l'activité protéolytique intracellulaire de bactéries propioniques laitières sur un substrat laitier (hydrolysat de caséine β).** La sélection des souches de bactéries propioniques est fondée sur la production de CO_2, d'acétate et de propionate. Cependant, un autre critère tel que l'activité protéolytique pourrait présenter un intérêt industriel afin de sélectionner des souches susceptibles de produire des précurseurs de flaveurs. En tenant compte du fait que les bactéries propioniques possèdent une capacité variable à s'autolyser et que

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l'activité protéolytique est localisée principalement dans la fraction cytoplasmique de la bactérie, nous avons évalué le potentiel protéolytique d'extrait intracellulaire de 37 souches de bactéries propioniques. Le substrat utilisé pour tester l'activité de manière globale était un hydrolysat trypsique/chymotrypsique de caséine β. L'hydrolyse était réalisée à 30 °C et à pH 5,7. L'augmentation de la teneur en groupements NH_2 libres était suivie sur 24 heures, ainsi que l'évolution des profils chromatographiques par analyse CLHP phase inverse. De plus, pour 14 souches, nous avons déterminé la composition en acides aminés libérés. Nous avons observé que toutes les souches étaient capables d'hydrolyser les peptides de la caséine β avec une activité protéolytique différente selon les quatre espèces de bactéries propioniques mais également au sein d'une même espèce. Cette variabilité s'exprimait plus en terme de nature de produits libérés (peptides et acides aminés) qu'en terme d'intensité de la protéolyse. À partir de ces résultats, une sélection des souches peut être envisagée, en utilisant comme substrat l'hydrolysat trypsique/chymotrypsique de caséine β et, comme critères, le degré d'hydrolyse à 24 h ainsi que la quantité et la nature des acides aminés libérés par les extraits intracellulaires. Une telle sélection pourrait donner d'utiles informations aux fromagers, d'autant plus si, en complément, la caractérisation des capacités autolytiques des souches en conditions fromagères est réalisée. © Inra/Elsevier, Paris.

propionibactérie / protéolyse / peptide / acide aminé libre / sélection

1. INTRODUCTION

Dairy propionibacteria species are mainly present in some fermented dairy products, particularly in hard cooked cheeses such as Gruyère and Emmental [4]. Their fermentation characteristics lead to typical flavor and aroma development but their involvement in the protein breakdown seems apparently low compared to that of the thermophilic lactic acid bacteria used as starters [33].

Various peptidase activities have been shown in the four dairy propionibacteria species (for review see [19]). They are mainly intracellularly located [12, 23, 27, 31]. Four of them identified in *P. freudenreichii* are supposed to play a major role in Swiss cheese flavor development: a proline iminopeptidase [25, 27], prolinase and prolidase [29] and two phenylalanine aminopeptidases [10]. The amino acids, Pro and Phe, are released at a high level during ripening and shown respectively as component participating to the sweetness and as a precursor of floral flavor of the cheese [3, 6, 17, 18]. The release of intracellular peptidase activities in cheese curd during ripening requires evidently an autolytic mechanism induced in propionibacteria cells, either physiologically or by environmental conditions. Such an autolysis was recently observed under in vitro conditions [20, 21, 23, 24] and in Emmental (Valence F., personal communication).

The aim of this work was to evaluate the cell-free proteolytic activity of 37 propionibacteria strains belonging to four different species, 15 of which were of industrial origin. This potential was determined on a substrate related to that encountered in cheese aqueous phase. Our choice was a tryptic/chymotryptic β-casein hydrolysate because: i) during Swiss-type cheese ripening, the main peptides arose from β-casein [1, 2]; ii) the substrate specificity of trypsin was equivalent to that of plasmin, which plays a major role in primary hydrolysis of para-casein network and iii) the chymotrypsin specificity towards C-terminus of amino acid residues such as Phe_{52}, Leu_{58}, Gln_{72} and Met_{93}, [26] allowed to have cleavage at

sites which have been identified in Swiss-type cheese [1, 2].

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

The strains, obtained from the CNRZ collection (Inra, 78352 Jouy-en-Josas, France), the CIP collection (Institut Pasteur, 75015 Paris, France), our own collection TL (Inra, 35042 Rennes, France) and from commercial origin (*table I*) were stored at –80 °C in yeast extract lactate (YEL) broth [22] containing 15% (v/v) glycerol. Two transfers at 1% (v/v) in YEL broth during 48 h at 30 °C were performed before the cells were grown statically in 1 L YEL broth at 30 °C. Growth was monitored at 650 nm (OD_{650}) using a model DU 7400 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, U.S.A.).

2.2. Preparation of the cell-free extracts

Cells harvested in early exponential growth phase (at an OD_{650} of 1; when the pH reached 6.0, corresponding to about 30 h of incubation) were washed twice with cold sterile distilled water, and recovered by centrifugation for 30 min at 5 000 *g*, at 4 °C. The cell pellets were stored at –20 °C in order to render the cells fragile before mechanical disruption at 138 MPa in a refrigerated French pressure cell (SLM-Aminco, Urbana, IL, U.S.A.) under the conditions described by Lemée et al. [21]. Undisrupted cells and cell debris were removed by centrifugation at 3 000 *g* for 30 min, at 4 °C

Table I. Origin of the strains used in this study.

Tableau I. Origine des souches utilisées dans cette étude.

Species	Source	Strains
P. freudenreichii subsp. *freudenreichii:*		
	industrial strains	A, B, C, D, E, F, G, H, I, J, K, L, M, N, O
	TL	TL 511, TL 213, TL 230, TL 234, TL 503, TL 501, TL 504A, TL 504B, TL 512, TL 510A, TL 510B, TL 510C
	CIP	CIP 5932, CIP 103026
	CNRZ	CNRZ 89
P. freudenreichii subsp. *shermanii:*		
	CIP	CIP 103027
	CNRZ	CNRZ 725, CNRZ 726
P. jensenii	CIP	CIP 103028
	TL	TL 222
P. thœnii	CIP	CIP 103029
P. acidipropionici	TL	TL 249

TL (Collection of the Laboratoire de recherches de technologie laitière, Inra Rennes, France); CNRZ (Collection of INRA, Jouy-en-Josas, France); CIP (Collection of the Institut Pasteur, Paris, France). A to O: Industrial strains.

TL (collection du Laboratoire de recherches de technologie laitière, Inra Rennes, France) ; CNRZ (Collection de l'Inra, Jouy-en-Josas, France) ; CIP (collection de l'Institut Pasteur, Paris, France). A à O : souches commerciales.

and the collected supernatant was filtered (0.45 µm, Sartorius, Laboratoires Humeau, La Chapelle S/Erdre, France), distributed in tubes (1 mL), and stored at –20 °C until use. For all the strains, the protein content of the cell-free extracts was determined according to the microprocedure of Bradford (Biorad SA, Ivry-sur-Seine, France) using bovine serum albumin (Sigma, Saint-Quentin-Fallavier, France) as standard. The protein content varied in the range 2.9 ± 0.8 mg eq. BSA per mL of cell-free extracts.

2.3. Preparation of the β-casein hydrolysate

β-Casein, kindly supplied by Eurial Poitouraine (Nantes, France), at 10 g/L in sterile distilled water, was hydrolyzed by a mixture of trypsin (5 000K, Novo Industry A/S, Copenhagen, Denmark) and chymotrypsin (Sigma), both at an enzyme/substrate ratio of 1/1 000 (w/w), at pH 7.2 maintained constant by adding NaOH 0.5 mol/L for 3 h at 37 °C. Then the enzymes were inactivated by heating at 80 °C for 20 min. The β-casein hydrolysate was freeze-dried (model CS 10-08, CIRP-Serail, Argenteuil, France) and stored at 4 °C until use.

The degree of β-casein hydrolysis was about 16%, determined from the ratio of the number of NH_2 groups released after the tryptic/chymotryptic hydrolysis to the total number of NH_2 groups of the initial β-casein. The NH_2 groups were detected by the reaction with fluorodinitrobenzene (FDNB) [15] using methionine (Sigma) as standard. This was in agreement with the theoretical peptide bond specificities of trypsin and chymotrypsin reported by Pélissier [26] and the number of final peptides (i.e., 28) susceptible to be produced with both enzymes.

2.4. Hydrolysis of β-casein peptides by the cell-free extracts

Cell-free extracts, 550 µg protein adjusted to 500 µL with sterile distilled water, were added to 3.5 mL of β-casein peptide solution (0.8 mg β-casein hydrolysate per mL of sodium phosphate buffer 50 mmol/L, pH 5.7). Duplicate samples (300 µL), withdrawn at zero time and after 0.5, 1, 3 and 24 h of incubation at 30 °C were inactivated by heating at 100 °C in a water bath for 10 min.

2.5. Measurement of the total proteolytic activity of the cell-free extracts

The proteolytic activity of the cell-free extracts was determined by analyzing the increase in N-terminal NH_2 groups of the β-casein peptides by reaction with fluorodinitrobenzene (FDNB) according to the method of Ghuysen et al. [15] and using methionine (Sigma) as standard. The results were expressed according to two parameters: i) the initial rate of free amino groups increase; and ii) the degree of proteolysis after 24 h. The initial rate was calculated during the first 30 min of incubation and was expressed in µmol equivalent Met per min, for one mg of cell-free extract protein. The degree of proteolysis corresponded to the difference between the NH_2 values determined at 24 h and at zero time and was expressed in µmol equivalent Met per mL of solution for a defined quantity of intracellular protein in the tested fraction. β-casein hydrolysate was incubated as a control as well as the cell-free extract of the strains, and the heat-treated cell-free extract mixed with the β-casein peptides.

2.6. Chromatographic analysis

β-casein peptide hydrolysates were acidified with 10% (v/v) trifluoroacetic acid (TFA, Pierce, Touzart et Matignon, Vitry-sur-Seine, France) to a final concentration of 0.1% (v/v), and passed through a filter of 0.45 µm pore size (Millex-HV_{13}, Millipore, Saint Quentin-en-Yvelines, France). One hundred µL were injected, without sample dilution, onto the analytical reverse-phase Lichrospher 100 RP C_{18} column (124 x 4 mm I.D.; Merck), equilibrated at a flow rate of 1 mL/min and at 40 °C with buffer A containing 1.06 mL/L TFA in water of HPLC grade (Millipore). The linear gradient was performed from 0 to 40% buffer B containing 1 mL/L TFA, 800 mL /L acetonitrile (Merck) and 200 mL/L water in 50 min, followed by 40 to 80% buffer B in 20 min. The absorbance was monitored at 214 nm with a

Spectra Physics SP 8490 UV detector (Thermo Separation Products, Les Ulis, France).

2.7. Analysis of the free amino acids

For 14 strains, the peaks eluted during the first 10 min of the 24 h RP-HPLC separations were collected in two distinct fractions named A and B as shown in *figure 2*. The samples, after freeze-drying were analyzed for their free amino acid content. They were resuspended in water of HPLC grade and directly derivatized with phenylisothiocyanate (Pierce, Touzart et Matignon). Quantitative analysis of the amino acid derivatives was performed by RP-HPLC on a Pico-Tag C_{18} column (150 × 3.9 mm I.D.; Waters, Saint-Quentin-en-Yvelines, France) according to Bidlingmeyer et al. [5]. Two controls were used: i) the β-casein peptides alone (0.7 mg/mL); and ii) a sample containing the heat-treated intracellular extract of CIP 103027 mixed with the β-casein peptides.

3. RESULTS

3.1. Time course of the β-casein peptide hydrolysis

3.1.1. Quantitative evaluation

Increase versus time of released NH_2 groups from ß-casein peptides by cell-free extracts obtained from the five typical dairy propionibacteria species and subspecies are shown in *figure 1*. No free amino group increase indicating no peptidase activity was observed in the control experiment. Activities were different in two aspects: initial rate and degree reached after 24 h hydrolysis. The highest peptidase activity was observed with *P. freudenreichii* subsp. *shermanii* CIP103027: initial rate determined for the first 30 min was 11.6 10^{-3} µmol eq. Met/min for 1 mg intracellular protein and degree of hydrolysis at 24 h, 1.86 µmol eq. Met/mL. *P. thoenii* CIP103029 and *P. freudenreichii* subsp. *freudenreichii* CIP103026 cell-free extracts had comparable initial rates and degree of hydrolysis whereas *P. acidipropionici* TL249 cell-free extracts showed practically no activity during the first 30 min and then a degree of hydrolysis close to that of *P. jensenii* CIP103028, i.e., somewhat below the other strains.

3.1.2. Qualitative evaluation

The more characteristic RP-HPLC-profiles obtained after 24 h hydrolysis with the cell-free extracts of the four species of propionibacteria are represented in *figure 2*. Initially, the β-casein hydrolysate contained 28 main peaks, numbered 1 to 28. Peaks 4, 5, 12, 13, 22, 25 and 26 completely disappeared for all the strains. Chromatographic profiles resulting from incubation with *P. acidipropionici* and *P. thoenii* extracts showed absence of peak 14 to 18 and 27 and 28, respectively. Peak 19 disappeared for both subspecies of *P. freudenreichii*. On the other hand, new peaks, numbered 29 to 35, appeared as a consequence of hydrolysis. Among them, peaks 29, 30 and 35 were present for all the species. In contrast, peak 31 was absent from the chromatographic profiles of *P. freudenreichii* including both subspecies, peak 32 from that of *P. thoenii*, peak 33 from those of *P. acidipropionici* and *P. freudenreichii* including both subspecies and peak 34 from that of *P. jensenii*. A specific 'fingerprint' is therefore observed for each of the four dairy propionibacteria species through the proteolysis of the tested β-casein hydrolysate by their cell-free extracts and there was no difference between two subspecies of *P. freudenreichii*.

3.2. Comparison of the proteolytic activity between the 37 strains

Figure 3 summarizes the proteolytic activity of the 37 tested strains of propionibacteria according to the initial rate (*figure 3a*) and the degree of hydrolysis

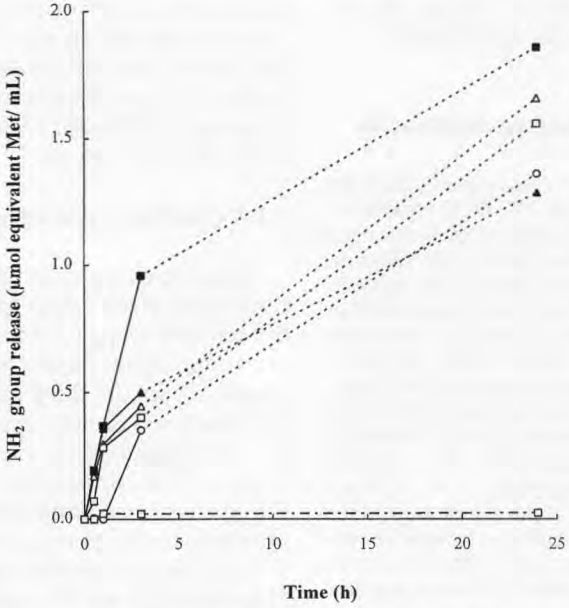

Figure 1. Time course of the β-casein peptide hydrolysis by the cell-free extract of the four species of propionibacteria: *Propionibacterium freudenreichii* subsp. *freudenreichii* CIP103026 (Δ) and subsp. *shermanii* CIP103027 (■), *P. jensenii* CIP103028 (▲), *P. thoenii* CIP103029 (□), and *P. acidipropionici* TL249 (○). The proteolytic activity is determined by the NH_2 group release and expressed in µmol eq. Met/mL. The hydrolysis was performed at 30 °C with 137.5 µg/mL intracellular protein for 0.7 mg of freeze-dried β-casein peptides/mL of 50 mmol/L sodium phosphate buffer at pH 5.7. Incubation of the β-casein peptides, without enzyme extract is represented by – · – ·□– · – ·. Peptide hydrolysis evolution between 3 and 24 h is schematically represented by dashed lines.

Figure 1. Évolution au cours du temps de l'hydrolyse des peptides de la caséine β par les extraits intracellulaires de souches de bactéries propioniques: *Propionibacterium freudenreichii* subsp. *freudenreichii* CIP103026 (Δ) et subsp. *shermanii* CIP103027 (■), *P. jensenii* CIP103028 (▲), *P. thoenii* CIP103029 (□), et *P. acidipropionici* TL249 (○). L'activité protéolytique était dosée par la libération des groupements NH_2 et exprimée en µmol eq. Met/mL. Les conditions d'hydrolyse étaient les suivantes : les extraits intracellulaires (137,5 µg eq. BSA/mL) étaient incubés à 30 °C en présence de 0,7 mg de peptides de caséine β lyophilisés/mL de tampon phosphate de sodium 50 mmol/L à pH 5,7. L'incubation des peptides de la caséine β en l'absence d'extraits intracellulaires est représentée par – · – ·□– · – ·. L'évolution de la protéolyse des peptides entre 3 et 24 h est représentée schématiquement par un trait en pointillés.

after 24 h (*figure 3b*). It appears clearly from this figure that there was no high correlation between the two enzymatic parameters for classifying the strains. Some *P. freudenreichii* strains (A, B, C, CNRZ 726, TL234, TL230 and TL501), one of *P. acidipropionici* (TL249) and one of *P. jensenii* (TL222) showed no evidence of peptide bond cleavage within the first 30 min, however, large variations in the degree of hydrolysis at 24 h were observed between them, from the lowest *P. jensenii* TL222 with 1.1 µmol eq. Met/mL to a value close to the highest *P. freudenreichii* TL230 with 1.70 µmol eq. Met/mL. The initial rate of proteolysis varied in general from 0 to about 12 10^{-3} µmol eq. Met/min for one mg intracellular

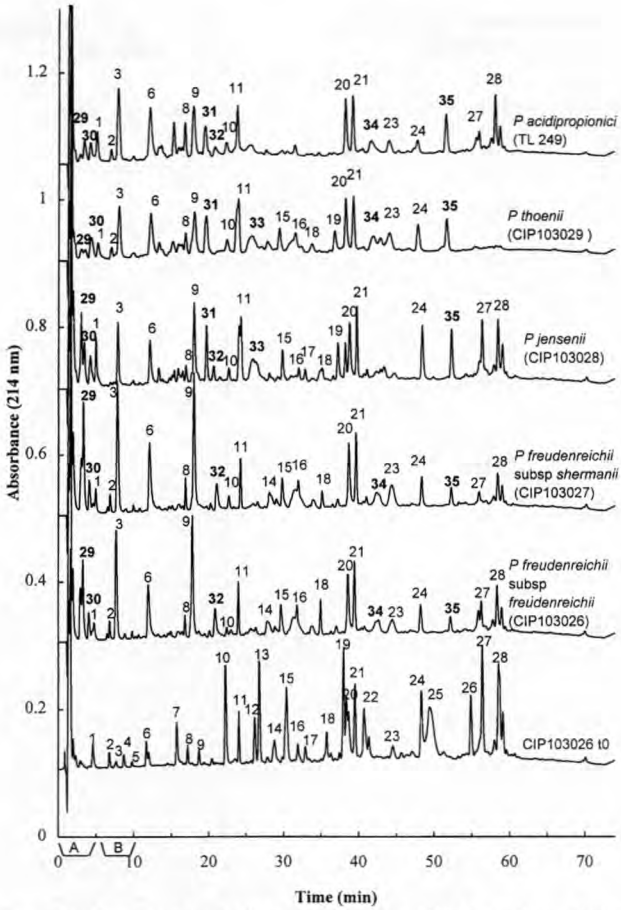

Figure 2. Comparison between the 24 h reversed-phase-HPLC profiles of the β-casein peptides hydrolyzed by the cell-free extracts of the four species of propionibacteria: *Propionibacterium freudenreichii* subsp. *freudenreichii* CIP103026 and subsp. *shermanii* CIP103027, *P. jensenii* CIP103028, *P. thoenii* CIP103029 and *P. acidipropionici* TL249. These profiles were obtained from hydrolysis performed under the same conditions as described in the legend to *figure 1*. Peaks, initially present in the RP-HPLC profile of the β-casein peptides at t = 0, were numbered 1 to 28 and the new peaks produced during hydrolysis were numbered 29 to 35. The RP-HPLC profile of the t = 0 sample (CIP103026), corresponds to the reaction mixture of the heat-treated cell-free extract of the CIP103026 strain and the β-casein peptides, collected at zero time of incubation. A and B correspond to the fractions collected for amino acid analyses of 14 strains.

Figure 2. Comparaison des profils chromatographiques (RP-HPLC) obtenus après 24 heures d'hydrolyse des peptides de caséine β par les extraits intracellulaires de quatre espèces de bactéries propioniques : *P. freudenreichii* subsp. *freudenreichii* CIP103026 et subsp. *shermanii* CIP103027, *P. jensenii* CIP103028, *P. thœnii* CIP103029 et *P. acidipropionici* TL249. Les conditions d'hydrolyse correspondaient à celles décrites sur la *figure 1*. Les pics présents initialement dans le profil RP-HPLC des peptides de caséine β à t = 0 sont numérotés de 1 à 28 et les nouveaux pics, produits pendant l'hydrolyse, de 29 à 35. Le chromatogramme du témoin noté CIP103026 to, correspond au mélange réactionnel suivant : l'extrait intracellulaire de la souche *P. freudenreichii* CIP103026 inactivé par la chaleur était ajouté aux peptides de la caséine β, et prélevé au temps t = 0. A et B correspondent aux fractions collectées pour l'analyse d'acides aminés réalisée sur 14 souches.

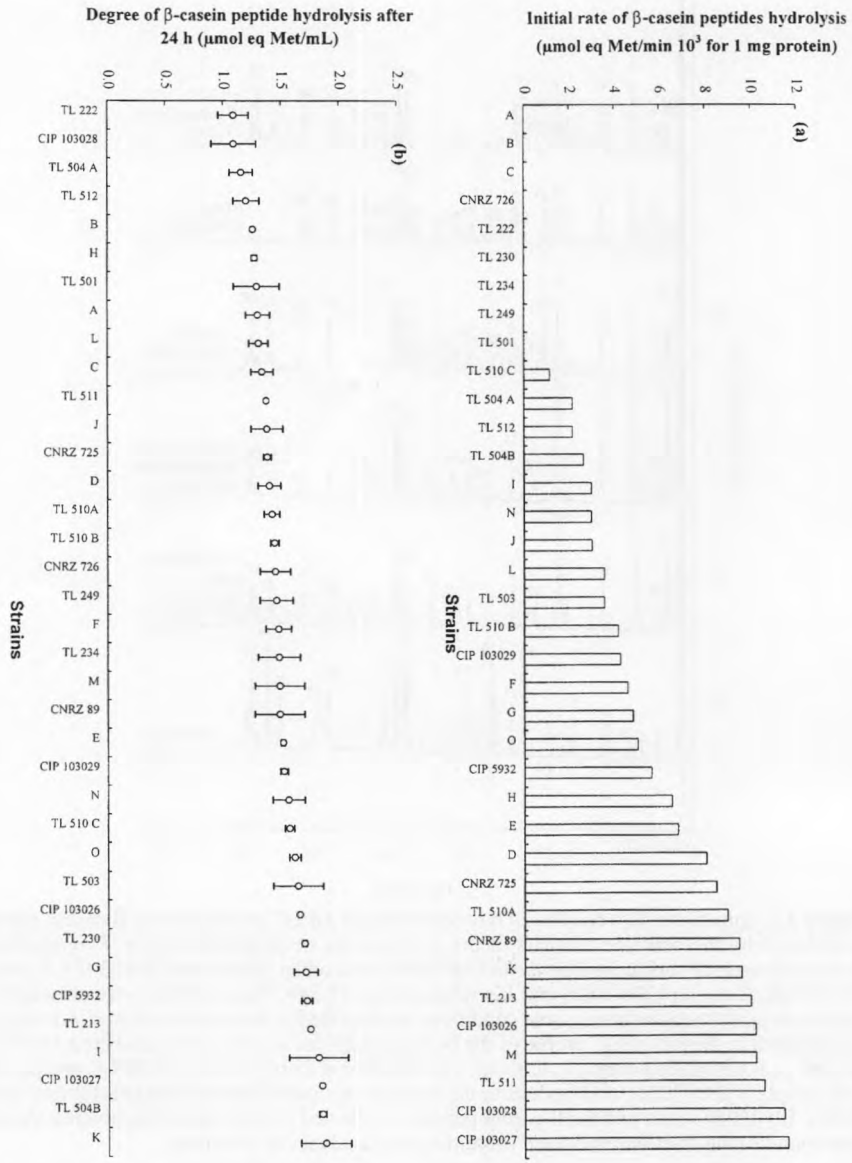

Figure 3. Comparison between the cell-free extracts of the 37 strains of propionibacteria of the initial rates of the β-casein peptide hydrolysis determined for the first 30 min (**a**) and the degree of hydrolysis obtained after 24 h of incubation (**b**). The enzymatic reaction was performed as described in the legend to *figure 1*. These results are the average of, at least, two experiments. The average error percentage for the initial rates was 23%.

Figure 3. Comparaison sur les 37 extraits intracellulaires de bactéries propioniques de la vitesse initiale d'hydrolyse des peptides de la caséine β, mesurée pendant les 30 premières min (**a**) et du degré d'hydrolyse après 24 heures d'incubation (**b**). Les conditions d'hydrolyse correspondaient à celles décrites sur la *figure 1*. Les résultats rendent compte de la moyenne de deux expérimentations au minimum. Le pourcentage d'erreur moyen sur les vitesses initiales était de 23 %.

protein, whereas the degree of hydrolysis in 24 h varied between 1.1 and 1.89 µmol eq. Met /mL.

From the data on degree of hydrolysis and by taking into account a 14% value as standard deviation of the experiment, only nine strains could be considered as having different proteolytic activities. They could be classified into two distinct groups: one containing five strains among the *P. freudenreichii* subspecies (TL213, I, K, TL504B and CIP103027) with degree of hydrolysis more than 1.75 µmol eq. Met/mL and the other containing two strains from *P. jensenii* (CIP103028 and TL222), and two from *P. freudenreichii* subsp. *freudenreichii* (TL504A and TL512) with degree of hydrolysis less than 1.20 µmol eq. Met/mL.

3.3. Free amino acids after 24 h hydrolysis of the β-casein peptides

In order to evaluate more closely the peptidase activities of the strains, free amino acids which were presumably eluted in fractions A and B in *figure 2* during the first 10 min of the RP-HPLC profiles, were collected and analyzed for 14 strains. The results are presented in *table II*.

The β-casein hydrolysate, analyzed as a control, only contained detectable traces of Pro and Lys, in agreement with the specificities of both enzymes used, trypsin and chymotrypsin [26]. The heat-treated cell-free extract of *P. freudenreichii* subsp. *shermanii* CIP103027 mixed with the β-casein peptides, was checked at t = 0 and t = 24 h as a second control. Presence of Glx, Pro, Thr and Val was observed in amounts comparable to those of Rolin et al. [30] for the intracellular fraction of this strain.

All the strains generated free amino acids, ranging from 7% of the total amino acid content of the β-casein for *P. jensenii* TL222 (corresponding to the release of 14.9 amino acid residues) to 24% (i.e., 49.5 amino acid residues) for *P. freudenreichii* subsp. *shermanii* CIP103027. Some amino acids were not produced by all the strains. Asx was released only by extracts of *P. freudenreichii* subsp. *freudenreichii* H, K, and CIP5932, *P. jensenii* CIP103028 and *P. thoenii* CIP103029. Gly was not produced by *P. freudenreichii* subsp. *freudenreichii* CIP103026 and subsp. *shermanii* CIP103027 and by *P. acidipropionici* TL249. Ile in fraction A was absent with *P. freudenreichii* subsp. *freudenreichii* CIP5932, K and subsp. *shermanii* CNRZ 725 and with *P. jensenii* CIP103028 and TL222. This last strain did not release Ser, Arg, His and Met.

From the total amount of the free amino acids released and expressed in µmol/L, the 14 strains can be classified roughly into three groups: the first group with the highest amount over 1 100 µmol/L contains the strains from *P. freudenreichii* subspecies (CIP103027, H, M, CIP5932, K) and *P. jensenii* CIP103028; the second group 900–1 000 µmol/L contains *P. freudenreichii* subsp. *freudenreichii* TL503, J and A, *P. acidipropionici* TL249 and *P. thoenii* CIP103029, and the remaining strains in the third group with values below 800 µmol/L, with *P. freudenreichii* subspecies CIP103026 and CNRZ 725 and *P. jensenii* TL222.

Inside the *P. jensenii* species, we noted that the strains TL222 and CIP103028 previously shown as having similar degree of hydrolysis, were quite different in amino acid release, i.e., 1189 and 435 µmol/L for CIP103028 and TL222, respectively. The nature of the amino acids released also differed. In *P. freudenreichii* species, differences between degree of hydrolysis and amino acid release was also shown. In *P. freudenreichii* subsp. *shermanii* strain, CIP103027 has the highest degree of hydrolysis and release of free amino acids compared to strain CNRZ725.

Table II. Analysis of the free amino acids (AA) released after 24 hours of β-casein peptide hydrolysis by the cell-free extracts of 14 propionibacteria strains.
Tableau II. Composition en acides aminés libres obtenus après 24 heures d'hydrolyse des peptides de la caséine β par les extraits intracellulaires de 14 souches de bactéries propioniques.

	Theoretical n° of AA in β-casein	β-casein peptides control	CIP 103027 heat-treated t 0 h/t 24 h[1]	CIP 103027	H	M	CIP 5932	K	CIP 103028	TL 249	CIP 103029	TL 503	J	A	CIP 103026	CNRZ 725	TL 222
		Free amino acids[2]															
Fraction A[3]																	
Asx	9	–	–	–	1.2	–	1.0	1.2	1.0	–	0.7	–	–	–	–	–	–
Glx	39	–	2.1/ 2.4	4.8	5.3	5.7	6.2	4.7	5.7	2.2	2.0	2.3	3.0	2.3	2.6	1.8	1.3
Pser + Ser	16	–	–	2.3	1.7	1.7	1.9	2.3	1.5	1.2	0.8	1.0	1.2	1.0	1.1	0.7	–
Gly	5	–	–	–	2.1	2.2	1.7	2.4	2.7	–	1.3	1.5	1.6	2.0	–	1.0	0.9
His	5	–	–	2.0	1.9	2.1	2.0	1.7	1.8	1.5	1.5	1.3	1.4	1.4	1.2	1.1	–
Arg	4	–	–	2.3	1.8	1.7	1.2	1.4	2.6	1.6	1.4	0.8	1.5	1.1	1.1	0.9	–
Thr	9	–	0.9/ 1	3.8	3.0	2.7	3.6	2.4	3.6	2.6	2.4	1.7	2.4	1.7	1.7	1.5	0.8
Ala	5	–	–	3.2	3.0	2.6	3.2	2.3	5.1	2.7	2.2	1.9	2.3	2.2	1.6	1.7	1.3
Pro	35	0.1	2.9/ 3.3	5.3	4.3	3.7	3.5	4.4	4.5	2.7	2.1	3.4	2.9	4.4	2.7	1.4	1.9
Val	19	–	0.7/ 0.7	5.5	4.2	4.5	4.8	4.2	4.1	4.0	3.6	3.2	3.2	3.1	3.3	2.0	1.6
Met	6	–	–	2.5	1.6	1.9	2.2	1.6	1.5	1.6	2.1	1.4	1.2	1.4	1.1	0.9	–
Ile	10	–	–	2.2	1.8	1.7	–	–	–	1.4	1.0	1.0	0.8	0.9	0.6	–	–
Lys	11	0.2	0/ 0.7	3.9	4.7	4.3	3.7	3.7	5.8	3.0	3.7	2.5	3.1	2.5	2.2	2.0	1.7
Fraction B :																	
Tyr	4	–	–	2.3	1.9	1.5	1.3	1.7	1.0	1.7	1.0	1.9	1.5	1.7	1.3	1.2	0.7
Ile + Leu	32	–	–	6.9	4.9	4.5	4.1	5.1	4.1	5.2	4.5	5.7	4.4	4.0	4.6	2.9	3.2
Phe	9	–	–	2.8	2.4	1.9	1.9	2.3	1.8	2.6	2.0	2.3	1.5	2.0	1.5	1.3	1.7
Total (µmol/L)	209			49.5 (1 445)	46.0 (1 344)	42.6 (1 245)	42.2 (1 233)	41.3 (1 207)	40.7 (1 189)	33.9 (991)	32.2 (941)	31.9 (932)	31.9 (932)	31.7 (926)	26.7 (780)	20.4 (596)	14.9 (435)

[1] Strain designations are referred to *table I*. The cell-free extract of CIP 103027 was heat-treated (100 °C, 10 min) before adding to the β-casein peptide solution. [2] The free amino acids are expressed as number of amino acid residues released per mole of ß-casein. [3] Samples were collected from the 24 h RP-HPLC profiles (fractions A and B; see text). The amino acids were analysed after 24 h of β-casein peptide hydrolysis by the cell-free extracts at 30 °C. (–), absence of free amino acids. Tryptophan was absent in all the samples.

[1] La nomenclature des souches correspond à celle du *tableau I*. L'extrait intracellulaire de la souche CIP 103027 était inactivé par la chaleur (100 °C, 10 min) puis additionné à la solution de peptides de caséine β. [2] Les acides aminés libres sont exprimés en nombre d'acides aminés libérés par mole de caséine β. [3] Les échantillons étaient collectés lors de l'analyse CLHP phase inverse selon les fractions A et B (cf texte). Les acides aminés libres étaient analysés après 24 h d'hydrolyse par les extraits intracellulaires à 30 °C. (-) Absence d'acides aminés libres. L'acide aminé tryptophane était absent de tous les échantillons.

Although *P. freudenreichii* subsp. *freudenreichii* CIP103026 possessed a higher degree of hydrolysis than strain H (1.66 vs. 1.27 µmol Met/mL), the latter strain released more free amino acids (1344 vs. 596 µmol/L).

Considering all the data it appears that Val, Pro, Glx and Lys in fraction A and Ile+Leu in fraction B were released to the greatest extents (*table II*). However, from the ratio of each amino acid released upon hydrolysis to their frequencies in β-casein, it was calculated that Ala always had the highest percentage of release (up to 100% release with CIP103028) followed by either Arg (up to 65% with CIP103028), Tyr (up to 57% with CIP103027), or Gly (up to 54% with CIP103028). The release percentage of proline was low, between 4% for CNRZ725 and 15% for CIP103027, that of phenylalanine was higher, between 14 and 31% for both strains.

4. DISCUSSION

As stated by Langsrud et al. [19] propionibacteria seem to be equipped with many of the enzymes necessary to release amino acids and small peptides from casein substrate. In this work, we showed that the dairy propionibacteria are able to hydrolyze β-casein peptides at pH 5.7 and at 30 °C. As mainly peptides from β-casein are present in Swiss-type cheese [1, 2], our results support the hypothesis that dairy propionibacteria may contribute during the later stages of proteolysis in Emmental ripening. There also appear to be agreement between the present results and those in reports on the prominent amino acids in cheese juice, i.e., glutamic acid, proline, lysine, leucine, valine [32], or, sometimes, phenylalanine in cheese [18].

The proteolytic activity, expressed with tryptic/chymotryptic peptides of β-casein as substrate, appears to be strongly strain-dependent among the dairy organisms in the genus *Propionibacterium*. It varied significantly among the four species and even within one species, as highlighted for *P. freudenreichii* strains. This variability exists not only in terms of level of activity but also in terms of nature of released products (peptides and free amino acids). Concerning the amino acids, the strain-dependent variability could also be the result of proteolysis and further degradation (including deamination, transamination, decarboxylation) which has been very little studied in propionibacteria. Brendehaug and Langsrud [7] showed that suspension of resting cells of two *P. freudenreichii* subsp. *shermanii* strains led to the total degradation of Asp, Ser, Gly and Ala and a lower or even no degradation of the other amino acids under aerobic conditions. This could explain the absence of some of these amino acids (Ser and Asp) found in our study. Concerning more particularly the free amino acid Asp, its absence in almost all the strains studied, might be due to an aspartase activity, which has been found in propionibacteria [18] and to the fact that Asp is the only amino acid metabolised by *P. freudenreichii* subsp. *shermanii* in a Swiss cheese environment according to Crow [9]. However, it implies the release of NH_3 which was not found in this study. On the other hand, to our knowledge, neither decarboxylase(s) have yet been reported in propionibacteria nor aminotranferase(s) specific for aromatic residues, which have recently been evidenced and characterized in lactococci [14, 36].

The amounts of total proline and glutamic acid include intracellular contents which were not negligible in *P. freudenreichii* subsp. *shermanii* CIP103027 strain (approximately 3 and 2.2 amino acid residues, respectively), and consequently their release is low. With regard to proline, such a low release from the tryptic/chymotryptic β-casein peptides can be attributed to its

central or ante-penultimate position in the peptide sequences [26], which perhaps should require previous proteolysis by other aminopeptidases for being released by the very specific enzyme activities already shown in propionibacteria (proline iminopeptidase [25, 27], prolinase and prolidase [29]), and X-prolyl dipeptidyl aminopeptidase [13]).

From our results, it can also be hypothesized that the sweet taste generally attributed to proline in Swiss type cheese may also be generated by the mixture of other sweet amino acids such as Gly, Ala, Ser and Thr [18], the release of which was effectively observed in this study.

Diversity in the proteolytic activity of the propionibacteria strains has already been observed but only for a few of them and by using amino acid β-naphthylamide derivatives, and specific dipeptides, tripeptides and oligopeptides [10–13, 27, 31, 35]. Amino acid β-naphthylamides are of course not natural substrates whereas selected di-, tri- and oligopeptides may represent some of the peptides in cheese.

From the obtained results, a multiple criteria distinction of strains with extreme characteristics can be proposed in order to check their respective effect on cheese flavor and texture. For example, it could be interesting to compare *P. jensenii* TL222 which had the lowest proteolytic activity in terms of degree of hydrolysis and amino acid release with either *P. freudenreichii* subsp. *shermanii* CIP103027 which had the highest or *P. jensenii* CIP103028 shown with a low degree of hydrolysis but a high activity for release of Tyr, Arg, Gly and Ala.

Such cheese experiments should be conducted first with the mini-Emmental cheese methodology [8] and then scaled up. According to the so-obtained results on cheese, a strain selection could be developed using as substrate the tryptic/chymotryptic β-casein hydrolysate and as criteria the degree of hydrolysis at 24 h with the amount and the nature of the free amino acids released by cell-free extracts. Such a selection will evidently require well-equipped laboratories, but it will give useful data for the cheesemakers, especially if characterization of autolytic ability in cheese environment is determined in parallel.

The results obtained in this study confirm indirectly the presence of intracellular endopeptidase(s) reported by Pripp et al. [28] and Tobiassen et al. [34], which could play a major role in the hydrolysis of hydrophobic peptides as shown in *figure 2*. Further work is nevertheless required before attributing the very rare frequency of bitterness defect in hard cooked cheese to enzymes released by autolysis of either propionibacteria or thermophilic lactic starters. However, recent studies showed that the proteolytic activities of lactic acid bacteria and propionibacteria could be complementary [16, 35]. Therefore, the determination of the respective action of the enzymes from both flora, which is likely sequential during Emmental ripening, appears to be crucial to control Swiss-type cheese ripening.

On the other hand, the release of phenylalanine observed at level up to 20% of the content in the β-casein peptides used leads to hypothesize a possible presence in the propionibacteria cell-free extracts of a carboxypeptidase. Indeed, because of the specificity of chymotrypsin [26], Phe has a C-terminal location in the chymotryptic peptides of β-casein, however, release of Phe may also result from a combined and sequential action of several peptidases.

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