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Properties and specificity of a cell-wall proteinase from *Lactobacillus helveticus*

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Summary

A cell-wall proteinase activity was extracted from cells of *Lactobacillus helveticus* grown on MRS medium. The enzyme was characterized after DEAE-Trisacryl chromatography and gel filtration on Ultrogel AcA 34 column. Two proteolytic fractions of different molecular weights were isolated. Their enzyme properties and specificity were clearly similar indicating that these fractions represented the same enzyme. It is a serine proteinase displaying optimum activity at pH 7.5-8.0 and 42 °C. Peptide bonds cleaved in αs1 and β-casein had no common feature and no clear specificity can be defined. The six main peptides obtained after β-casein hydrolysis were identified (Arg_1-Leu_6, His_{106}-Phe_{119}, Lys_{176}-Gln_{182}, Arg_{183}-Phe_{190}, Leu_{191}-Val_{209}, Tyr_{193}-Val_{209}) as well as the two peptides liberated from αs1-casein (Arg_1-Ile_6, Arg_1-Glu_9). These peptides whose size varies between 6 and 19 residues have probably to be hydrolysed by membrane-bound peptidases prior to their transport through the cytoplasmic membrane. Some of those released from the C-terminal part of β-casein are potentially bitter.

Key words : Lactobacillus helveticus - Proteinase - Cell-wall.

Résumé

Propriétés et spécificité d'une protéase de paroi de Lactobacillus helveticus

Une activité protéolytique a été extraite de la paroi de cellules de *Lactobacillus helveticus* cultivées sur milieu MRS. L'enzyme était caractérisée après chromatographie d'échange d'ions (DEAE-Trisacryl) et filtration sur gel (Ultrogel AcA 34). Deux fractions protéolytiques de poids moléculaire différents ont été isolées. L'étude des propriétés enzymatiques et de la spécificité indique clairement que ces fractions représentent la même enzyme. C'est une protéase à sérine dont les optima d'activité sont pH 7,5-8,0 et 42 °C. Les liaisons peptidiques rompues sur les caséines αs1 et β n'ont pas de point commun et les règles gouvernant la spécificité de l'enzyme ne peuvent être clairement définies. Six peptides obtenus après hydrolyse de la caséine β ont été identifiés : Arg_1-Leu_6, His_{106}-Phe_{119}, Lys_{176}-Gln_{182}, Arg_{183}-Phe_{190}, Leu_{191}-Val_{209}, Tyr_{193}-Val_{209} et deux peptides ont été libérés après hydrolyse de la caséine αs1 : Arg_1-Ile_6, Arg_1-Glu_9. Ces peptides dont la taille varie entre 6 et 19 résidus sont probablement hydrolysés par les peptidases de membranes avant d'être transportés à travers la membrane cytoplasmique. Certains étaient issus de la partie C- terminale de la caséine β et sont potentiellement amers.

Mots clés : Lactobacillus helveticus - Protéase - Paroi.

Introduction

The nutritional requirements of lactic acid bacteria are extensive and depend on the supply of exogenous amino acids. THOMAS and MILLS (1981) have shown that the proteolytic system plays a determining role in the optimum growth of mesophilic streptococci on milk culture and that caseins are hydrolysed by cell-wall associated proteinases. The liberated peptides are then broken down by peptidases located in the plasma membrane into free amino acids and peptides small enough to be transported through the membrane (EXTERKATE, 1975 ; LAW and KOLSTAD, 1983). Cell-wall proteinases from *Streptococcus cremoris* and *S. lactis* have been characterized by EXTERKATE and DE VEER (1985), GEIS *et al.* (1985), MONNET *et al.* (1987) and VISSER *et al.* (1986). They are serine proteinases with an optimum pH close to 6.0. Some of them seem to be mainly active towards β-casein and the specificity of the enzyme from *S. lactis* on this casein has been characterized by MONNET *et al.* (1986).

Only a few data are available on the proteinase of lactobacilli. The presence of cell-wall proteinases has been observed in *L. helveticus, L. lactis* and *L. bulgaricus* by ARGYLE *et al.* (1976), EZZAT *et al.* (1985, 1987), VESCOVO and BOTTAZZI (1979), as well as in *L. casei* and *L. plantarum* by EL SODA *et al.* (1986). Little is known about the properties of these proteinases. However, it has been established that the enzyme of *L. helveticus* hydrolyses αs1 and β-caseins. Electrophoresis in sodium dodecyl sulfate (SDS)-acrylamide gel showed that the major breakdown product of β-casein had a slightly lower molecular weight than intact casein, the molecular weight of the other products ranging between 2,500 and 19,000 according to EZZAT *et al.* (1985). The optimum pH for casein hydrolysis by the enzyme of *L. bulgaricus* is approximately 6.0 according to ARGYLE *et al.* (1976) and EZZAT *et al.* (1987).

MORELLI *et al.* (1986) have detected slow-coagulating variants in *L. helveticus* whose proteolytic activity is lower than that of fast-coagulating ones. Analysis of plasmid profiles suggests a possible relationship between casein hydrolysis and the presence of a plasmid of 3.5 MDa.

Beside their role in the growth of lactic acid bacteria, cell-wall proteinases play a determining part in cheese-making technology because they are involved in cheese ripening. MILLS and THOMAS (1980) and STADHOUDERS *et al.* (1983) have observed that they contribute in some cases to the occurrence of bitter taste during ripening.

The present study was made to determine the properties of the cell-wall proteinase from *L. helveticus* and to define its specificity on αs1 and β-caseins.

I. Materials and methods

A. *Bacteria and culture conditions*

Lactobacillus helveticus CNRZ 303 was obtained from the collection of the INRA Research Centre (Jouy-en-Josas, France). The organism was routinely

maintained frozen in litmus milk at −20 °C. For the preparation of the cells, 2 l of MRS medium (DE MAN *et al.*, 1960) supplemented with 20 mM $CaCl_2$ was inoculated with 1 % of a freshly grown culture in the same medium. $CaCl_2$ is added in order to limit the spontaneous release of proteinase in the medium. The temperature was 42 °C and the pH was maintained at 6.0 ± 0.1 by automatic addition of 10 N NaOH. The medium was gently stirred to ensure rapid dispersion of the alcali. After 9 h incubation (i.e. at the end of the exponential phase (cell number = 1.5×10^9/ml) the cells were harvested by centrifugation at 10.000 g for 15 min and washed twice at 4 °C in 50 mM β-glycerophosphate buffer pH 7.0 containing 20 mM $CaCl_2$. In some experiments cells were grown at 42 °C in low heat milk (Nilac, NIZO, Netherlands) as described by MILLS and THOMAS (1978). The pH was maintained constant at 6.0 ± 0.1 and the cells were collected at the end of the exponential phase after 10 h culture.

B. *Cell wall proteinase extraction*

The centrifuged cells were resuspended 3 successive times in 50 mM Tris-HCl buffer pH 7.8 (1/20 of the culture volume). Each incubation lasted 1 h and was carried out at 30 °C. The 3 supernatants were pooled and filtered (0.22 μm filters, Millipore). They represented 25 mg of proteins determined by the method of BRADFORD (1976). Lysis of cells during proteinase extraction was estimated by following the release of lactate dehydrogenase according to the method of THOMAS (1975). Total lysis observed during the 3 extractions was less than 3 %.

C. *Proteolytic activity assays*

Proteolytic activity was measured using ^{14}C methylated casein (specific activity = 43.5 mCi/mmol) prepared according to DONNELLY *et al.* (1980). Unless otherwise stated the activity was tested by mixing 100 μl of enzyme solution with 100 μl of 0.1 % ^{14}C casein in phosphate buffer pH 7.5. The incubation took place at 42 °C and the reaction was stopped by addition of 200 μl trichloroacetic acid (TCA) to 6 % final concentration. One unit of proteolytic activity (PU) was defined as the amount of enzyme yielding 1 % of total initial casein radioactivity as TCA soluble radioactivity after 10 min hydrolysis.

Aminopeptidase and dipeptidase activities were determined at 42 °C and pH 7.5 using respectively L-leucine paranitroanilide from SIGMA, St-Louis, USA and L-Gly-L-Tyr from Interchim, France according to RABIER and DESMAZEAUD (1973).

D. *Purification of the proteinase*

Preliminary purification experiments revealed that enzyme stability was improved in the presence of 10 % glycerol, 1 mM dithiothreitol and 100 mM ammonium sulfate. These 3 reagents were added to buffers used during the purification.

1. *DEAE-Trisacryl ion exchange chromatography*

The crude extract (300 ml) first dialysed at 4 °C for 24 h against 20 mM imidazole buffer pH 7.5 containing the 3 above reagents. It was then applied to

a column (1.2×14 cm) of DEAE-Trisacryl M from IBF, France that had been equilibrated with the same buffer. The enzyme activity was eluted by a linear gradient of 0-1 M NaCl.

2. *Gel chromatography on Ultrogel AcA 34*

The pooled active fractions from DEAE-Trisacryl M were concentrated by ammonium sulfate precipitation at 80 % saturation. The precipitate was separated by centrifugation and dissolved in 5 ml of 20 mM imidazole buffer pH 7.5 containing the 3 reagents required.

This fraction was applied to an Ultrogel AcA 34 (IBF, France), column (2×98 cm) equilibrated and eluted with the same buffer. Elution was carried out at a flow rate of 40 ml/h and 5 ml fractions were collected.

E. *Electrophoresis*

Electrophoresis were performed on a 1 mm thick polyacrylamide gel including a running gel (9.7 % acrylamide, 0.3 % bisacrylamide) and a stacking gel (4.85 % acrylamide, 0.15 % bisacrylamide) as described by LAEMMLI (1970). Migration was performed in a Tris-glycine buffer pH 8.8, for 2 h at 4 °C under a voltage of 25 Vcm. Protein staining was performed with Coomassie blue R 250 after fixation with 20 % trichloroacetic acid. SDS gel electrophoresis was performed as described above but buffers contained 0.1 % SDS. Before electrophoresis samples were treated with 1 % SDS and 0.1 % β-mercaptoethanol for 2 min at 100 °C.

F. *Specificity towards αs1 and β-caseins*

Bovine αs1 and β-caseins were prepared by DEAE-cellulose chromatography as described by MERCIER *et al.* (1968).

1. *Hydrolysis conditions*

Hydrolysis was performed in 0.05 M phosphate buffer pH 7.5 containing 0.1 % sodium azide (w/v). αs1 and β-casein concentration was 3 % (w/v) and the reaction mixture contained the same amount of proteolytic activity for fraction I and II. Aliquots were taken after 30 min, 2, 4 or 24 h and the reaction was stopped by lowering the pH by adding trifluoroacetic acid (TFA, Pierce, Rockford, IL, USA) to 1.1 % final concentration. The peptide fraction remained soluble, while the casein and the larger hydrolysis products were precipited.

2. *Peptide purification*

The 1.1 % TFA-soluble fraction was analysed by reversed phase HPLC with a Spectra-Physics (San José, CA, USA) apparatus. The peptides were separated on a reverse-phase MICRO BONDAPAK C18 column (4.6 mm ID×250 mm, SFCC, Gagny, France) and detected at 220 nm. The initial solvent A was 0.11 % TFA. Peptides were eluted by a linear gradient from solvent A to solvent B (acetonitrile/water/TFA 600/399/1, v/v/v) at 1.5 ml/min flow rate.

3. *Peptide identification*

The amino acid composition of the peptides was determined after acid hydrolysis under vacuum in the presence of 5.7 N HCl for 24 h at 110 °C and hydrolysates were run through an amino acid analyser (Biotronik LC 5000, Munich, FRG). The C-terminal amino acids were identified after action of carboxypeptidase A (Boehringer, Mannheim, FRG) or carboxypeptidase Y (Sigma, St-Louis, MO, USA). The N-terminal amino acids were identified after recurrent Edman degradation as described by TARR (1982).

II. Results

A. *Purification*

The proteolytic activity extracted from cells decreased with the number of extractions. It was 98.000, 41.000 and 8.500 PU, respectively, at the first, second and third extractions. The activity measured in the culture medium after centrifugation of cells represented 120.000 PU, i.e. was similar to that extracted from cell-wall. This fraction released into the medium was not studied.

During DEAE-trisacryl chromatography a single peak of activity followed by a tail was eluted at about 0.35 M. NaCl. Proteins from this peak were concentrated by precipitation with ammonium sulfate and then subjected to chromatography on an Ultrogel AcA 34 column. A peak of activity was eluted in the exclusion volume of the column which corresponded to proteins with a molecular weight of more than 350.000. This peak was followed by a large tail extending to the separation volume of the column (fig. 1). Proteins of the excluded peak

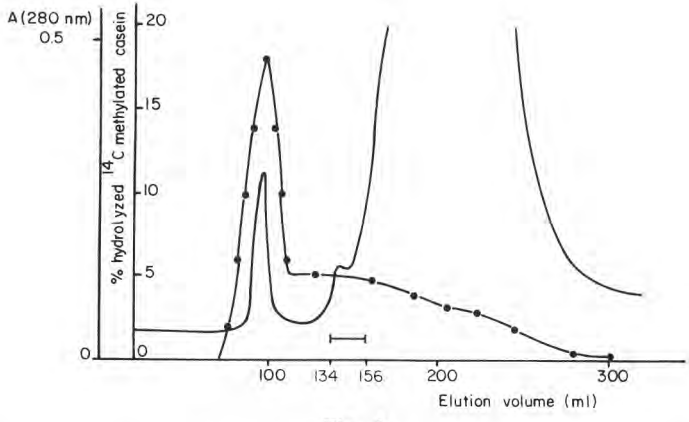

Fig. 1

Gel filtration of the cell wall extract from L. helveticus *CNRZ 303 on Ultrogel AcA 34.*
—— *A 280 nm*
•—• *Proteolytic activity (% hydrolysis of ^{14}C methylated casein).*
Filtration sur Ultrogel AcA 34 de l'extrait de paroi du L. helveticus *CNRZ 303.*
—— *A 280 nm*
•—• *Activité protéolytique (% d'hydrolyse de la ^{14}C caséine).*

were pooled and called fraction I. Further purification trials resulted in loss of activity due to high enzyme instability. Consequently enzyme properties were studied on fraction I. In order to compare the properties of the enzyme activity present in the tail with that of the main fraction, fractions 134-156 were pooled and called fraction II. Fractions I and II were devoid of aminopeptidase and dipeptidase activities. Purification trials were also made from cells grown on low-heat milk kept at pH 6.0 ± 0.1. Results obtained after DEAE-Trisacryl and Ultrogel AcA 34 chromatography were similar to those obtained after culture on MRS. The major peak observed on Ultrogel column was excluded from the gel and also was followed by a tail in the volume of separation of the column. After electrophoresis in the presence or absence of SDS, fraction I was identified by the presence of a diffuse tail without any specific band. Fraction II exhibited a single protein band.

B. *General properties*

1. *Effect of pH and temperature*

The optimum pH of activity of fraction I on ^{14}C-labelled casein was 7.5-8.0 and the optimum temperature 42 °C. Fraction II activity required the same optima.

2. *Effect of proteinase inhibitors* (table 1)

Fraction I was inhibited by diisopropylfluorophosphate (DFP) and by phenyl methane sulfonyl fluoride (PMSF) which are the specific inhibitors of serine pro-

TABLE 1

Effects of protease inhibitors. The enzyme was pre-incubated 30 min at 22 °C with inhibitor in 0.05 M Tris-HCl, pH 7.5. The remaining proteolytic activity of protease — fractions I and II was measured on methylated ^{14}C casein at 42 °C in the same buffer and was expressed as a percentage of the control

Effets des inhibiteurs de protéases. L'enzyme était pré-incubée avec l'inhibiteur pendant 30 mn à 22 °C en Tris-HCl 0,05 M pH 7,5. L'activité protéolytique de la protéase — fractions I et II était mesurée sur la ^{14}C caséine méthylée, à 42 °C dans le même tampon et exprimée en pourcentage du témoin

	Concentration mM	Relative activity Fraction I	Relative activity Fraction II
Control	—	100	100
EDTA	1.0	92	100
Parachloromercuribenzoate	1.0	100	95
Pepstatin	1.0	100	94
4 amino benzamidine	1.0	91	83
Phenyl methane sulfonyl fluoride	1.0	11	17
Diisopropylfluorophosphate	1.0	4	0
N-Tosylphenylalanine chloromethylketone	1.0	3.5	0
α_2-macroglobulin	0.1 mg/ml	96	100

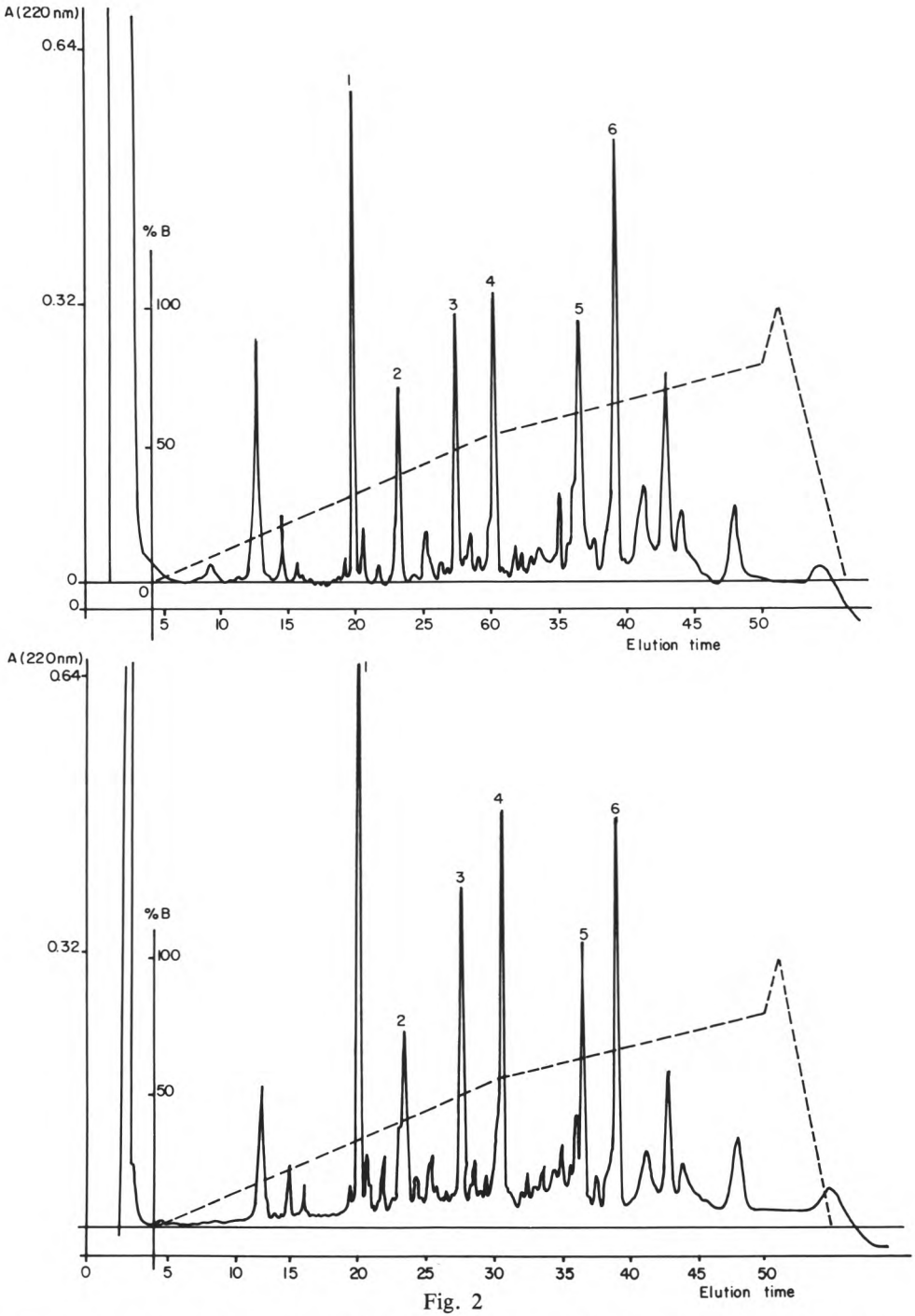

Fig. 2

Separation by reverse phase HPLC of the peptides from the 0.11 % TFA — soluble fraction obtained after 4 h of hydrolysis of β-casein by the cell wall proteinase of L. Helveticus. *a : fraction I, b : fraction II.*

Séparation par HPLC sur phase inverse de peptides solubles en TFA 0,11 % obtenus après 4 h d'hydrolyse de la caséine β par la protéase de paroi du L. helveticus. *a : fraction I, b : fraction II.*

TABLE 2

Amino acid composition and determination of the N- and C-terminal amino acids of the peptides corresponding to peaks 1-6 from β-casein hydrolysate.
Composition en acides aminés et détermination des acides aminés N- et C- terminaux des peptides correspondants aux pics 1-6 de la caséine β hydrolysée.

	1	2	3	4	5	6
Asx	—	—	1.2 (1)	—	—	—
Thr	—	—	—	—	—	—
Ser	—	—	—	—	—	—
Glx	1.1 (1)	2.7 (3)	1.2 (1)	1.9 (2)	1.8 (2)	2.0 (2)
Pro	2.4 (2)	—	—	3.3 (4)	3.6 (4)	4.3 (4)
Gly	—	—	—	—	1.8 (2)	2.9 (2)
Ala	0.9 (1)	—	0.9 (1)	—	—	—
Val	1.1 (1)	—	—	1.5 (1)	2.5 (3)	2.5 (3)
Met	—	—	0.6 (1)	0.5 (1)	—	—
Ile	—	—	0.9 (1)	—	1.0 (2)	0.8 (2)
Leu	—	1.7 (2)	—	—	1.0 (1)	2.5 (3)
Tyr	1.0 (1)	—	—	1.0 (1)	0.8 (1)	0.6 (1)
Phe	—	—	1.0 (1)	1.7 (2)	1.0 (1)	1.0 (1)
His	—	—	—	1.0 (1)	—	—
Lys	1.0 (1)	—	—	1.4 (2)	—	—
Arg	—	1.0 (1)	1.0 (1)	—	0.7 (1)	0.8 (1)
N-terminal sequence	Lys-Ala-Val	Arg-Glu	X-Asp-Met	X-Lys-X Met	Tyr	Leu-Leu-Tyr
C-terminal sequence CPY	Gln, Pro, Tyr					
C-terminal sequence CPA	N.R.	Leu, Glu	Phe, Ala	Phe	Val, Ile	Val, Ile
Peptide identified	176-182	1-6	183-190	106-119	193-209	191-209
% of hydrolyse	8	11	11	7	8	9

N.R. = no reaction

teinases. It was also inactivated by N-Tosylphenylalanine chloromethylketone (TPCK) (chymotrypsin inhibitor), but was rather insensitive to the action of p-aminobenzamidine which inhibits serine proteinases whose specificity is close to that of trypsin. The conventional inhibitors of thiol proteinases parachloromercuribenzoate (PCMB), metalloproteinases (EDTA) or aspartyl proteinases (pepstatin) were ineffective. The behaviour of fraction II towards these different inhibitors was similar to that of fraction I (table 1).

3. *Specificity towards αs1 and β-caseins*

Chromatograms of TFA soluble fractions obtained after hydrolysis of β-casein by fraction I or fraction II exhibited the same retention time, height or kinetics of appearance of the various peaks (fig. 2a and b). After 30 min hydrolysis, only peaks 1, 3 and 6 were present in measurable amounts. After 4 h 8 major peaks were detected and chromatograms observed after 24 h were only slightly different from those obtained after 4 h incubation. Peptides corresponding to the major peaks 1 to 6 were identified and localized in the casein sequence (table 2 and fig. 3). Their size varied from 6 to 19 residues. Four of these peptides came from the hydrophobic C-terminal part and one of them from the hydrophilic N-terminal part of casein. After 4 h hydrolysis the yield of different peptides obtained corresponded to the hydrolysis of 7 to 11 % of the initial casein.

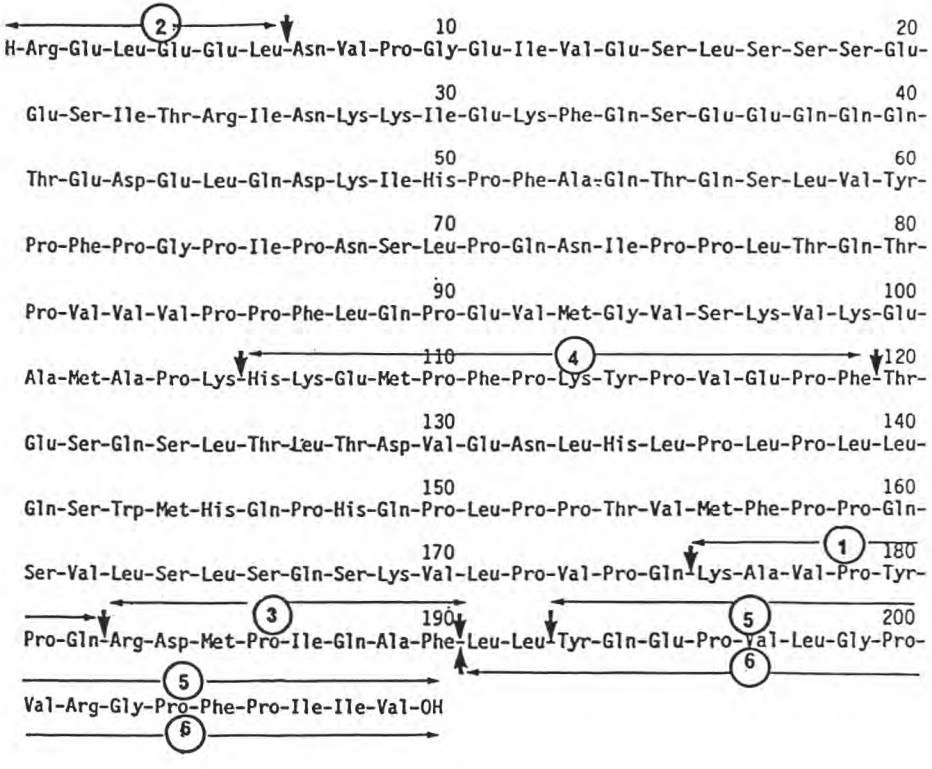

Fig. 3

Sites of hydrolysis of β-casein by the cell wall proteinase of L. helveticus — *fractions I and II — and localization of peptides 1-6.*
Amino-acid sequence of bovine β-casein was determined by Ribadeau-Dumas et al. *(1972) and corrections of* Bayev et al. *(1987) were taken into account.*
Sites d'hydrolyse de la caséine β par la protéase de paroi du L. helveticus — *fractions I et II — et localisation des peptides 1-6.*
La séquence en acides aminés de la caséine β bovine a été déterminée par Ribadeau-Dumas et al. *(1972) et corrigée par* Bayev *et al.* (1987).

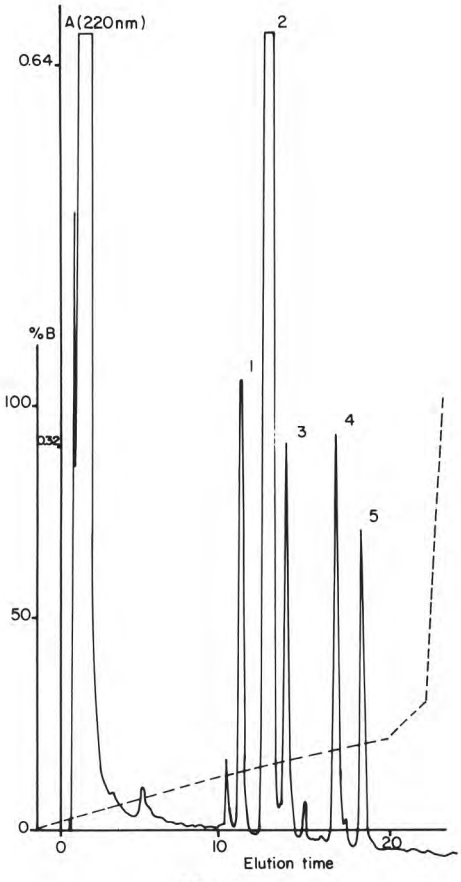

Fig. 4

Separation by reverse phase HPLC of the peptides from the 0.11 % TFA soluble fraction obtained after 4 h hydrolysis αs1 casein by the cell wall proteinase of L. helveticus *— fraction I.*
Séparation par HPLC sur phase inverse de peptides solubles en TFA 0,11 %, obtenus après 4 h d'hydrolyse de la caséine αs1 par la protéase de paroi du L. helveticus *— fraction I.*

After 4 h hydrolysis αs1-casein by fraction I or fraction II, only 4 major peaks were obtained from the TFA-soluble fraction (fig. 4). After 24 h, the chromatograms did not reveal any new peak. Peptides 2 and 3 exhibited the same composition (table 3) and corresponded to N-terminal peptide 1-9. Peptides 4 and 5 had also the same composition and corresponded to peptide 1-6. The presence of two peaks for a same peptide might be attributed to a partial blockage of the N-terminal amino acid (Arg) due to the presence of carbamates. One of the two cleaved bonds (Gln-Gly) was rapidly hydrolysed (48 % yield after 4 h incubation), while the other one (Ile-Lys) more slowly (14 % yield).

TABLE 3

Amino acid composition and determination of the N- and C-terminal amino acids of the peptides corresponding to peaks 2-5 from αs1-casein hydrolysate
Composition en acides aminés et détermination des acides aminés N- et C- terminaux des peptides correspondants aux pics 2-5 de la caséine αs1 hydrolysée

	2	3	4	5
Asx	—	—	—	—
Thr	—	—	—	—
Ser	—	—	—	—
Glx	0.4 (1)	0.9 (1)	—	—
Pro	2.1 (2)	2.0 (2)	2.1 (2)	1.9 (2)
Gly	—	—	—	—
Ala	—	—	—	—
Val	—	—	—	—
Met	—	—	—	—
Ile	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)
Leu	—	—	—	—
Tyr	—	—	—	—
Phe	—	—	—	—
His	2.0 (2)	2.3 (2)	1.1 (1)	1.2 (1)
Lys	2.0 (2)	2.2 (2)	1.0 (1)	1.1 (1)
Arg	1.2 (1)	1.0 (1)	1.2 (1)	1.0 (1)
N-terminal sequence	Arg-Pro	—	—	—
C-terminal sequence CPY	Gln	Gln	Ile	Ile
C-terminal sequence CPA	N.R.	—	—	—
Peptide identified	1-9	1-9	1-6	1-6
% of hydrolyse	45	3.5	8	5.8

N.R. = no reaction

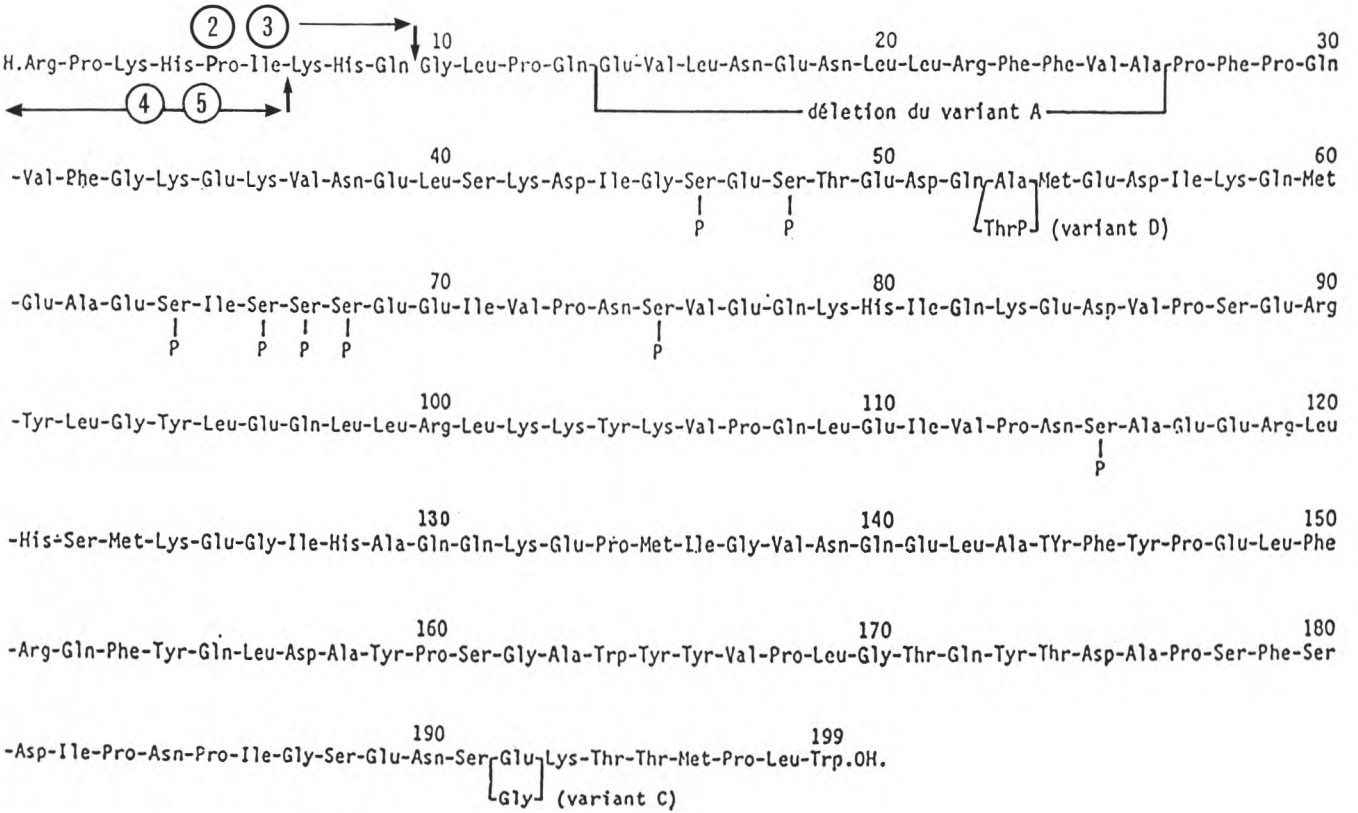

Fig. 5

Sites of hydrolysis of αs1-casein by the cell wall proteinase of L. helveticus *— fraction I — and localization of peptides 2-5.*

Sites d'hydrolyse de la caséine αs1 par la protéase de paroi du L. helveticus *— fraction I — et localisation des peptides 2-5.*

III. Discussion

The extraction process used for recovering the proteolytic activity was mild and it has already been observed that in the case of mesophilic streptococci it allowed the liberation of cell-wall enzymes without altering cells (MILLS and THOMAS, 1978). In the case of *L. helveticus* the level of LDH activity recovered in crude extracts was also very small (3 %) indicating that the liberation of intracellular enzymes is very limited. This suggests that the detected proteolytic activity is located in the cell-wall, as already observed for other strains of thermophilic and mesophilic lactobacilli (EL SODA *et al.*, 1986 ; EZZAT *et al.*, 1985 ; VESCOVO and BOTTAZZI, 1979). The amount of enzyme recovered from cells decreased with the number of extractions. Such a cascade-like liberation of cell-wall proteinases has also been observed in *S. cremoris* (EXTERKATE and DE VEER, 1985), *S. lactis* (MONNET *et al.*, 1987) and *L. bulgaricus* (EZZAT *et al.*, 1987). The amount of activity recovered in the culture medium (120.000 PU) was similar to that extracted from cells probably because of a marked liberation of the enzyme into the medium during culture. This is in agreement with the significant decrease in cell-wall proteinase observed in *L. helveticus* after the exponential phase by EZZAT *et al.* (1985).

Comparison of the properties of the major fraction (fraction I) after chromatography on Ultrogel AcA 34 with a fraction (fraction II) collected from the tail suggests that they represent the same enzyme and that there is only one major cell-wall proteinase in *L. helveticus* CNRZ 303. Indeed, the two preparations displayed the same behaviour towards pH, temperature and inhibitors and had also the same specificity towards caseins. It is quite unlikely that two different proteinases exhibit exactly the same specificity towards such a complex substrate as β-casein.

The action of proteinase specific inhibitors clearly showed that it is a serine proteinase. Moreover, their alkaline optimum pH (7.5-8.0) corresponded to the properties of this enzyme category. Cell-wall proteinases from mesophilic streptococci are also serine proteinases, but their more acid optimum pH (5.5-6.0) is more favourable to casein hydrolysis during acidification in milk-grown cells.

It is difficult to determine the rules governing proteinase specificity. The amino acid in position P1 (according to BERGER and SCHECHTER, 1970) may be either hydrophobic (Leu), aromatic (Phe), basic (Lys) or hydrophilic (Gln). Likewise, the other positions close to the cleaved bond are not characteristic of a given amino acid. The first bonds of β-casein to be cleaved were located in the highly hydrophobic C-terminal part of this molecule. Nevertheless, this enzyme is able to cleave bonds in hydrophilic parts since N-terminal peptides of αs1 and β-caseins were liberated. Therefore, the enzyme of *L. helveticus* seems to have a large specificity. The cell-wall proteinase from *S. lactis* also exhibits a wide specificity and the liberated peptides are located in the C-terminal part of β-casein (MONNET *et al.*, 1986).

Liberated peptides have a too large size (from 6 to 19 residues) to be transported through the plasma membrane according to LAW and KOLSTAD (1983). It is most likely that membrane-bound peptidases previously degrade them into free amino acids and peptides small enough to be transported through the membrane.

Peptides issued from the C-terminal part of β-casein are probably bitter because of their hydrophobicity, size and amino acid composition. Production of bitter peptides has also been observed after β-casein incubation in the presence of cell-wall from lactic acid streptococci by VISSER *et al.* (1983).

The genetic determinants of the studied proteinase do not seem to be plasmid linked since no plasmid could be detected with strain CNRZ 303 (MERCENIER, personal communication). However, the relationship between casein hydrolysis and the presence of a plasmid has been suggested for *Lactobacillus* HLM 1 by MORELLI *et al.* (1986).

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