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Partial characterization and comparison of cell wall proteinases from 5 strains of *Streptococcus lactis*

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Summary

Cell wall proteinases from 5 strains of *Streptococcus lactis* including one *S. lactis* subsp. *diacetylactis* were compared and partially characterized. They shared some common enzymatic features : only one activity optimum at 30-40°C, at pH 5.5-6 on casein and 4-4.5 on haemoglobin. Crude cell wall extracts from the *S. lactis* strains were fully inhibited by DFP, but this inhibition was only partial for the *S. lactis* subsp. *diacetylactis* strain. Electrophoregrams of α_{s1} , β and K-casein hydrolysates obtained with the cell-wall extracts of these strains were quite similar for each protein substrate. β -casein was preferentially broken down; α_{s1} and K-caseins were hydrolysed to a lesser extent.

The production of proteolytic cell-wall activity was highly affected by the culture medium. The higher the peptide and/or calcium content of the medium, the lower the release of cell-wall proteinase.

Key words : *Streptococcus lactis* - Cell wall - Proteinase.

Résumé

Caractérisation partielle et comparaison des protéases de la paroi de 5 souches de Streptococcus lactis

Les protéases de paroi de 5 souches de *Streptococcus lactis* dont une de *S. lactis* subsp. *diacetylactis* sont comparées et partiellement caractérisées. Pour les cinq souches, un seul optimum d'activité est mis en évidence à 30-40 °C, à pH 5,5-6 sur la caséine et 4-4,5 sur l'hémoglobine. Les extraits bruts de paroi des souches de *S. lactis* sont totalement inhibés par le DFP, mais cette inhibition n'est que partielle pour la souche de *S. lactis* subsp. *diacetylactis*. Les schémas d'électrophorèses des hydrolysats des caséines α_{s1} , β et K par les extraits de paroi de ces souches sont très similaires pour chacun des substrats. La caséine β est préférentiellement hydrolysée ; les caséines α_{s1} et K sont aussi attaquées mais plus faiblement.

Le milieu de culture des cellules influe fortement sur la production d'activité protéolytique de paroi. Plus la concentration en peptides et/ou en calcium du milieu est élevée, plus le relargage des protéases de paroi est faible.

Mots clés : *Streptococcus lactis* - Paroi - Protéase.

Introduction

The existence of a proteolytic activity in the cell-wall of lactic acid bacteria was evidenced in *S. lactis* in 1974 by THOMAS *et al.* This proteolytic activity is essential for cell growth as it releases peptides used as such by the cell or after hydrolysis by cell-wall or membrane peptidases (EXTERKATE, 1976; THOMAS and MILLS, 1981). Furthermore, it was found that cell-wall proteinases play an important role in cheese technology because they are able to release bitter peptides (MILLS and THOMAS, 1980).

Several studies on *S. cremoris* and *S. lactis* strains have revealed the existence of a complex enzyme system. Using different methods, up to 4 distinct components have been described in this cell-wall associated proteolytic system (HUGENHOLTZ *et al.*, 1984; CLIFFE and LAW, 1985). From activity measurements at different temperature and pH values, EXTERKATE (1975, 1976) evidenced three different proteinases in crude cell-wall extracts of milk-grown *S. cremoris*. The proteinase equipments of the tested strains were different, including one, two or three enzymes. Such a comparison has never been performed in the case of *S. lactis*. This paper reports the characterization of the cell-wall associated proteinases of 5 *S. lactis* strains including one strain of *S. lactis* subsp. *diacetylactis*.

The influence of culture medium composition and physiological age of cells on the production of these proteinases was also studied.

I. Material and methods

A. Cultures

Strains of *S. lactis* NCDO 606, NCDO 763 and NCDO 823 were obtained from the National Collection of Dairy organisms (Reading, Great Britain). Strains of *S. lactis* CNRZ 261 and CNRZ 377 came from the collection of the Research Center of INRA (Jouy-en-Josas, France).

Cells were grown in a low-heat milk (NILAC, NIZO, Netherlands) as described by MILLS and THOMAS (1978) or in M17 medium (TERZAGHI and SANDINE, 1975), or in a commercial UHT milk. Cell growth at 30 °C was assessed by pH and optical density (O.D.) measurements. Optical density was determined in conditions which allow solubilisation of casein micelles and prevent bacterial cells aggregation: 1/10 dilution of milk in 0.2 % EDTA, pH 12 (THOMAS and TURNER, 1977). Bacteria were harvested by centrifugation at the end of the exponential phase and washed twice in 0.05 M sodium glycerophosphate buffer, pH 7, at 4 °C in the presence of 20 mM CaCl₂; calcium and a low temperature prevented the release of cell-wall proteinases (MILLS and THOMAS, 1978).

B. Proteinase extraction

The centrifuged cells were resuspended three successive times in 50 mM triethanolamine buffer, pH 7, containing 0.2 mM 1-6 diphosphate fructose (1/10 of the culture volume). These buffer conditions were required for estimating lysis by lactate dehydrogenase (LDH) assay according to the method of THOMAS (1975). Each incubation lasted 1 h and was carried out at 25°C in the absence of calcium. The three crude extracts were then filtered (0.22 μ m filters, Millipore, Bedford, U.S.A.). Protein determination were carried out by the method of Bradford (1976); protein content of the crude extract being complex and unknown, an average of the values obtained with ovalbumin and serumalbumin was arbitrarily chosen as standard.

C. Proteolytic activity assay

Proteolytic activity was measured on 14 C labeled casein or haemoglobin according to the method of DONNELLY *et al.* (1980). The specific activity of these substrates was 43.5 and 80 mCi/mM, respectively and the final substrate concentration was 0.05 %. When nothing else is mentioned, the incubation took place at pH 6.0 and 35°C and the reaction was stopped by addition of TCA to 6 %, final concentration. The release of TCA-soluble radioactivity was linear with time until it reaches 10 % of the initial radioactivity of the labeled casein substrate. One proteolytic unit was the enzyme activity which released 1 % of the initial radioactivity in the TCA-soluble fraction after 10 min incubation. For each experiment, it has been checked that no spontaneous hydrolysis of casein (without any enzymic extract) occurred.

D. Electrophoresis

Enzymatic digests of whole or purified caseins were studied by electrophoresis on agarose acrylamide gels according to URIEL's method (1966) modified by GRIPON *et al.* (1975).

II. Results and discussion

A. Preparation of crude cell-wall extracts

The five *S. lactis* were grown on low-heat milk. The growth curve shapes were the same for the different strains, but the amounts of proteinase released per O.D. unit and thus per cell were clearly different (table I). The amounts of cell-wall associated proteinases produced by strains CNRZ 377, NCDO 606 and NCDO 763 were equivalent while NCDO 823 (a *S. lactis* subsp. *diacetylactis* strain) seemed to release more activity and CNRZ 261 less.

The extent of cell lysis occurring during extraction was shown to be below 1 % by LDH determination in the extracts.

TABLE I

Growth and cell wall proteinase production parameters of five strains of S. lactis

Paramètres rendant compte de la croissance et de la production de protéases de paroi de cinq souches de S. lactis

STRAIN PARAMETERS	CNRZ 261	CNRZ 377	NCDO 606	NCDO 763	NCDO 823
INITIAL pH	7.26	7.02	7.15	7.15	7.00
FINAL pH	6.37	5.86	6.03	6.27	6.04
Δ pH	0.89	1.16	1.12	0.88	0.96
INITIAL O.D.	0.14	0.17	0.09	0.16	0.17
FINAL O.D.	0.45	0.57	0.44	0.54	0.62
Δ O.D.	0.31	0.4	0.35	0.38	0.45
CULTURE TIME (HOURS)	4.5	6	6	5.5	4.75
TOTAL ACTIVITY UNITS	14815	34285	26493	33600	56160
ACTIVITY UNITS/ Δ O.D.	47790	85712	75694	88421	124800

B. Characterization of cell-wall crude extracts

For the five tested extracts, only one activity optimum was detected, at pH 5.5 - 6 on casein and 4 - 4.5 on haemoglobin (fig. 1 and 2). Two of the three cell-wall proteinases found by EXTERKATE (1975, 1976) in *S. cremoris* crude extracts were also more active at acid pH. This feature was confirmed for two cell wall proteinases purified from *S. cremoris* (GEIS *et al.*, 1985) and *S. lactis* (MONNET *et al.*, 1986), respectively. One optimal temperature of action was evidenced between 30°C and 40°C for all the five strains (fig. 3).

The effect of different inhibitors on the crude cell-wall extracts was tested. Serine proteinase inhibitors proved to be particularly efficient. However, the *S. diacetylactis* strains NCDO 823 was only partially inhibited by diisopropyl fluorophosphate (DFP) while a total inhibition was observed in the four *S. lactis* strains. Copper (II) was fully inhibitory on the five extracts. The effect of other compounds was less marked and variable according to the extracts (table II). THOMAS *et al.* (1974) only tested some inhibitors on *S. lactis* cell wall crude extracts and found an almost total inhibition by EDTA suggesting the presence of a different enzyme. On the other hand, an inhibition similar to that we observed in the presence of DFP was obtained with purified proteinases (GEIS *et al.*, 1985 ; MONNET *et al.*, 1986).

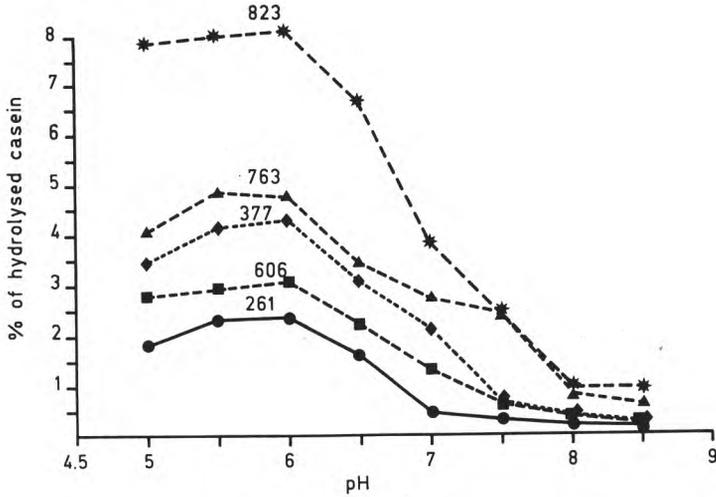


Fig. 1

pH optima at 35° C for the proteolytic activity of cell wall crude extracts from five strains of S. lactis using ¹⁴C-casein as substrate.

pH optima d'activité protéolytique des extraits bruts de paroi des cinq souches de S. lactis en utilisant la ¹⁴C-caséine comme substrat.

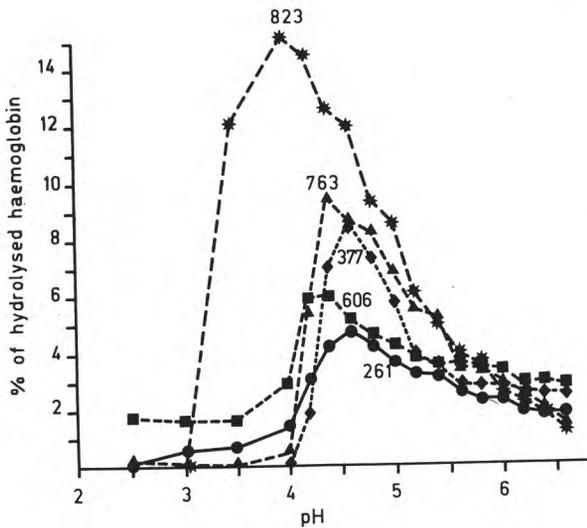


Fig. 2

pH optima at 35° C for the proteolytic activity of cell wall crude extracts from five strains of S. lactis using ¹⁴C-haemoglobin as substrate.

pH optima d'activité protéolytique des extraits bruts de paroi des cinq souches de S. lactis déterminés à 35° C en utilisant de la ¹⁴C-hémoglobine comme substrat.

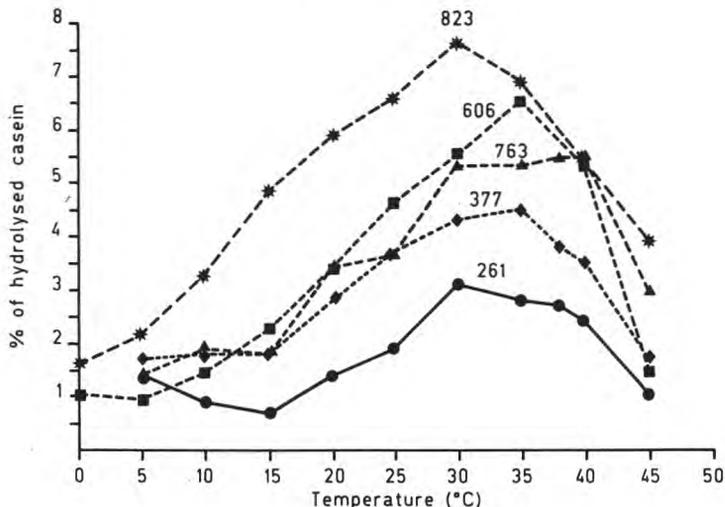


Fig. 3

Temperature optima at pH 6.0 for the proteolytic activity of cell wall crude extracts from five strains of S. lactis using ^{14}C -casein as substrate.

Température optima de l'activité protéolytique des extraits bruts de paroi des cinq souches de S. lactis déterminés à pH 6 en utilisant de la ^{14}C -caséine comme substrat.

The action of the 5 cell-wall extracts on the main caseins (α_{s1} , β , K) was analysed by electrophoresis after 2 h digestion at pH 6 and 35°C (fig. 4). β -casein appeared to be the preferential substrate for all cell-wall extracts. This was also observed by EXTERKATE and DE VEER (1985) using a partially purified *S. cremoris* extract. The reduction of the β -casein band was marked and many breakdown products were visible; α_{s1} and K caseins were digested to a lower extent.

The electrophoretic patterns obtained with the 5 extracts were quite similar for each substrate. Patterns obtained with strains CNRZ 377, NCDO 606 and NCDO 763 were almost identical while those obtained with strains CNRZ 261 and NCDO 823 were somewhat different. In the case of strain CNRZ 261, this could be due to a low activity of the extract, leading to primary degradation products which may be degraded further by more active extracts. If this is the case, it is noteworthy that products with a low electrophoretic mobility proceeding from β -casein breakdown appeared later than the others. Two hypotheses may be put forward in the case of the extract from strain *S. lactis* subsp. *diacetylactis* NCDO 823, i.e. the hydrolysis was either stronger or slightly different.

From the results mentioned above it may be assumed that the 4 *S. lactis* strains under study have similar cell-wall associated proteolytic systems. Contrary to EXTERKATE studying *S. cremoris* (1975 and 1976), we have only found one single proteolytic activity in each of the tested strains. The homologies we have observed between the various extracts may be compared to the similitude

TABLE II

Effect of several inhibitors (1 mM final concentration) on the proteolytic activity of cell wall crude extracts from five stains of *S. lactis* using ^{14}C -casein as substrate, at pH 6.0 and 35° C

Action de différents inhibiteurs (concentration finale 1 mM) sur l'activité protéolytique des extraits bruts de paroi de 5 souches de *S. lactis* déterminé en utilisant la ^{14}C -caséine comme substrat, à pH 6 et à 35° C

ND : inhibition < 10 %.

DAN : diazoacetyl-norleucine methyl ester ; EDTA : ethylene diamine tetraacetic acid ; MIAA : mono iodoacetic acid ; PCMB : p-chloromercuribenzoic acid ; DTT : dithiothreitol ; PMSF : phenylmethylsulfonyl fluoride ; DFP : diisopropylfluorophosphate ; TLCK : tosyl-lysine chloromethyl ketone ; TPCK : tosyl-phenylalanine chloromethyl ketone.

ND : inhibition < 10 %.

DAN : diazoacétyl-norleucine méthyl ester ; EDTA : acide éthylène diamine tétraacétique ; MIAA : acide mono iodoacétique ; PCMB : acide p-chloromercuribenzoïque ; DTT : dithiothréitol ; PMSF : phénylméthylsulfonyl fluorure ; DFP : diisopropylfluorophosphate ; TLCK : tosyl-lysine chlorométhyl cétone ; TPCK : tosyl-phénylalanine chlorométhyl cétone.

	261	377	606	763	823
DAN	ND	ND	ND	ND	ND
PEPSTATIN	ND	ND	ND	ND	ND
EDTA 1 mM pH 6	ND	10 %	ND	ND	ND
EDTA 10 mM pH 6	ND	ND	ND	ND	ND
EDTA 1 mM pH 5	38 %	30 %	-	ND	ND
EDTA 10 mM pH 5	61 %	35 %	-	ND	ND
O-PHENANTROLINE	ND	ND	ND	ND	ND
PHOSPHORAMIDON	20 %	ND	ND	ND	ND
MIAA	ND	ND	25 %	11 %	ND
PCMB	ND	ND	25 %	18 %	20 %
DTT	ND	ND	ND	28 %	ND
β-MERCAPTOETHANOL	21 %	ND	ND	ND	ND
PMSF	75 %	71 %	65 %	61 %	46 %
DFP	85 %	95 %	92 %	85 %	37 %
TLCK	20 %	ND	18 %	18 %	ND
TPCK	ND	ND	20 %	ND	ND
LEUPEPTIN	ND	ND	ND	ND	25 %
α ₂ MACROGLOBULIN	ND	ND	ND	ND	ND
Cu ⁺⁺ (CUSO ₄) 1 mM	80 %	95 %	96 %	93 %	91 %
Ca ⁺⁺ (CaCl ₂) 1 mM	ND	ND	ND	ND	ND

of casein breakdown patterns obtained in experimental Gouda cheeses which did not contain any active rennet and were manufactured with different *S. cremoris* strains (VISSER and DE GROOT-MOSTERT, 1977). The *S. lactis* subsp. *diacetylactis* NCDO 823 extract had many points in common with those of the *S. lactis* strains. However its pH optimum of activity on haemoglobin was slightly lower and the inhibition by DFP only partial suggesting the existence of some differences for this strain which does not belong to the same subspecies.

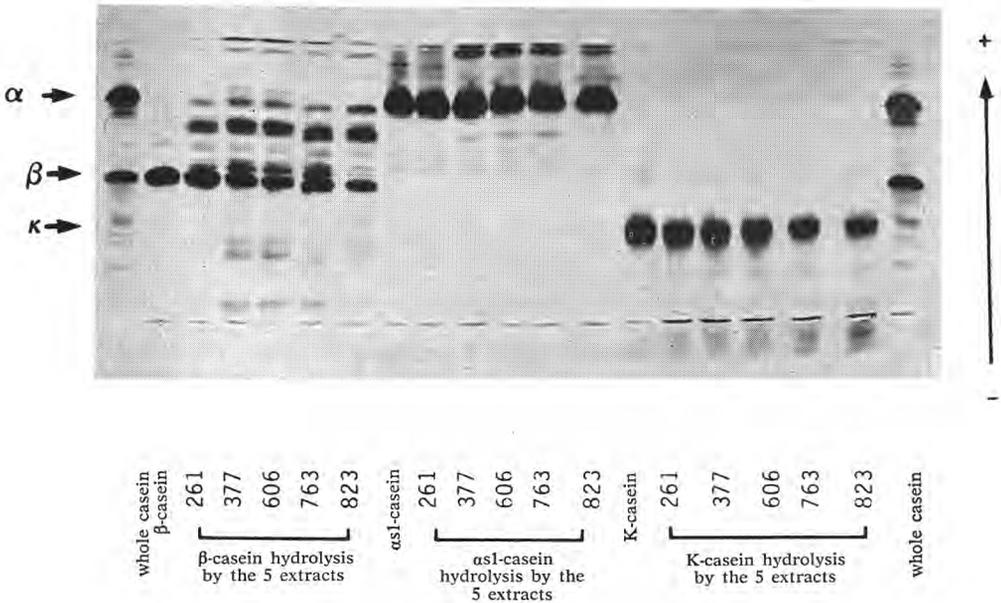


Fig. 4

Acrylamide-agarose gel electrophoresis of α_{S1} , β and K-casein digests obtained with the five cell wall crude extracts. Digestions were carried out at 35° C, pH 6.0 for 2 hours.

Electrophorèses en gel d'acrylamide-agarose d'hydrolysats des caséines α_{S1} , β et K par les cinq extraits bruts de paroi. Les hydrolyses ont été effectuées à 35° C, pH 6 pendant 2 h.

TABLE III

Proteolytic activities of 3 successive extracts obtained from *S. lactis* NCDO 763 grown on 3 media : low-heat milk, UHT milk and M17. RUHT and RM17 values are the ratio between the proteolytic activity units obtained from the cells harvested from these media and those obtained from cells harvested from low-heat milk

Activités protéolytiques contenues dans les 3 extractions successives obtenues à partir de cellules de *S. lactis* NCDO 763 cultivées sur 3 milieux : lait « low-heat », lait UHT et milieu M17. RUHT et RM17 sont les rapports entre les unités d'activité protéolytique obtenues à partir de cellules cultivées sur lait UHT et milieu M17 et celles obtenues à partir de cellules cultivées sur lait « low-heat »

Extraction number	LOW-HEAT MILK	U. H. T. MILK	R. U. H. T.	M17 + Ca ⁺⁺ 5mM Medium	RM17
1	1 U/10 ⁹ ç	0,4 U/10 ⁹ ç	2,5	0,3 U/10 ⁹ ç	3,3
2	2,6 U/10 ⁹ ç	1,4 U/10 ⁹ ç	1,8	0,5 U/10 ⁹ ç	5,2
3	1,8 U/10 ⁹ ç	1,5 U/10 ⁹ ç	1,2	0,2 U/10 ⁹ ç	9
\bar{M}	1,7 U/10 ⁹ ç	1,1 U/10 ⁹ ç	1,8	0,3 U/10 ⁹ ç	5,8

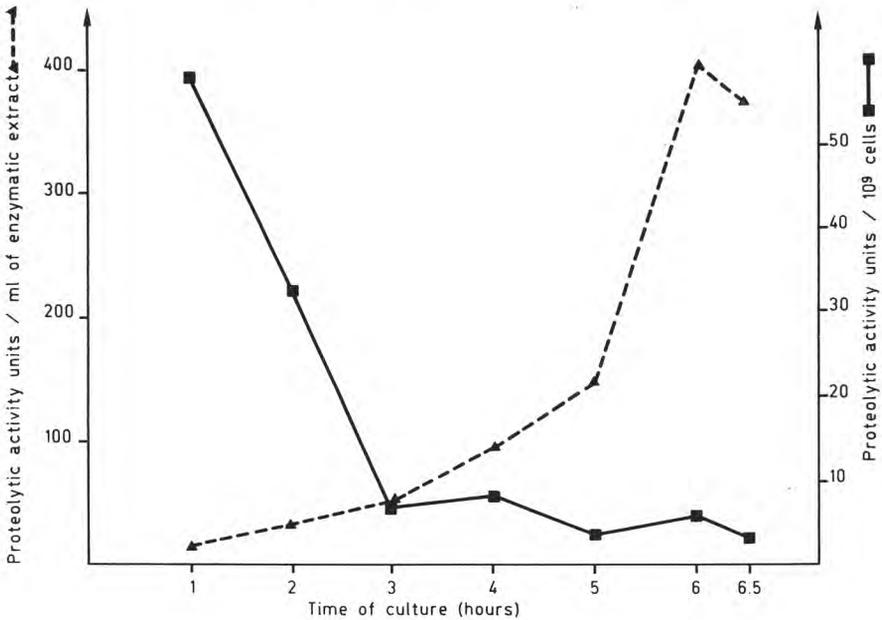


Fig. 5

Production of cell wall proteolytic activity by S. lactis NCDO 763 cells during their growth on low-heat milk

- ▲ ——— ▲ *total proteolytic activity units.*
- ——— ■ *proteolytic activity units/10⁹ cells.*

Production de l'activité protéolytique de paroi par les cellules de S. lactis NCDO 763 pendant leur croissance sur lait « low-heat »

- ▲ ——— ▲ *unités d'activité protéolytique totales.*
- ——— ■ *unités d'activité protéolytique/10⁹ cellules.*

C. Proteinase production

The amount of cell-wall proteinase produced on milk being rather small, one of the strains (*S. lactis* NCDO 763) was grown on two other media easier for high volume culture and allowing a good cell growth: UHT milk, and M17 medium admixed with 5 mM CaCl₂, required for the accumulation of proteinases in the cell-wall (EXTERKATE, 1979). The use of a higher calcium concentration led to precipitation of the medium.

Table III shows the proteolytic activity recovered during 3 successive extractions from cells grown on the 3 media. RUTH and RM17 ratios show that cells grown on UHT milk produced almost 2-fold less enzyme than cells grown on low-heat milk while the amount produced on M17 was 5 times lower.

Two factors may account for these differences:

— On one hand, the amount of peptides in the medium; it was indeed noticed that the proteinase production decreased when cells were grown on a

medium including more peptides than the low-heat milk used as a control. The high peptide content of M17 and the fact that commercial UHT milk contains at least 8 % additional non protein nitrogen (due to the heat treatment) as compared to low-heat milk (ALAIS, 1984) may explain our results. Our observations are in keeping with the hypothesis of EXTERKATE (1985) concerning the inhibition of cell-wall proteinase synthesis in the case of an excessive supply of amino acids and peptides in the culture medium.

— On the other hand, the calcium concentration of the medium which was only 5 mM in M17 whereas it was 32 mM in the milk.

The influence of physiological cell age on cell-wall proteinase production was investigated during bacterial growth on milk (fig. 5). It appeared that the proteolytic activity released by the cells increased with culture time. However, it remained lower than 40 units/ml of enzymatic extract during the first 3 h whereafter it rapidly increased to 400 units/ml of extract after 6 h of culture. On the other hand, the shape of the curve representing the number of proteolytic units released per 10^9 cells as a function of time was different in these 2 periods. Thus, during the first 3 h the values decreased from 57 to 7 units/ 10^9 cells whereafter they became stabilised between 3 and 8 units/ 10^9 cells. It may be assumed that during early culture the cells have to produce proteinases capable of releasing enough peptides for their growth requirements, the medium (milk) being poor in peptides. The bacterial multiplication leading to a higher peptide production, the amount of synthesised proteinases will then be adjusted according to the number of bacteria and their nitrogen requirements.

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