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Cell-wall associated peptide hydrolase and esterase activities in several cheese-related bacteria

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Cell-wall associated proteinases, peptidases and esterases of *Lactobacillus helveticus*, *Pediococcus* sp., *Leuconostoc mesenteroides* ssp. *mesenteroides*, *Brevibacterium linens*, *Propionibacterium acidipropionici* and *Bifidobacterium infantis* were assayed. Proteolytic activity was measured using C14-labelled casein. Aminopeptidase, dipeptidase and esterase activities were measured using chromogenic substrates. All the cheese related bacteria tested exhibited cell-wall proteinase activities with highest being detected in *Leuconostoc mesenteroides* ssp. *mesenteroides*, *Pediococcus* sp. and *Brevibacterium linens*. *Lactobacillus helveticus* and *Brevibacterium linens* exhibited greater cell-wall esterase and aminopeptidase activities, but little of the total activities of these enzymes were associated with the cell envelope. Greater properties of total cellular dipeptidase activities were associated with the cell envelope, with *Lactobacillus helveticus* and *Brevibacterium linens* exhibiting highest specific activities.

The optimum pH of the crude cell-wall proteinase of different strains ranged from pH 5·5 to 8·0 while the optimum temperature ranged from 20 to 40°C. The impact of proteinase inhibitors tested differed between species.

INTRODUCTION

A cell envelope associated proteinase has been shown to be essential for the optimal growth of lactococci and lactobacilli in milk. Since milk contains insufficient amounts of free amino acids and small peptides, the cell envelope proteinase must degrade caseins to provide peptides as nitrogen sources to the cell. These peptides are further hydrolyzed by intracellular peptidases until their size allows translocation through the plasmic membrane by transport systems. Hydrolysis of caseins by the cell envelope proteinase is, therefore, considered to be the first and essential step in the nitrogen metabolism by lactococci and lactobacilli. Several studies have described the cell wall proteinase of cheese related bacteria. This activity in *Lactococcus lactis* ssp. *lactis* was reported initially by Thomas *et al.* (1974) in New Zealand. Later, Cliffe & Law (1985) detected four proteolytic activities in polyacrylamide gels using solubilized cell wall fractions of *Lc. lactis* ssp. *lactis*. More recently, Monnet *et al.* (1987*a*) detected cell-wall proteinases in five strains of this species. The proteolytic activity was affected by the culture medium. Higher peptide and/or calcium contents of the medium lowered the release of cell-wall proteinase activity.

Cell bound proteinases are also present in *Lc. lactis* ssp. *cremoris*. In extensive studies, Exterkate (1975, 1976, 1985) demonstrated the presence of two cell bound proteinase activities. The *de novo* synthesis of cell-wall proteinase is subjected to regulation by amino acids or peptides at the level of mRNA translation.

In studying the metabolism of nitrogenous compounds by lactic acid bacteria in milk, Mills & Thomas (1978) detected cell-wall proteinase activity in *Lc. lactis* ssp. *cremoris* AM2 and E_8. They also demonstrated that milk protein becomes an increasingly important source of nitrogen for growth as the cell density increases. *X*-Prolyl–dipeptidyl–aminopeptidase activities were also detected in the cell-wall fraction of *L. lactis* ssp. *cremoris* (Kiefer-Partsch *et al.*, 1989).

Several studies have described the cell wall proteinase activities in lactobacilli. Argyle *et al.* (1976) described a cell-wall associated proteinase activity in *Lactobacillus*

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delbruckii ssp. *bulgaricus*. The action of the enzyme on caseins was also reported. A cell bound aminopeptidase from *Lb. delbruckii* ssp. *lactis* was isolated by Eggiman & Bachman (1980). The enzyme was purified and characterized. The cellular location of these last two enzymes was, however, criticized (Marshall & Law, 1984) because of the lack of evidence on possible intracellular enzyme release. Cell bound activity was also demonstrated in *Lb. delbruckii* ssp. *bulgaricus*, *Lb. delbruckii* ssp. *lactis*, *Lb. helveticus* (Ezzat *et al.*, 1985), *Lb. casei* and *Lb. plantarum* (El Soda *et al.*, 1986*a*). Significant differences in the cell bound activities were demonstrated between species and between strains of the same species. It was also shown that cells grown in milk exhibited higher levels of activities as compared to cells grown in MRS medium. Cell-wall peptidase activity in *Propionibacterium shermanii* was also described (Sahlstrom *et al.*, 1989). The cell wall fractions were obtained after cell lysis.

Many of the previously described enzymes were isolated, purified, and characterized. Most of the purified enzymes exhibited an optimum pH in the acid range, and optimum temperature 90 000 to about 150 000 daltons. All the enzymes are inhibited in the presence of phenylmethylsulfonyl fluoride (PMSF) and diisopropylfluorophosphate (DFP) and are considered to be serine proteinases (Geis *et al.*, 1985; Exterkate & De Veer, 1987; Ezzat *et al.*, 1987; Monnet *et al.*, 1987*b*; Ezzat *et al.*, 1988; Zevaco & Gripon, 1988, Bockelmann *et al.*, 1989; Kiefer-Partsch *et al.*, 1989; Laloi *et al.* 1991; Naes *et al.*, 1991). Some exceptions were noted. The optimum pH for *Lb. casei* and *Lb. helveticus* was close to neutrality (Ezzat *et al.*, 1988). The *Lb. bulgaricus* ssp. *delbruckii* enzyme was a metalloproteinase rather than a serine proteinase (Ezzat *et al.*, 1987). It is now well established that there are two groups of proteinases based upon specificity. The P I group hydrolyzes only β-casein, and the P III group hydrolyzes α_s- and K-caseins, in addition to β-casein (Exterkate & De Veer 1989).

The previous literature on the cell-wall associated enzymic activities in cheese related bacteria clearly indicates the lack of information in this area for several technologically important microorganisms involved in the manufacture of cheese and other fermented food products. The objective of the present paper is, therefore, to assay for the presence of cell-bound enzymic activities in *Brevibacterium linens*, *Propiombacterium acidipropionici*, *Bifidobacterium infantis*, *Leuconostoc mesenteroides* ssp. *mesenteroides*, *Pediococcus sp.* and *Lb. helveticus*. The characterization of the proteinase activity will also be considered.

MATERIALS AND METHODS

Bacterial strains

The strains used in this study were: *Bre. linens* CNRZ 944, *Propionibacterium acidipropionici* CNRZ 80, *Pediococcus* sp. LR, *Leu. mesenteroides* ssp. *mesenteroides* CNRZ 1019, *Bf. infantis* 4038 and *Lactobacillus* sp. CNRZ 303. Cultures were maintained by subculturing in MRS broth and stored at −30°C with the exception of *Bre. linens*, which was stored in a medium of the following composition: meat extract (4 g), yeast extract (3 g), tryptone (10 g), monopotassium hydrogen phosphate (4 g), sodium hydroxide (1 g) and glucose (5 g), dissolved in 1 litre of distilled water at pH 7·5.

The cultures were grown at 30°C in MRS broth in a Biolaffite (Biolaffite LSL Group, UK) 2 liter fermentor equipped to control pH (the pH was maintained at 6·0 ± 0·1 by adding 10 N NAOH). Growth was assessed by absorbance measurements. *Bre. linens* was grown in the same medium used for subculturing this organism. The pH was maintained at 7·5 + 0·1.

Preparation of the total extract

The total extract was prepared according to the method described by El Soda & Desmazeaud (1982). The extract obtained after cell grinding was centrifuged at 400 × *g* to remove whole cells and alumina.

Extraction of the cell bound enzymic activities

Late logarithmic phase cells were harvested from the growth medium by centrifugation at 4500 × *g* for 30 min. The resulting pellet was washed with 0·05M β-glycerophosphate buffer at pH 7·0 containing 20 mM $CaCl_2$. The pellet was then suspended at 30°C in 0·05 M Tris–HCl buffer (pH 7·8) for 1 h. The supernatant fluid obtained after centrifugation contained the cell bound enzymic activities. Three successive extractions were performed. The release of lactate dehydrogenase (LDH) from cells incubated in buffer was used to estimate cell lysis (Thomas, 1975).

Determination of enzymic activities

Cell-wall proteinase activity was determined using [^{14}C]methylated casein (Monnet *et al.* 1987*a*). The substrate (0·05% final concentration) was incubated in 0·05M sodium phosphate buffer at pH 6·0 and 35°C. One unit of proteolytic activity is defined as that amount of enzyme releasing 1% of the total initial casein radioactivity after 10 min of hydrolysis. Results were also expressed as a percentage of the total activity.

The aminopeptidase and dipeptidase activities were determined and the specific activity defined as described by El Soda & Desmazeaud (1982). Esterolytic activity was measured according to El Soda *et al.* (1986*b*).

Partial purification of the cell-wall proteinase

The cell-wall fraction from different microorganisms was loaded on a 26/40 Pharmacia LKB (Uppsala) column of Sephacryl S300 (Pharmacia LKB) equilibrated with 0·01 M potassium phosphate buffer at pH 7·0. For *Bre. linens*, a 0·01M Tris–borate buffer (pH 8·0) was used. The column was eluted with the same buffer at 5°C. The flow rate was 2·5 ml/min and 3 ml fractions were collected.

Table 1. Detection of cell-wall associated peptide hydrolase and esterase activities[a] in cheese-related microorganisms

Microorganism	Enzymatic activities			
	Proteinase	Aminopeptidase	Dipeptidase	Esterase
Lb. helveticus	14·10 (25)[b]	2·13 (20)	12·20 (66)	7·09 (6)
Leu. mesenteroides ssp. *mesenteroides*	20·05 (48)	0·16 (3)	5·22 (36)	0·00 (0)
Pediococcus sp.	19·50 (48)	0·41 (3)	5·60 (36)	0·46 (5)
Pro. acidipropionici	11·16 (30)	0·15 (8)	6·26 (36)	0·12 (7)
Bre. linens	17·12 (51)	5·35 (0·6)	11·29 (51)	7·20 (13)
Bf. infantis	12·20 (13)	0·33 (8)	6·50 (13)	0·10 (6)

[a] Results are expressed as specific activities.
[b] Results in parentheses are percentage of the total extract activity for each enzyme class.

Protein determination

The method of Lowry *et al.* (1951), using bovine serum albumin as a standard, was used to measure the protein concentration of the total extracts.

RESULTS AND DISCUSSION

The results in Table 1 indicate the presence of variable amounts of cell-wall associated peptide hydrolase activities and esterolytic activities in all the species tested with the exception of *Leuconostoc mesenteroides* ssp. *mesenteroides*, which did not exhibit any cell-bound esterase. The highest specific activities of cell-wall associated proteinases were observed in the *Leuconostoc, Pediococcus* and *Brevibacterium* strains. *Bifidobacterium* and *Propionibacterium* species represented a group with relatively lower activity. Under the experimental conditions used in this study, very little intracellular enzyme release could be measured. The percentage lactate dehydrogenase (LDH) in the extraction medium varied from 0·01 to 1·2% of total cellular LDH activity, with the exception of *Leu. mesenteroides* where 6% release was detected.

The highest levels of aminopeptidase were found in *Bre. linens* where activity was more than 10-fold greater than all other species except *Lb. helveticus*.

In the case of dipeptidase, *Bre. linens* and *Lb. helveticus* formed a group that was distinguished by its relatively high cell wall associated dipeptidase activity. The specific activity for the dipeptidase of the other organisms varied from 5 to 6. The esterolytic activity of *Bre. linens* and *Lb. helveticus* was 15 to 70-fold higher than that of the other species.

When the results were expressed as percentage of the total cell activity (Table 1), about one-half of proteinase activity of *Bre. linens*, *Pediococcus* sp. and *Leuconostoc* sp. were associated with the cell wall. For *Propionibacterium* sp., *Lb. helveticus* and *Bf. infantis*, only 30, 25 and 13%, respectively, of the activities had a cell-wall location. Relatively low levels of aminopeptidases and esterases were bound to the cell wall, while larger proportions of the dipeptidase activities were cell bound.

Characterization of the cell-wall proteinases

The gel filtration procedure used in this study separated the cell bound proteinase activity from dipeptidase, aminopeptidase and esterase activities. The cell-wall proteinase fractions were pooled for enzyme characterization. No further purification steps were undertaken.

Table 2 indicates that the optimum temperature for the cell-wall proteinase activities of the different organ-

Table 2. Characterization of the cell-wall proteinase from several cheese-related microorganisms

Microorganism	Optimum temp.	Optimum pH[a]	Inhibition[b] by		
			EDTA[c] (%)	PHMB[c] (%)	PMSF[c] (%)
Lb. helveticus	40	7·0	0	0	100
Leu. mesenteroides ssp. *mesenteroides*	40	7·0	13	40	0
Pediococcus sp.	30	7·0	1	20	100
Pro. acidipropionici	30	5·5	25	15	35
Bre. linens	40	6.5	70	50	20
Bf. infantis	20 and 40	7·0	65	0	15

[a] Optimum pH was determined in Tris buffer.
[b] Rate of hydrolysis in the absence of inhibitor was taken as 100%.
[c] Concentration of inhibitors was 1×10^{-3} M.
PHMB = *p*-hydroxymercurybenzoate.
PMSF = phenylmethylsulfonyl fluoride.
EDTA = ethylenediaminetetraacetic acid.

isms varied from 20 to 40°C. The optimum pH for casein hydrolysis was close to neutrality for most species with the exception of the *Propionibacterium* sp., which exhibited an optimum pH of 5·5.

The cell bound proteinases isolated from *Pediococcus* sp. and *Lb. helveticus* were totally inhibited in the presence of PMSF, which indicates the involvement of a serine residue in the catalytic action of the enzyme. The enzymes from *Bre. linens* and *Bf. infantis* were strongly inhibited by the metal chelator EDTA. In the case of *Leu. mesenteroides*, *p*-hydroxymercurybenzoate was the most effective inhibitor, although 60% of the activity remained after treatment.

This study reveals for the first time the presence of cell-bound peptide hydrolase activity in *Bf. infantis*, *Bre. linens*, *Leu. mesenteroides* ssp. *mesenteroides* and *Pediococcus* sp. It also confirms the work of Ezzat *et al.* (1985) and Zevaco & Gripon (1988), concerning the presence of cell-wall associated proteinase in *Lb. helveticus*.

The characterization of the cell-wall proteinase of the microorganisms described in this study is comparable to those previously described microorganisms (Kok, 1990). Some differences were, however, detected. At the concentration used, PMSF did not exhibit any inhibition on the cell-bound proteinase from *Leu. mesenteroides*, and only slight inhibition was observed for *Brevibacterium* and *Bifidobacterium* species. Serine residues, apparently, are not important to the catalytic site of those enzymes, which differentiates them from the cell-bound proteinases isolated from the lactococci.

The cell-wall location of the esterolytic activity of the cheese-related bacteria described in this paper was previously demonstrated in *Bre. linens* (Sorhaug & Ordal, 1974), in several lactic acid bacteria (Oterholm *et al.*, 1968) and in lactobacilli isolated from meat (Papon & Talon, 1988, 1989). The role of these enzymes is, however, not well defined and more research in this area is needed.

Although the primary utility of *Propionibacterium*, *Bifidobacterium* and *Leuconostoc* species does not reside in their proteolytic and esterolytic activities, these organisms exhibit intracellular and cell-bound activities that would contribute to proteolysis and flavor or off-flavor production in cheeses or other fermented foods. More attention should, therefore, be given to their potential role in the cheese ripening process and to the selection of the most interesting activities exhibited by those microorganisms, which could then be incorporated into cheese.

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