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NATURAL ISOLATE *ENTEROCOCCUS FAECALIS* BGPM3 PRODUCES AN INDUCIBLE EXTRACELLULAR PROTEINASE

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Abstract - Enterococcus faecalis BGPM3 produces a proteinase that hydrolyses total casein as well as α_{S1}-, β-, and κ-casein fractions. This proteinase was also able to hydrolyse gelatine, but not denatured bovine serum albumin and haemoglobin. The optimal pH of casein hydrolysis was 6.5 (determined at 30° C). Maximum caseinolytic activity was obtained at 37° C. The presence of proteolytic activity in cell-free supernatant strongly indicated that *E. faecalis* BGPM3 produces strictly extracellular proteinase. Proteinase production occurred through the growth cycle reaching a maximum at stationary phase. Pretreatment of the BGPM3 proteinase with metal ion chelators resulted in a total loss of proteolytic activity. Restoration of activity (75%) was obtained only with Zn^{2+} suggesting that the BGPM3 proteinase is zinc-metalloenzyme. Cu^{2+} even in the presence of Zn^{2+} inhibited proteolytic activity. It seems that production of proteinase is induced by oligopeptides (casitone), since 10-fold higher proteolytic activity was obtained in the cell-free supernatant prepared from the cultures containing casitone. The molecular mass determination revealed that extracellular BGPM3 proteinase has a molecular mass of about 29 kDa.

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INTRODUCTION

Production of extracellular proteases is widespread among bacterial species. They have various functional roles. Thus, *Bacillus subtilis* secretes proteinases during the early stationary phase of growth. Some of them, such as alkaline serine protease, are involved in the process of sporulation (P r i e s t 1977). The subtilisin together with a metalloproteinase or neutral proteinase are major proteinases (encomprise 98% of proteolytic activity) found in the medium used for the growth of sporulating *B. subtilis* (K a w a m u r a and D o i 1984). Extracellular metalloproteases involved in degradation of dietary proteins in human large intestine are produced by *Clostridium bifermentans.* Synthesis of these proteases is markedly influenced by nutritional and culture conditions in which bacteria grew. In addition, analysis of culture supernatant revealed that *C. bifermentans* produces a mixture of proteases with molecular masses ranging from 36 to 125 kDa (M a c f a r l a n e and M a c f a r l a n e 1992). Lysostaphins are zinc(dependent proteinases produced by Staphylococcus species responsible for hydrolysis of interpeptide bridges in the peptidoglycan of the staphylococcal cell wall (R e c s e i *et al.* 1987).

Plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris* produces two extracellular proteases. One of them is serine protease (PRT1) and the other zinc-dependent protease (PRT2). Mutants of *X. campestris* pv. *campestris* that didn't produce proteases showed reduced pathogenic effects suggesting a role of proteases in black rot pathogenesis (D o w *et al.* 1990).

Lactic acid bacteria are components of starter cultures for variety of fermented products. Important feature of these bacteria is their proteolytic system which enables them to grow in milk and other protein-containing media. Lactococci depend on the efficient extracellular degradation of casein by proteinases to satisfy their requirement for essential amino acids, while growing in the milk. However, majority of lactococci do not synthesise strictly extracellularly secreted proteinases. They are producing the cell envelope(associated serine proteinases involved in the beginning of casein utilisation by them. In addition, an efficient system of peptidases and peptide-transport system are also involved in the utilisation of casein degradation products (P r i t c h a r d and C o o l b e a r 1993). It has been shown in the recent report that extent of proteinase

biosynthesis in *Lactococcus lactis* is regulated by the components of culture medium. Specific dipeptides were involved in the control of transcription initiation of proteinase genes (M a r u g g *et al.* 1995). The cell envelope(associated proteinases were also discovered in various lactobacilli and *Streptococcus thermophilus* (K o j i ć *et al.* 1991; 1995; L a l o i *et al.* 1991; M a r t i n-H e r n a n d e z *et al.* 1994; S h a h b a l *et al.* 1993).

Other bacterial species are sometimes incorporated into a dairy starter culture, such as *Streptococcus faecium* that is used during the manufacture of modified Cheddar cheese in the USA. The late formation of CO_2 has been ascribed very often to amino acid decarboxylation by enterococci (formerly *Streptococcus faecalis*) (C h a p m a n and S h a r p e 1981; T a m i m e 1981). On the other hand, enterococci are very often present in artisanal cheeses (A r i z c u n 1997). The occurrence of these organisms in cheese could be explained by their ability to survive pasteurisation and relatively high salt levels in comparison with lactic acid bacteria. Enterococci could also become prevalent microflora during long ripening period of cheese (d e F e r n a n d o *et al.* 1991; O r d ó ñ e z *et al.* 1978).

The objective of this study was the characterisation of natural isolate *Enterococcus faecalis* BGPM3 that produces extracellular proteinase. The effects of growing conditions, pH, temperature and metal ions on proteolytic activity are presented.

MATERIAL AND METHODS

Bacterial strains and media. Enterococcus sp. was isolated from traditionally prepared homemade smoked ham (generic name "pršuta") that contains approx. 4% of salt. A final species identification as an *Enterococcus faecalis* was performed by courtesy of LMG, Culture Collection Service, Gent, Belgium. *E. faecalis* BGPM3 was grown in different media including MRS broth (Difco, Detroit, Mich., U. S. A.), M17 medium (T e r z a g h i and S a n d i n e 1975) supplemented with glucose (0.5%, w/vol) (GM17 broth), LB broth (D a v i e s *et al.* 1980), Basal Collins medium (BCM) (d e F e r n a n d o *et al.* 1991), and minimal medium E (D a v i e s *et al.* 1980). Agar plates were prepared by the addition of agar (1.5%, w/vol) to each broth. The milk-citrate agar (MCA plates) containing 4.4% reconsti-tuted skim milk (RSM), 0.8% Na-citrate, 0.1% yeast extract, 0.5% glucose and 1.5% agar (w/vol) or MRS broth containing casitone were used to test possible proteinase inducibility. Inhibitory action of PM3 bacteriocin was tested against lacococcal strains (*Lactococcus iaetis* subsp. *lactis* MG1363 and NP45, *Lactococcus laciis* subsp. *cremoris* NS1 and natural isolates *Lactococcus* sp. BGJAV11, BGV2 and BGSJM2) and agianst *Lactobacillus plantoarum* A112.

Assay of proteinase activity. Proteolytic activity of *E. faecalis* BGPM3 was assayed as described previously (K o j i ć *et al.* 1991). For enzymatic assays, the BGPM3 strain was grown on MCA plates for 48 *h* at 30 °C prior to cell collection. Collected cells (5 *mg*) were resuspended in 100 mM Na(phosphate buffer (pH 7.2) to an approximate density of 10^{10} cells/mL. The cell suspension was mixed with the substrate dissolved in the same buffer at a 1:1 volume ratio, if not indicated otherwise. The resulting mixtures were incubated at 30 °C, for various time intervals. As a substrates, α_{S1}-, β-, κ-casein fractions (5 *mg*/mL) and total casein (12 *mg*/mL) (Sigma Chemie GmbH, Deisenhofen, Germany) were used. Alternatively, to obtain the crude proteinase extract the cells were washed twice in 100 mM Na(phosphate buffer (pH 7.2) by centrifugation and two extracts were pooled. Protein concentrations in crude extracts were measured by the method of L o w r y *et al.* (1951) with bovine serum albumin (BSA) (Sigma) as a standard. Denatured BSA, haemoglobin or gelatine were used as alternative substrates to test the proteinase specificity. For this purpose, 10 *mg* of the cells were collected from MCA plates, resuspended in 50 μL of 100 mM Na-phosphate buffer (pH 7.2) and incubated with 50 μL of each substrate (5 *mg*/mL) for 1 *h* at 30 °C unless otherwise stated. Proteinase activity was also determined by measuring the concentration of TCA-soluble products of substrate hydrolysis using the method of L o w r y *et al.* (1951). One unit (U) of activity was defined as the amount of enzyme that hydrolyses 1mg of β-casein for 30 min.

Effect of ions and inhibitors. Crude proteinase extract was dialysed against 100 mM Na-phosphate buffer (pH 7.2) at 1:500 volume ratio for 24 *h* at 4 °C with one replacement of the buffer. Ions or proteinase inhibitors were added to dialysed crude extract and the mixture was incubated 15 min at 30 °C prior to the addition of β-casein (5 *mg*/mL) dissolved in Na-phosphate buffer (pH 7.2) in 1:1 volume ratio. Resulting mixtures were incubated for 30 min at 30 °C. The reaction was stopped by the addition of trichloroacetic acid (TCA) and TCA-soluble hydrolytic products were determined by the method of L o w r y *et al.* (1951). The effects of mono- and divalent cations, as well as inhibitors on the proteinase activity were tested at concentrations indicated in Table 1.

Determination of pH and temperature optima. To test the influence of pH conditions on proteinase activity, the reaction mixtures containing dialysed crude proteinase extract and β-casein were incubated in 100 mM Na-phosphate buffer (pH 5.4 to 7.2) or in 100 mM

Tris-HCl buffer (pH 8 and 8.7) for 30 min at 30 °C. Similarly, to determine the temperature optimum of proteolytic activity, the reaction mixtures described above were incubated at the temperatures indicated in Fig. 7B. In both cases, hydrolytic products were quantified by the method of L o w r y *et al.* (1951).

Table 1. Effect of cations and proteinase inhibitors on the BHPM3 proteinase activity.

Preparation		Relative proteolytic activity (%)
Proteinase extract		100
Proteinase extract + ion		
Na^+	(5 mM)	101
K^+	(5 mM)	104
Mg^{2+}	(5 mM)	75
Zn^{2+}	(5 mM)	93
Mn^{2+}	(5 mM)	78
Ca^{2+}	(2mM)	100
	(5 mM)	101
	(10 mM)	98
	(20 mM)	96
Cu^{2+}	(2 mM)	25
	(5 mM)	22
	(10 mM)	16
	(20 mM)	11
Proteinase extract + inhibitor		
EDTA	(5 *mg*/mL)	67
	(10 *mg*/mL)	53
	(20 *mg*/mL)	41
EGTA	(5 *mg*/mL)	68
	(10 *mg*/mL)	50
	(20 *mg*/mL)	45
PMSF	(5 *mg*/mL)	45
	(10 *mg*/mL)	42
	(20 *mg*/mL)	34

All ions were used as chloride salts.

The results are the means of two independent experiments.

Test of the BGPM3 proteinase inducibility. The inducibility of the BGPM3 proteinase was tested by inoculating MRS broth and MRS broth containing casitone (0.5 %, w(vol) with the BGPM3 strain (1 % inoculum). Cultures were grown at 30 °C and the sam-ples were taken at A_{600} of 0.2, 0.4 and 0.8 as well as from overnight culture (16 *h*). The samples were centri-fuged and total proteins in supernatants were concentrated 40-fold by NH_4-sulphate (25%, w/vol) precipitation. Proteolytic activity of resuspended, unwashed pelleted cells and concentrated supernatants were test-ed by hydrolysis of β-casein (5 *mg*/mL) for 10 min at 30 °C. The efficiency of β-casein hydrolysis was analysed by Sodiumdodecyl-sulphate polyacrilamide gel electr-ophoresis (SDS-PAGE). In each case the concentrtion of acrylamide was 15%.

Non-denaturing SDS-PAGE. SDS-PAGE was pe-rformed by a modified procedure of M a c f a r l a n e and M a c f a r l a n e (1992). Crude proteinase extract was dissolved in sample buffer (1% SDS, 25% glycerol, 0.06% bromphenol blue and 125 mM Tris-HCl, pH 6.8). The samples were run on polyacrylamide gels containing 0.25% (w/vol) of total casein. After running, the gels were renatured for 2 *h* at room temperature in buffer containing 25 mM Tris-HCl (pH 8), 0.5% Tween 80 and 0.5% Triton X-100. Molecular mass of proteinase was determined by using Rainbow^{TM} Protein Molecular Weight Standard (Amersham, Buckinghemshire, UK).

Bacteriocin assay. To test bacteriocin production, GM17 plates were overlyied by 3 mL of appropriate soft agar (0.7%) containing 0.1 mL of 10^{-2} diluted fresh culture of the indicator strain. Wells were made in the lawn of harden soft agar. Aliquots (50 μL) of supernatant collected from overnight culture of the BGPM3 strain were poured into wells. The plates were incubated overnight at 30 °C and the appearance of inhibition zone around wells indicated bacteriocin production.

DNA/DNA hybridisation. The proteinase gene probes, Q1, Q6 and Q92 from *L. lactis* subsp. *cremoris* Wg2, were kindly provided by Dr J. Kok. Labelling of probes and hybridisation experiments have been carried out essentially as described previously (K o j i ć *et al.* 1991).

RESULTS

General characteristics of the strain Enterococcus faecalis BGPM3. The strain BGPM3 was able to grow in MRS broth at 30 °C, 37 °C and 45 °C. In addition, this strain exposed very good growing characteristics on MRS, GM17 or LA plates at 30 °C, 37 °C and 42 °C. It was also able to grow in the presence of 4%, but not in the presence of 6.5% NaCl. The strain BGPM3 does not produce catalase. Milk clotting by the strain BGPM3 was not very efficient. A tiny curd was formed after 48 *h* of incubation at 37 °C. The pH of the obtained curd was 4.92. In addition, less consistent curd (pH 5.94) was also obtained during incubation of the strain BGPM3 in milk for 48 *h* at 45 °C. In both cases, milk was inoculated (1% inoculum) with the culture pregrown in MRS broth.

Strain BGPM3 produces bacteriocin. Bacteriocin exposed inhibitory action on the growth of lactococcal strains (*Lactococcus lactis* subsp. *lactis* MG1363, nisin producer *L. lactis* subsp. *lactis* NP45, *L. lactis* subsp. *cremoris* NS1 and natural isolates *Lactococcus* sp. BGJAV11, BGV2, and BGSJM2), and on the *Lactobacillus plantarum* A112 (data not shown). Plasmid analysis revealed the presence of one plasmid of approx. 20 kb in *E. faecalis* BGPM3 (data not shown).

Proteolytic activity. The strain *E. faecalis* BGPM3 produces proteinase that gives visible halo around the bacterial colonies on MCA plates. To evaluate whether the BGPM3 proteinase exerts protein-substrate specificity, whole cells were collected after growing on MCA plates and using various proteins as substrates their proteolytic activity was tested. For this purpose, total casein, purified casein fractions (α_{S1}, β and κ) as well as denatured BSA, gelatine or haemoglobin were used. Results of these tests revealed that the BGPM3 proteinase intensively hydrolysed caseins. Hydrolysis of α_{S1}-, β- and κ-casein fractions by BGPM3 proteinase was complete within one hour of incubation at 30 °C. However, testing of the casein hydrolysis within 10 min at 30 °C revealed that activity of BGPM3 proteinase towards different caseins varied in efficiency. Thus, the BGPM3 proteinase showed the highest efficiency in degradation of β-casein. Degradation of α_{S1} - casein and κ-casein was also pretty good but not as intense as that of β-casein (Fig. 1). One major product was observed in hydrolysis of κ-casein in contrast to degradation patterns of α_{S1} - and β-caseins. It is therefore plausible that BGPM3 proteinase recognised relatively small number of cleavable sites in the κ-casein. The BGPM3 proteinase was able to hydrolyse total casein, as well. Degradation products of casein fractions can be recognised within the pattern of total casein hydrolysis (Fig. 1). The BGPM3 proteinase also hydrolysed gelatine, but it was not possible to detect hydrolytic effect of the BGPM3 proteinase on either denatured BSA or haemoglobin under applied experimental conditions even with the prolonged time of incubation or with variations of enzyme/substrate ratio (data not shown).

Kinetics of β-casein degradation was followed by using the crude proteinase extract of the strain BGPM3. It appeared that this extract was much more efficient in β-casein hydrolysis than that prepared from *Lactococcus lactis* subsp. *cremoris* Wg2. Hydrolysis of β-casein by the crude proteinase extracts prepared exactly in the same way from the strain BGPM3 and *L. lactis* subsp. *cremoris* Wg2 was of a similar extent despite of much higher enzyme/substrate volume ratio used in the test of the strain BGPM3 (1:1 for Wg2 and 1:4 for BGPM3).

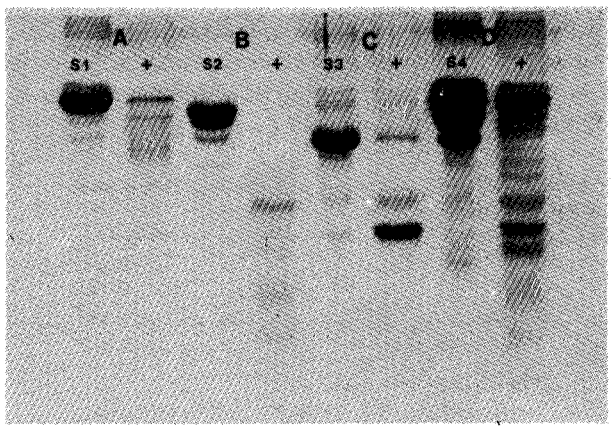

Fig. 1. Casein hydrolysis by whole cells of *E. faecalis* BGPM3. Hydrolysis of α_{s1}-casein (A), β-casein (B), κ-casein (C), and total casein (D). s1, s2, s3 and s4 are non-digested substrates; (+) denotes hydrolysis of respective substrate after incubation with whole cells for 10 min at 30 °C.

In addition, patterns of β- casein hydrolysis differed between the two strains (Fig. 2).

Localisation of the BGPM3 proteinase. Isolation of cell-envelope associated proteinases from lactococci and some lactobacilli was achieved by repeated washing of the cells with Ca^{2+}-free buffer. To test whether the BGPM3 proteinase belongs to the class of cell-envelope associated proteinases the strain BGPM3 was pregrown on MCA plates. To obtain proteinase extracts, the cells were collected and a half of pelleted cells was washed with Ca^{2+}-free and another half with Ca^{2+}-containing buffer. Both prepared proteinase extracts were tested for the ability to degrade β-casein. The results revealed that these extracts hydrolysed β-casein with almost identical efficiency regardless of the presence or the absence of Ca^{2+}-ions. It is therefore plausible that the release of the BGPM3 proteinase from the cell, its stability, as well as proteolytic activity towards casein is not Ca^{2+}-dependent.

To test whether the BGPM3 proteinase is an extracellular enzyme, the cells were collected and washed in Na-phosphate buffer. The resulting extract, as well as washed cells were tested for proteolytic activity. Whole-unwashed cells were also used in the test as a control. It appeared that hydrolysis of β-casein could be detected only by unwashed cells and crude extract, but not by washed cells preparation (Fig. 3). Definitive proof that the BGPM3 proteinase is strictly extracellular enzyme came from the detection of proteolytic activity in cell-free supernatant of BGPM3 culture, but not in respective pelleted cells (see below).

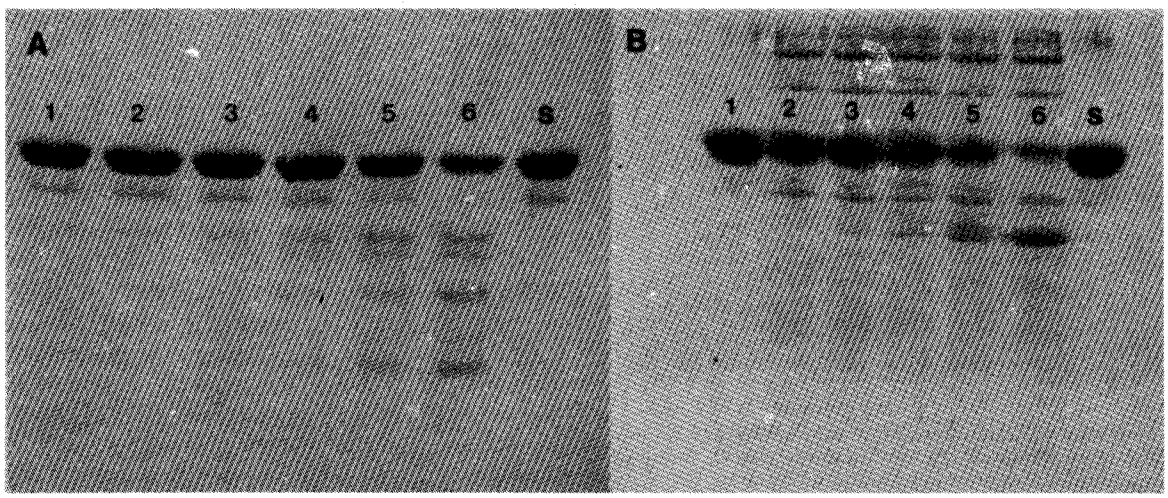

Fig. 2. Comparative kinetics of β-casein degradation by crude proteinase extract of *E. faecalis* BGPM3 (A) and *Lactococcus lactis* subsp. *cremoris* Wg2 (B). The samples were taken from reaction mixture for SDS-PAGE analysis at: Lane 1, 0 min; Lane 2, 15 min; Lane 3, 30 min; Lane 4, 1 *h;* Lane 5, 2 *h*; Lane 6, 4 *h.* Lane s - starting substrate. The incubation of the reaction mixtures was performed at 30 °C. The volume ratios of crude proteinase extracts and the substrate were 1:4 and 1:1 for the strain BGPM3 and the strain Wg2, respectively. Total protein concentration of crude extracts were 2 *mg*/mL for the strain BGPM3 and 2.7 *mg*/mL for the strain Wg2.

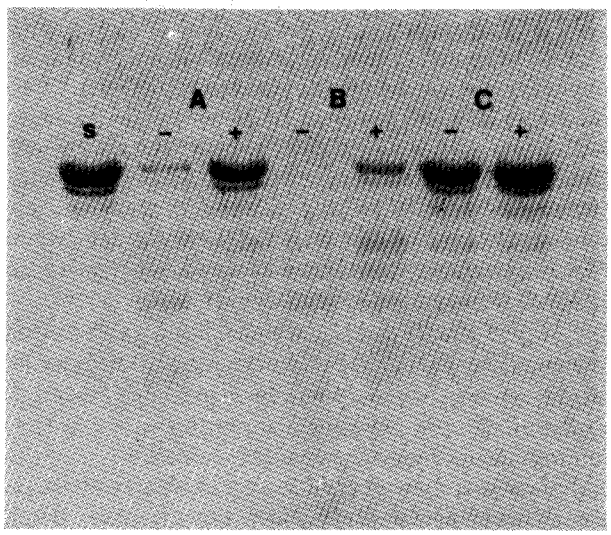

Fig. 3. Localisation of the proteolytic activity in *E. faecalis* BGPM3.
As a substrate, β-casein (lane s) was digested by unwashed whole cells for 10 min at 30 °C (A), by crude proteinase extract for 10 min at 30 °C (B) and by washed whole cells for 1 *h* at 30 °C (C) in the presence (+) or in the absence (-) of the serine proteinase inhibitor PMSF (final concentration 10 mg/mL).

Size determination of the BGPM3 proteinase. To determine the size, *i.e.*, molecular mass of the BGPM3 proteinase, a specific, non-denaturing polyacrylamide gel containing total casein was prepared (Material and Methods). Extracts obtained by cells washing were run on this gel and the hydrolysis of casein was obtained only at one position. Zone of the casein hydrolysis occurred at similar position in the gel consistent with the migration behaviour expected of a 29 kDa protein (Fig. 4). It could be therefore concluded that the BGPM3 proteinase has a molecular mass of about 29 kDa. Exactly the same result was obtained when proteinase from cell-free supernatant was analysed in the same manner (data not shown).

Inducibility of proteinase production. To test the influence of media and growing conditions on proteinase production, the strain BGPM3 was grown on the surface of different solid media (MRS, MCA or LA plates) or in liquid culture [MRS broth or MRS broth containing casitone (0.5%, w/vol)]. Testing of the β-casein hydrolysis by whole cells collected from MRS, MCA or LA plates revealed that no significant β-casein hydrolysis could be detected with the cells collected from either LA or MRS plates. In contrast, the cells collected from MCA plates degraded β-casein and hydrolytic products could be visualised on the SDS-PAGE gels (Fig. 5). These results suggested that the production of BGPM3 proteinase might be inducible.

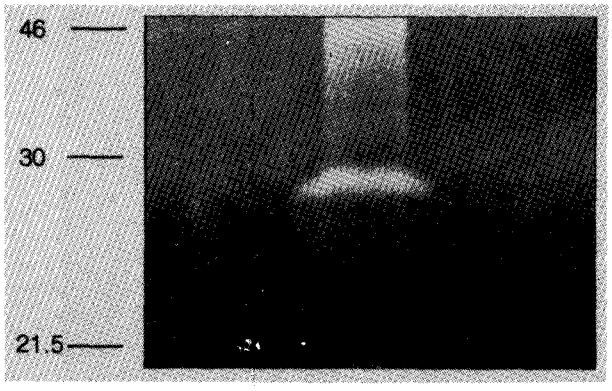

Fig. 4. Molecular size determination of the BGPM3 proteinase. Molecular mass standards are ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and trypsin inhibitor (21.5 kDa).

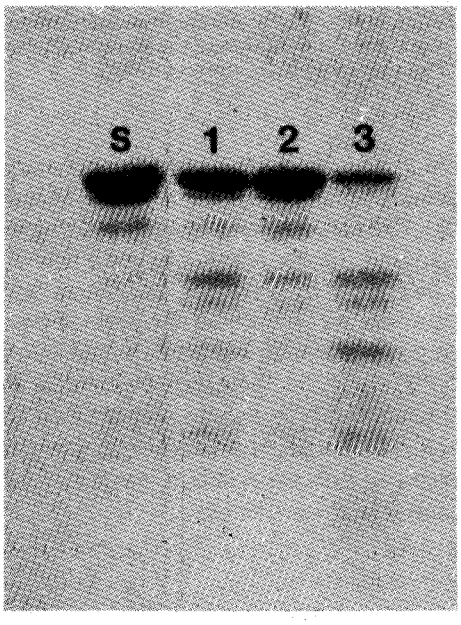

Fig. 5. Proteinase activity towards β-casein of *E. faecalis* BGPM3 grown on solid media.
Lane S, starting substrate; Lane 1, whole cells from LA plates; Lane 2, whole cells from MRS plates; Lane 3, whole cells from MCA plates. Hydrolysis of β-casein was performed for 10 min at 30 ° C in each case.

Possible inducibility of the BGPM3 proteinase production was tested in liquid culture, as well. For this purpose, the samples were taken during the growth of *E. faecalis* BGPM3 strain in either MRS or MRS+casitone broth. Supernatants and respective pelleted cells from each sample were assayed for the presence of proteolytic activity. The results revealed that hydrolysis of β-casein could be obtained in the cell-free supernatants of both MRS culture and MRS+casitone culture, although hydrolysis was incomparably more intensive in the presence of supernatant recovered from MRS+casitone culture. Pelleted cells regardless of the culture from which they were collected did not show any significant proteolytic activity (Fig. 6). In addition, increasing the quantity of casitone in MRS cultures (0.1%, 0.5%, 1% and 2% w/vol) resulted in enhancement of proteolytic activity in culture supernatants towards β-casein. Maximal activity was observed in the supernatant of culture growing in the presence of 1% casitone. No significant increment of proteolytic activity was observed in the presence of 2% casitone (data not shown). It seems that the presence of casitone that contains small peptides (about 80%) induces the production of BGPM3 extracellular proteinase. Interestingly, the strain BGPM3 was not able to grow either in BCM medium that contains only amino acids or in the minimal medium (E). At least, both media have in common the absence of peptides or proteins. However, the strain BGPM3 grew in BCM containing yeast extract (0.1%, w/vol) and NaCl (0.5%, w/vol), although the growth was pretty slow. Comparative analysis of proteinase production by the strain BGPM3 in MRS and BCM (both containing casitone) revealed that inducibility was much higher in the latter medium. Thus, the strain BGPM3 grown in BCM containing casitone has shown a 10-fold increase in proteinase activity if compared to the detected proteinase activity when this strain grew in BCM lacking casitone. In contrast, only 2-fold increase in proteinase activity was observed when MRS containing casitone was used for the strain BGPM3 propagation instead of pure MRS.

Effect of cations, pH and temperature on proteolytic activity. The BGPM3 proteinase activity present in crude extract towards β-casein degradation was followed in 100 mM Na-phosphate buffer (pH 7.2) containing various mono- or divalent cations. None of the tested cations showed significant stimulatory effect on the BGPM3 proteinase activity. On the other hand, only Cu^{2+} ions exerted an inhibitory effect on the BGPM3 proteinase activity (89% of inhibition when added at a final concentration of 20 mM). It seems also that the BGPM3 proteinase does not belong to serine-proteinases, since its activity was only partially inhibited by PMSF (Table 1 and Fig. 3). In addition, the BGPM3 proteinase activity was also reduced up to 60% in the presence of EDTA or EGTA (Table 1). Besides, pretreatment of the BGPM3 proteinase with 2 mM EDTA followed by dialysis for 90 min resulted in a total loss of

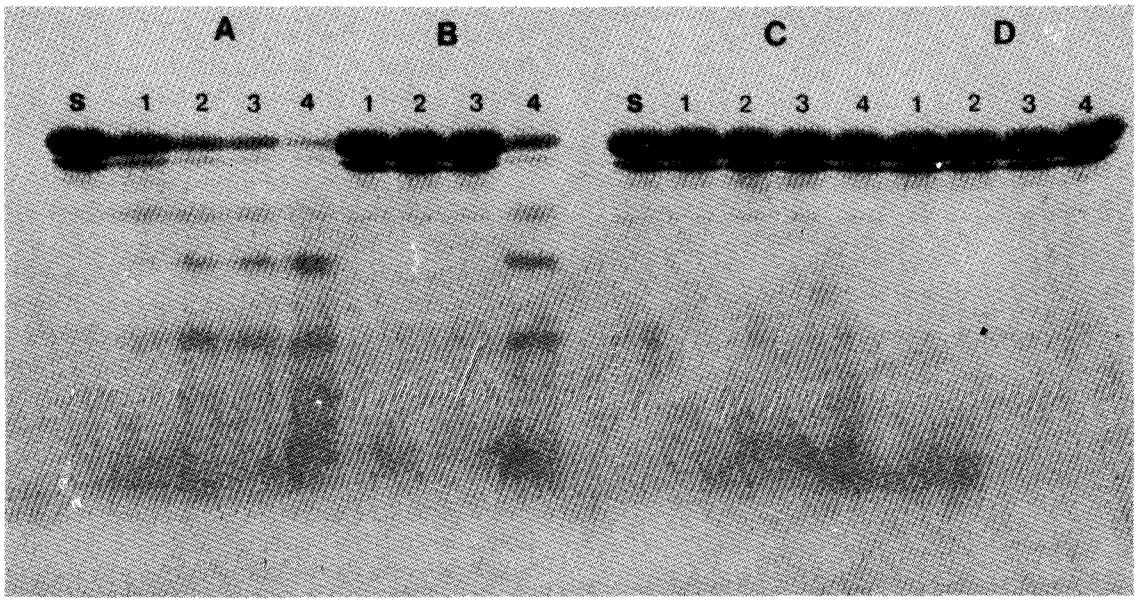

Fig. 6. Testing of the inducibility of BGPM3 proteinase.
(A) supernatant of MRS broth culture containing casitone (0.5% w/v), (B) supernatant of MRS broth culture, (C) pelleted cells from MRS broth culture containing casitone (0.5% w/v) and (D) pelleted cells from MRS broth culture. Lanes 1, 2, 3 and 4, are samples taken from the growing culture at A_{600} = 0.2, 0.4, 0.8 and overnight culture (16 *h*), respectively. Lane s, non-digested β-casein. Hydrolysis of β-casein was performed for 10 min at 30 °C in each case.

Table 2. Effect of metal ions on reactivation of EDTA-treated BGPM3 proteinase.

Preparation	Relative proteolyric activity (%)
Proteinase extract	100.0
EDTA-pretreated extract	0.0
EDTA-pretreated extract +	
Zn^{2+}	75.0
Cu^{2+}	3.9
Ca_{2+}	5.7
Mn^{2+}	7.7
Mg^{2+}	0.0
Na_{+}	1.9
K^{+}	5.8

The BGPM3 proteinase extract was treated with 2 mM EDTA for 30 min at 30 °C before being dialysed against 100 mM Na-phosphate buffer, pH 7.2, for 90 min at 4 °C. Dialysed extracts were preincubated with metal ions (5 mM) for 15 min before addition of β-casein. The results are the means of two independent experiments.

proteolytic activity towards β-casein. Proteolysis of β-casein by dialysed EDTA-pretreated BGPM3 proteinase was restored by preincubation for 15 min with Zn^{2+} (75% at 5 mM) prior to addition of the substrate. No significant recovery of proteolytic activity was observed with Ca^{2+}, Mg^{2+}, Mn^{2+}, Na^{+} or K^{+} ions (Table 2).

Degree of reactivation dialysed EDTA-pretreated BGPM3 proteinase depended on the duration of incubation with Zn^{2+}. Results showed that 25.5%, 48.5% and 75% of proteolytic activity were regained after proteinase preincubation with 5 mM Zn^{2+} for 5 min, 10 min, and 15 min, respectively. The reactivation of the BGPM3 proteinase only by Zn^{2+} ions strongly suggested that this enzyme is a zinc-requiring metalloprotease. However, Zn^{2+} had no significant effect on the activity of the native enzyme (Table 1).

To elucidate the relationship between stimulatory Zn^{2+} effect and an inhibitory Cu^{2+} effect on proteolytic activity, the dialysed EDTA-pretreated BGPM3 proteinase was preincubated for 15 min with Zn^{2+} (5 mM). After addition of Cu^{2+} (5 mM) to the same reaction mixture, the incubation was continued for additional 15 min prior to addition of β-casein. The results showed that only 35% of potential proteolytic activity towards β-casein was restored. Similar reactivation of the BGPM3 proteinase (31%) was observed when the order of ion addition was changed under the same experimental conditions. These results suggested that Zn^{2+} for some reason could not reactivate the proteinase in the presence of Cu^{2+} at the level as it does in the absence of Cu^{2+}.

The optimal pH and temperature for hydrolysis of β-casein by the BGPM3 proteinase was 6.5 (elaborated at 30 °C) and 37 °C (elaborated at pH 7.2), respectively (Fig. 7).

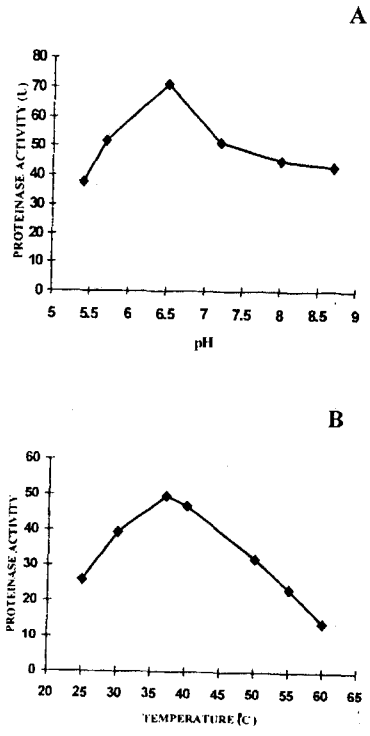

Fig. 7. Determination of the pH (A) and temperature (B) optima for the BGPM3 proteinase.

Hybridisation experiments. Total DNA isolated from the strain BGPM3 did not hybridise to the specific proteinase gene probes (Q1, Q6 or Q92) originating from *Lactococcus lactis* subsp. *cremoris* Wg2 at either 65 °C or 45 °C (data not shown). The absence of hybridisation suggests that the gene(s) encoding the extracellular BGPM3 proteinase have quite different primary structure than that in lactococcal strains.

DISCUSSION

The ability to produce one or more extracellular proteinases is a very common feature among bacterial species (D o w *et al.* 1990; M a c f a r l a n e and M a c f a r l a n e 1992; S t r y d o m *et al.* 1986). The natural isolate *Enterococcus faecalis* BGPM3 also produces extracellular proteinase. Proteolytic activity was detected in the supernatant of the culture. In addition, pelleted cells did not retain any proteolytic activity suggesting that the strain BGPM3 indeed secreted proteinase in the medium. On the other hand, the presence of only one protein band on SDS-PAGE showing proteolytic activity strongly indicated that the strain BGPM3 produces a single type of extracellular proteinase. The apparent molecular mass of this proteinase seems to be about 29 kDa (Fig. 4). Similar results have been obtained with *Micrococcus caseolyticus* in which the extracellular proteolytic activity was also attributed to the presence of one protease (about 38 kDa) isolated from the culture supernatant (D e s m a z e a u d and H e r m i e r 1968a).

Metal ions exerted different effects on bacterial proteases. Thus, the complete inhibition of the BGPM3 proteinase activity by metal chelators such as EDTA strongly indicated that the enzyme belongs to the metalloproteases. A satisfactory restoration of proteolytic activity (75%) was obtained only when EDTA-treated BGPM3 proteinase was incubated with Zn^{2+} ions prior to addition of the substrate suggesting that the BGPM3 proteinase is zinc-metalloenzyme. The same results were obtained in analysis of the PRT2 proteinase from *Xanthomonas campestris*. It was found that Zn^{2+} ions are required for optimal PRT2 activity (D o w *et al.* 1990). It was also observed that in *E. facealis* subsp. *liquefaciens* the formation of active extracellular proteinase requires the presence of Zn^{2+} and Ca^{2+} ions (H e g a z i 1991). Interestingly, Cu^{2+} ions inhibited the BGPM3 proteinase even in the presence of Zn^{2+} ions. It has been shown recently that Cu^{2+} ions are inhibitors of the cell envelope-associated proteinase in *Streptococcus thermophilus,* while divalent cations Ca^{2+}, Mg^{2+} and Mn^{2+} actedas activators (S h a h b a l *et al.* 1993). Similar results were obtained in analysis of proteinase production by *Bacteroides fragilis* NCDO2217. This strain produced three major proteases of molecular masses 73, 52 and 34 kDa. Testing the effect of protease inhibitors on these proteases revealed that protease P1 and P2 belong to serine and metalloproteases classes respectively, while protease P3 was a cysteine class of protease. In addition, the proteolytic activity of whole cells was stimulated by divalent cations (Ca^{2+}, Mn^{2+} and Mg^{2+}), but was inhibited by about 95% by Cn^{2+} G i g s o n and M a c f a r l a n e 1988). However, the proteinases of both *S. thermophilus* and *B. fragilis,* in contrast to extracellular proteinase of *E. faecalis*, were inhibited by Zn^{2+} ions, too. Partial ingibition of BGPM3 proteinase was also detected in presence of PMSF and ingibitor of serine proteinases (Fig. 3). Although it may suggest that BGPM3 proteinase belongs to that class of proteinases, this is probably not the case, bacause PMSF also inhibits other classes of proteinases (Beynon ad Bond 1994).

It has been also shown that Ca^{2+} ions play an important role in cell wall-associated proteinase release from whole cells of lactococci and some lactobacilli. In addition, Ca^{2+} ions are involved in the control of proteinases autocatalytic processing (K o k 1990; K o j i ć *et al.* 1991). In contrast, Ca^{2+} ions are not crucial either for proteinase release or for proteolytic activity of the proteinase produced by *E. faecalis* BGPM3 con-

firming that this proteinase does not belong to the class of classical cell wall-associated proteinases. However, Ca^{2+} as well as Sr^{2+} ions induced partial reactivation of EDTA-treated extracellular proteinase of *Micrococcus caseolyticus* (Desmazeaud and Hermier 1968a).

Production of extracellular proteinases largely depends on culture conditions. Great variations in the ability to produce extracellular proteinases was detected among bacterial species even within the same genus. For example, extracellular protease production by *Clostridium sporogenes* occurred at the end of active growth, and was inhibited by glucose, phosphate, ammonia or some amino acids. On the other hand, protease production in saccharolytic species *C. perfringens* occurs through the growth cycle and is stimulated by carbohydrates and high growth rates. Recent studies have shown that *C. botulinum* synthesises an intracellular and an extracellular proteases during active growth that were repressed by arginine and ammonia but not by glucose (Allison and Macfarlane 1990). Proteinase production by *E. faecalis* BGPM3 occurred through the growth cycle. Proteinase activity increased during the exponential phase reaching maximal value at the stationary phase. In addition, it appeared that proteinase production was induced by casitone. Induction was detected in minimal (BCM) and rich medium (MRS) both containing casitone, although induction was 10-fold higher in the former. Strain *E. faecalis* subsp. *liquefaciens* failed to produce proteinase when grown in original BCM. However, proteinase was produced in all other media containing proteins. In addition, beginning of extracellular proteinase production occurred at the end of exponential growth (de Fernando *et al.* 1991). Peptides were also found to be inducers of extracellular proteinase in *Micrococcus caseolyticus,* and proteins are not inducers in resting cells. Amino acids are very poor inducers of proteinase production (Desmazeaud and Hermier 1968b). Similarly, increased proteinase production was observed in *Streptococcus thermophilus* CNRZ703 grown in skim milk (containing casein) as compared with proteinase production when the same strain has grown in rich medium (M17). In contrast, such medium-dependent difference in proteinase production was not exhibited with another *S. thermophilus* strain CNRZ385. In fact, the culture medium expressed no effect on proteinase production in the latter *S. thermophilus* strain (Shahbal *et al.* 1993).

It has been reported that the regulation of proteinase gene expression in *Lactococcus lactis* subsp. *cremoris* SK11 is medium-dependent. The results showed that the peptide content in the growth medium has an effect on gene expression. Increasing concentration of casitone in growth medium (whey permeate) resulted in a gradual repression of proteinase gene expression. The lowest activity was observed in the presence of 2% casitone in the growth medium (Marugg *et al.* 1995). This effect of casitone on proteinase gene expression in *L. lactis* SK11 is just opposite to the effect of casitone on proteinase production in *E. faecalis* BGPM3.

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ПРОИЗВОДЊА ИНДУЦИБИЛНЕ ЕКСТРАЦЕЛУЛАРНЕ ПРОТЕИНАЗЕ ПОМОЋУ ПРИРОДНОГ ИЗОЛАТА *ENTEROCOCCUS FAECALIS* BGPM3

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Enterococcus faecalis BGPM3 производи протеиназу способну да хидролизује укупан казеин као и фракције α_{S1}-, β- и κ-казеина. Ова протеиназа, такође, хидролизује желатин, али не делује на денатурисани говеђи серум албумин или хемоглобин. Установљено је да се оптимална хидролиза казеина у присуству BGPM3 протеиназе постиже на pH 6.5, а његова максимална хидролиза на 37 °C. Присуство протеолитичке активности у супернатанту, који не садржи живе ћелије, указује да изолат *E. faecalis* BGPM3 производи екстрацелуларну протеиназу. Синтеза ове протеиназе се одиграва током целокупног циклуса раста бактерије, при чему се максимум производње постиже у стационарној фази. Третман BGPM3 протеиназе са хелаторима металних јона доводи до потпуног губитка протеолитичке активности . Међутим, могуће је повратити протеолитичку активност (до 75%) ако се третираном ензиму додају јони Zn^{2+} што указује да је BGPM3 протеиназа металоензим. Протеолитичка активност овог ензима је инхибирана јонима Cu^{2+}, чак и у присуству јона Zn^{2+}. Експериментални резултати указују да је производња BGPM3 протеиназе индуцибилна, тј., долази до повећања њене синтезе када бактерија расте у присуству смеше олигопептида (казитона). Тако се добија десетоструко повећање протеолитичке активности у безћелијском супернатанту када се припреми из културе која садржи казитон. Одређивање молекулске масе BGPM3 протеиназе је покаало да је то протеин од око 29 kDa.