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Autodegradation of the extracellular proteases of *Brevibacterium linens* ATCC 9172

Wolfgang Buchinger [a], Jarmila Tomaschová [b], Jaroslav Zemanovic [b], Werner A. Hampel [a,*]

[a] *Institute of Biochemical Technology and Microbiology, Vienna University of Technology, Getreidemarkt 9/1722, A-1060 Vienna, Austria*

[b] *Department of Milk, Fat and Food Hygiene, Faculty of Chemical Technology, Slovak University of Technology, 812 37 Bratislava, Slovak Republic*

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Abstract

Brevibacterium linens ATCC 9172 produces multiple forms of extracellular proteolytic enzymes as shown by polyacrylamide gel electrophoresis (PAGE) and activity staining. Four main bands with strong proteolytic activity, showing proteins with molecular weights of 280, 220, 130 and 43 kDa, were distinguished from several bands of lower activity. The formation of smaller entities on incubation at 38°C from the isolated main species of proteases was demonstrated, showing specific sequences in autodegradation. The cell wall-associated proteases were completely released by incubation of the cells at 50°C for 2 h and showed a similar isoenzyme pattern. There is evidence that the multiplicity of proteases is a result of aggregation from subunits and of autocatalytic degradation. The enzymes were identified as serine proteases by specific inhibition.

1. Introduction

Brevibacterium linens is the main component of the surface flora of orange-red smear cheese such as Limburger, Romadour, Münster, Appenzeller and Brick (Kelly, 1937; Valdes-Stauber, Scherer & Seiler, 1997). On the surface of the cheese it is associated with several yeasts. The yeast cells grow initially and raise the pH of the curd by lactate catabolism and ammonia formation from amino acids (Kelly & Marquardt, 1939). The reduced acidity, together with the release of growth factors (vitamins), enables *B. linens* to grow and subsequently to dominate the surface microflora (Purko, Nelson & Wood, 1951). The growth of *B. linens* on the surface is essential in the development of the characteristic flavour, aroma, colour and texture of the cheese. By the action of extracellular enzymes (proteases, aminopeptidases and esterases), secreted by the bacterial cells and by cell-associated lipases and proteases on casein and milk fat, several low molecular weight substances are formed (Ades & Cone, 1969). Subsequently some of them are converted into aroma compounds by metabolic reactions, thus forming the typical flavour of the cheese (Hosono, 1968; Hosono & Tokita, 1969; Tokita & Hosono, 1968). The application of proteolytic enzymes of *B. linens* was reported for the accelerated ripening of hard cheese such as Cheddar (Hayashi, Revell & Law, 1990).

Extracellular proteases of different strains of *B. linens* have been the subject of several experiments described in the literature. Foissy (1973, 1974) demonstrated, by electrophoretic separation techniques, the existence of strain-specific isoenzyme patterns for intra- and extracellular proteases, according to wide interstrain differences within the species. The subsequent isolation, purification and characterisation showed a broad spectrum in molecular mass of proteases ranging from 37 to 325 kDa and a considerable diversification in biochemical properties, such as enzyme stability and optimal activity. Moreover, inhibition experiments revealed that almost all of them could be classified as serine proteases (Ezzat, El-Soda & El-Shafei, 1994; Hayashi, Cliffe &

* Corresponding author. Tel.: +43-1-58801-17220; fax: +43-1-58801-17299.

E-mail address: whamp@mail.zserv.tuwien.ac.at (A. Hampel).

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Law, 1990a; Juhasz & Skarka, 1990; Rattray, Bockelmann & Fox, 1995; Tomaschová, Buchinger, Hampel & Zemanovic, 1998)

There is some indication that the isoenzyme pattern may arise from autocatalytic degradation of the molecule. Strauss, Kopecky, Zemanovic and Hampel (1994) demonstrated changes in the isoenzyme pattern on incubation of a crude enzyme preparation at room temperature. We report in this paper a detailed study of the autodegradation of the multiple extracellular proteases of *B. linens* ATCC 9172.

2. Material and methods

2.1. Organisms and growth conditions

B. linens ATCC 9172 was obtained from the American Type Culture Collection (Rockville, USA). The cultivation was performed in a highly instrumented laboratory bioreactor (Model WA250, Chemap AG, Volketswil, Switzerland) at a stirrer speed of 800 rpm and an aeration rate of 0.33 VVM in 10 l of medium. Temperature was maintained at 28°C and pH was regulated by addition of 1 M H_2SO_4 to a constant level of 7.0. The culture media contained [g/10 l]: 2.7 KH_2PO_4, 8.9 $Na_2HPO_4.2H_2O$, 4.6 $MgSO_4.7H_2O$, 0.051 $FeSO_4.7H_2O$, 0.046 $CaCl_2.2H_2O$, 300 NaCl, 15 $(NH_4)_2SO_4$, 62.2 Na-L-lactate (equal to 5 g/l L-lactic acid) and 15 peptone from soymeal (papain-digested, Merck AG, Darmstadt, Germany). Additionally (10 ml/l) trace element solution (Wolin, Wolin & Wolfe, 1963) as well as Ca-pantothenate (6 mg/l) and biotin (10 μg/l) were sterilised by filtration and added aseptically. For inoculation, the organism was grown on an orbital shaker in 1-l Erlenmeyer flasks at 250 rpm and 28–30°C for 30 h. A total volume of 1 l cell supension was used to inoculate the fermenter.

For comparative studies on isoenzyme pattern the strains *B. linens* ATCC 9174 (American Type Culture Collection; Rockville, USA) and *B. linens* Lactoflora 200 (Prague, Czech Republic) were additionally used.

2.2. Recovery of protease from culture medium

After attaining the stationary phase of growth (ca. 34 h of cultivation) the cells were removed by a laboratory separator (Model LWA 205, Westfalia Separator AG, Oelde, Germany) at a flow rate of 6 l h^{-1}. To avoid membrane clogging the supernatant was prefiltered on a cellulose nitrate filter (0.45-μm pores, Whatman, England). The clear solution was concentrated by cross flow ultrafiltration in a Minisette cell (Filtron Techn. Corp., Clinton, MA, USA) using a Filtron cartridge (Type alpha, Screen Channel, molecular weight cut off: 30 kDa) at 4°C. Further concentration was performed in a cooled (ice bath), stirred ultrafiltration cell (Model 202, Amicon Inc., Beverly, MA, USA) using a DIAFLO membrane (Type PM 10, molecular weight cut off: 10 kDa). The crude enzyme concentrate was dialysed against Tris/HCl buffer (10 mM, pH 8.0) at 6–10°C.

2.3. Determination of proteolytic activity

The proteolytic activity was measured according to Lin, Means and Feeney (1969) using N,N-dimethylcasein as the substrate. The enzyme reaction was carried out at pH 7.5 in 0.1 M phosphate buffer at 48°C for 30–60 min. The reaction was stopped by immersing the test tubes in boiling water for 5 min. In the case of a cell suspension as enzyme sample, the tubes were centrifuged prior to boiling the supernatant. For inhibition experiments the enzyme solution was preincubated together with the inhibitor at 30°C for 1 h and the reaction was carried out in presence of the respective inhibitor.

2.4. Determination of lactate dehydrogenase activity

Lactate dehydrogenase activity was determined by the method described by Thomas (1975).

2.5. Electrophoretic separations

2.5.1. Native electrophoresis

The discontinuous electrophoresis system of Ornstein (1964) was used to analyse the fragments after autodigestion of the proteases. Electrophoresis was carried out in a Mighty Small II SE 260 electrophoresis unit (Hoofer Scientific Instruments, San Francisco, CA, USA) at a constant current of 16 mA on cooling by tap water. The gel was stained for protein with Coomassie Brilliant Blue R250 in methanol–acetic acid–water (40:10:50) and destained in the same solvent.

2.5.2. Nondenaturating SDS-electrophoresis

Modifications for the determination of the proteolytic isoenzyme pattern according to Lacks and Springhorn (1980) and Brown, Yet and Wold (1982) were used. The separating gel contained 0.1% gelatin as a protease substrate. To avoid interaction between the substrate and the enzymes, the electrophoresis was carried out in the presence of 0.1% SDS. Samples were mixed with sample buffer containing 3% SDS at room temperature. SDS addition did not influence the migration of the proteases in comparison to native electrophoresis. After electrophoretic separations, the gels were gently shaken in 50 ml of 2.5% Triton X-100 to remove SDS (30°C, 0.5–2 h) and, depending on the concentration of the proteases, incubated in phosphate buffer (0.1 M, pH 7.5) at 30–48°C for up to 2 h. After staining and destaining the proteolytic enzymes appeared as clear bright bands on a blue background.

2.6. Autodigestion experiments

Studies were carried out at a temperature of 38°C. To observe the sequence of autodigestion, the gel pieces (six for each species), containing the respective protease, were incubated in tightly closed 0.5 ml Eppendorf vials for 0.5, 1, 2, 4, 6 and 17 h. The reaction was stopped by freezing (−20°C). For fragment analysis, the gel pieces were put into the wells of another separating gel and submitted to electrophoresis. To detect all fragments formed, the gel was subsequently stained with Coomassie Blue, whereas the identification of proteolytic active fragments was done in another gel by activity staining.

2.7. Extraction of cell wall associated proteases

Cells were harvested by centrifugation at 4°C (5000×*g*; 15 min) after cultivation for 37.5 h in shaker flasks, as described above. The pellet was washed twice in cold (4°C) phosphate buffer (pH 7.5, 0.1 M) and resuspended in the same buffer.

To extract the cell-associated proteases, the cell suspension was incubated at ambient temperatures of 4, 30, 40, 50 and 60°C for a period of 2 h. Samples were withdrawn after 20, 40, 60 and 120 min of incubation and the cells removed by centrifugation (5000×*g*; 5 min). The proteolytic activity, as well as the electrophoretic protease pattern in the supernatant, were determined. Additionally, the activity of an intracellular marker enzyme (lactate dehydrogenase) was measured to detect lysis of the cells during incubation.

3. Results

3.1. Isoenzyme pattern

The electrophoretic isoenzyme pattern (Fig. 1) of the strains ATCC 9172 and L 200 show several bands of different intensities and are very similar; the only differences are detectable with protease species of higher mobility (Fig. 1). In contrast, strain ATCC 9174 obviously forms only one protease, an enzyme which has been purified and characterised by Rattray et al. (1995).

The protease profile of a submerged cultivation of *B. linens* ATCC 9172 is shown in Fig. 2. There are minor changes in the prorease pattern during active growth, i.e. within the first 40 h of cultivation whereas, during the stationary phase, protease A as well as proteases B1 and B2 clearly decrease between 65 and 161 h. Nevertheless, the low molecular weight isoenzyme seems to increase consistently. The changes in the protease pattern in the stationary growth phase are similar to the observations made by Strauss et al. (1994), where active proteases of higher molecular weight disappeared during incubation of a crude enzyme solution at ambient temperature, whereas the intensity of those with lower molecular weight increased. The main bands, after separation by native electrophoresis, were classified as protease isoenzymes A, B, C and D. The exoproteolytic activity of L-leucine-aminopeptidase (LAP), an enzyme which is also formed by *B. linens*, is not detectable by the procedure used for activity staining.

3.2. Autodigestion studies

In order to suppress the formation of LAP by *B. linens* ATCC 9172, the organism was cultivated in the presence of 3% NaCl (Hayashi, Cliffe & Law, 1990b). The enzyme shows an electrophoretic mobility similar to the proteases B1 and B2. Suppression of aminopeptidase

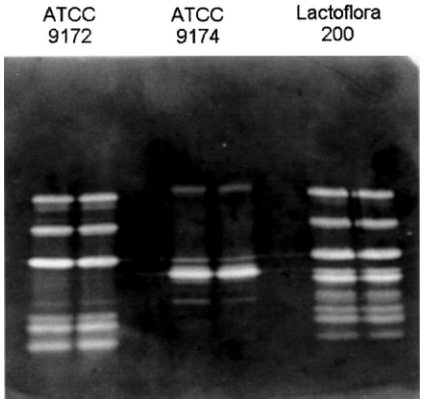

Fig. 1. Isoenzyme pattern from the culture filtrate of several strains of *Brevibacterium linens* by nondenaturating electrophoresis (11% T) with subsequent activity staining. Cells were cultivated in 1-l flasks with 250 ml of medium containing 5 g/l Hy-Case and 1 g/l soy-peptone on a rotary shaker (250 rpm, 28–30°C, 48 h).

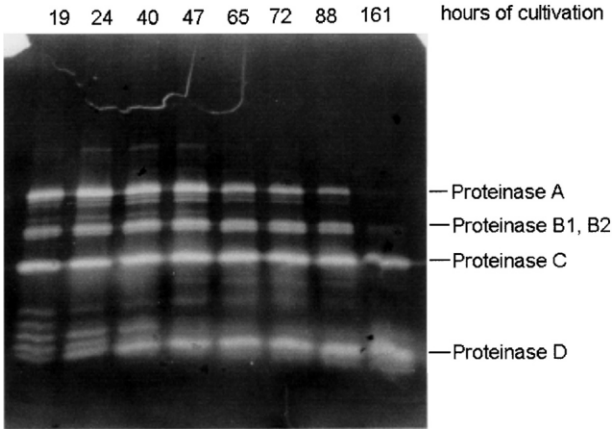

Fig. 2. Isoenzyme pattern during the cultivation of *Brevibacterium linens* ATCC 9172 [non-denaturating electrophoresis (11% T) with subsequent activity staining]. Cells were cultivated in 1 l flasks with 250 ml of medium containing 5 g/l Hy-Case and 1 g/l soy-peptone on a rotary shaker (250 rpm, 28–30°C, 161 h).

was achieved at a level of about 95%, thus enabling the detection of the proteases B1 and B2 in native electrophoresis and subsequent staining for proteins (Coomassie Blue) without any interference.

The proteases A and C could be separated and isolated by preparative slab gel electrophoresis and by excision of the relevant gel piece, whereas proteases B1 and B2 could not be separated from each other and were excised and treated together in the autodigestion studies.

For detecting the sequence of enzyme autodigestion, the gel pieces containing the relevant protease were incubated at 38°C and the protein fragments formed were separated by both, native and non-denaturating electrophoresis, and detected by activity and protein staining.

Protease A (Fig. 3), a protein of approximately 280 kDa as determined by gel filtration, was decomposed within 2–4 h into a proteolytically active fragment of approximately 130 kDa molecular mass, which is equivalent to protease C in the crude proteolytic enzyme preparation (CF). Further degradation formed only one smaller proteolytic fragment named protease D, after native electrophoresis and staining for proteins, whereas up to three main bands (Fig. 3b, PF) are formed after non-denaturating SDS-electrophoresis and staining for proteolytic activity. The higher sensitivity of the activity staining may perhaps explain this phenomenon. Protease D is not decomposed further and is still active within 17 h of incubation, indicating it to be stable against autodigestion. There are only small amounts of that protease species detectable in the CF.

Incubation of a mixture of the proteases B1 and B2 showed similar results (Fig. 4). The bands of B1 and B2 disappeared within 4 h of incubation, forming a fragment equivalent in mobility to protease C, which is further decomposed into a proteolytic fragment comparable to protease D and two other proteolytic fragments (Fig. 4b, PF) as described for proteinase A.

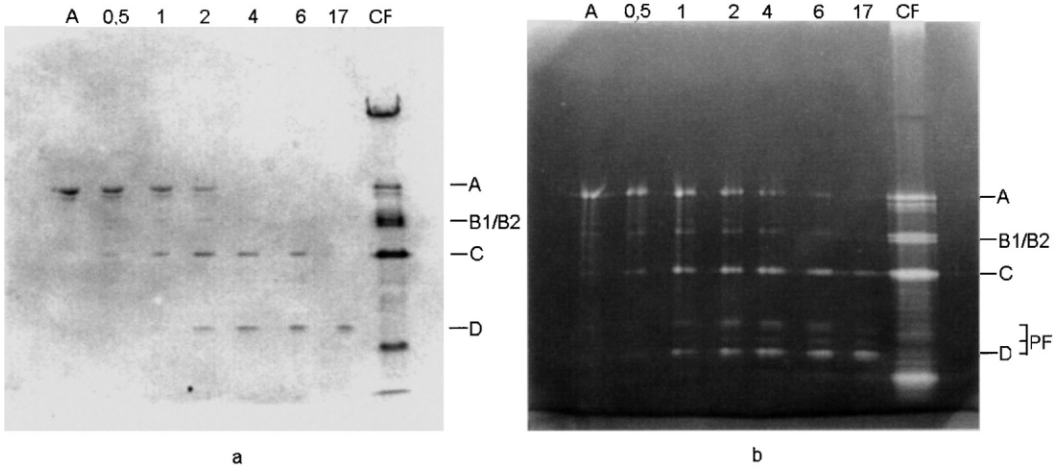

Fig. 3. Native (a) and non-denaturating (b) electrophoresis (11% T) with subsequent protein (a) and activity (b) staining. A: isolated proteinase A; autodigestion products after x hours of incubation at 38°C, CF: concentrated culture filtrate; PF, proteolytic fragments.

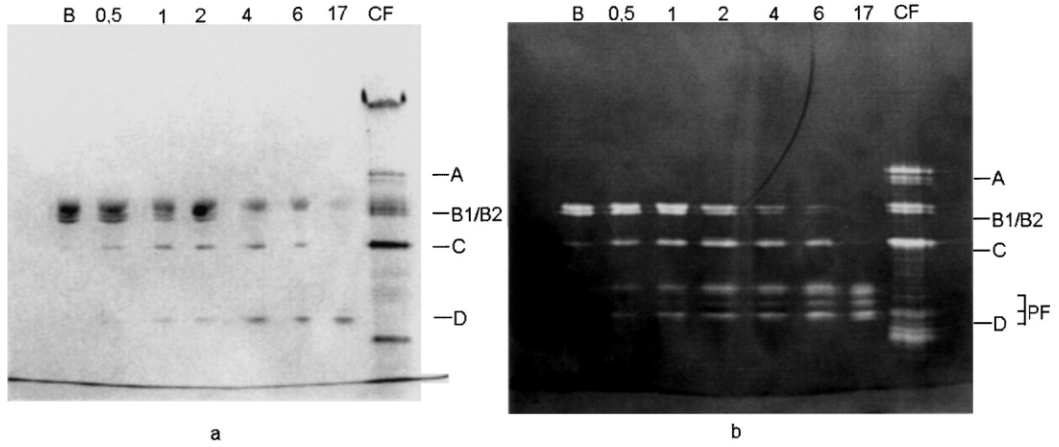

Fig. 4. Native (a) and non-denaturating (b) electrophoresis (11% T) with subsequent protein (a) and activity (b) staining. B isolated proteases B1 + B2; autodigestion products after x hours of incubation at 38°C; CF, concentrated culture filtrate; PF, proteolytic fragments.

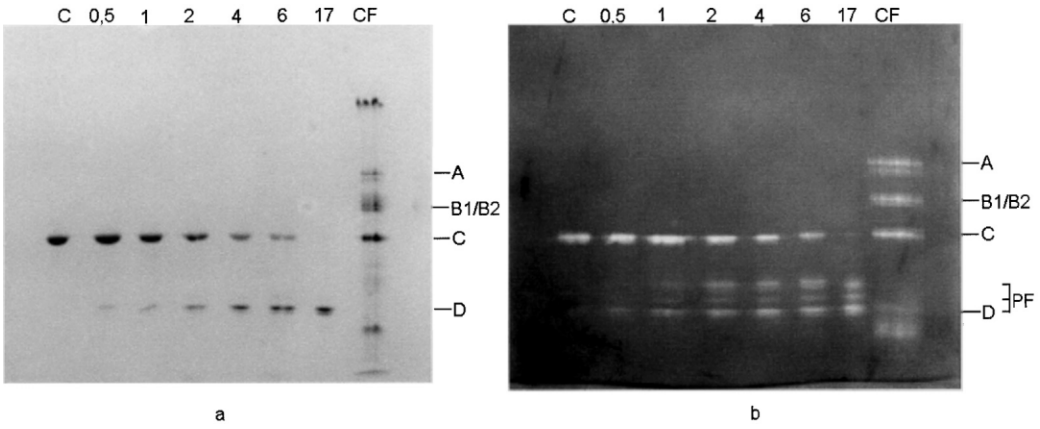

Fig. 5. Native (a) and non-denaturating (b) electrophoresis (11% T) with subsequent protein (a) and activity (b) staining. C: isolated protease C; autodigestion products after x hours of incubation at 38°C, CF: concentrated culture filtrate; PF, proteolytic fragments.

Protease C (Fig. 5) seems to be the most stable protease of the isoenzymes studied. Bands of the enzyme did not disappear within 6 h of incubation and fragments formed revealed a pattern on electrophoresis (Fig. 5a; Fig. 5b, PF) similar to that described above for proteinase A (Fig. 3b, PF) and proteinases B1 and B2 (Fig 4b, PF). Isolation of these fragments (PF, Fig. 5b), according to the procedure described for the other isoenzymes, could not be achieved, due to their small differences in electrophoretic mobility.

There seems to be an ordered sequence in the autodegradation of proteases from species of high molecular weight to fragments of low molecular weight. Nevertheless, the decay of protease A does not obviously occur via the step of B1 and B2 fragments.

For detecting subunit components and the estimation of molecular weights of the isolated species, i.e. proteases A and C, the mixture of proteases B1 and B2, as well as the product of autodigestion of protease C (= protease D), the relevant gel pieces were treated with SDS and ß-mercaptoethanol for 5 min at 100°C and separated by standard SDS-PAGE according to Lämmli (1970). The results (Fig. 6) indicate that proteinase A as well as protease B1 and B2 form aggregates which are composed of several subunits (5–6) of different size. The molecular weights of the individual subunits range from 45 to 68 kDa. Aggregates of proteinase C contain only three different subunits, the 53 kDa species being the main component. The product of autocatalytic degradation of protease C, which was classified as protease D, showed only one component of approximately 43 kDa molecular mass.

3.3. Cell wall associated proteases

Determination of proteolytic activity in the culture broth and the cell free medium revealed that about 50% of the extracellular proteases of *B. linens* ATCC 9172 is associated with the cell wall (Table 1). The extraction of the proteases from the cells could be achieved, if the cells were incubated in phosphate puffer (0.1 M; pH 7.5) at elevated temperatures (Fig. 7). At 4°C and ambient temperature (25°C) no activity was found in the supernatant, indicating that the proteases remain on the cell surface. An almost complete release of protease from the cells can be achieved by incubation of cells at 50°C for 2 h. No activity was found on incubation at 60°C due to the denaturation of the proteases according to

Table 1
Distribution of extracellular proteolytic activity

	Biomass	Protease activity (PA)	PA related to biomass
	(g/l)	(U/l)	(U/g)
Supernatant	–	33.7	10.4
Culture broth	3.2	70.4	21.8
Cell suspension	11.6	100.8	8.7

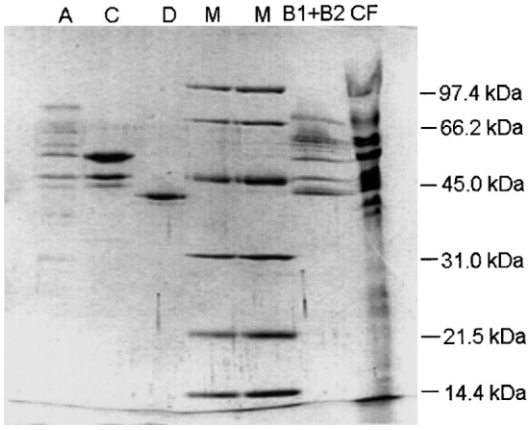

Fig. 6. Denaturating SDS-PAGE (12% T) of the purified proteases. M, molecular weight marker; CF, concentrated culture filtrate; A, protease A, B, protease B; C, protease C; D, protease D, formed by incubation of protease C at 38°C for 17 h).

the studies of Tomaschowá et al. (1998). There was no lysis of cells during incubation at all temperatures, as seen by the complete absence of the marker enzyme lactate dehydrogenase in the supernatant.

The isoenzyme pattern of the extracted proteases (Fig. 8) was comparable with that of an enzyme preparation from the cell-free culture filtrate. At higher temperatures, and for longer incubation, the intensities of bands of the low-size fragments increased, whereas those of the higher molecular enzyme species decreased. The results support the autocatalytic sequence elucidated by the autodigestion studies.

3.4. Specific inhibition

Chelating agents as EDTA and phenanthroline (1 mM) inhibited crude protease preparations to an extent of about 50% (Table 2), whereupon higher concentrations did not increase inhibition, indicating a stabilisation by divalent metal ions. The enzyme was almost completely inhibited by PMSF and 3,4-DCI (1 mM), suggesting them to be serine proteases. Although 3,4-DCI is covalently bound to the active site (Harper, Hemmi & Powers, 1985), the enzyme can be reactivated to an extent of about 90% by the addition of hydroxylamine (0.5 M) within 150–180 min. All other inhibitors tested did not affect proteolytic activity.

Inhibition experiments were also carried out with isolated isoenzymes using the relevant gel piece after electrophoretic separation. The protease preparation was preincubated in the presence of an inhibitor for 1 h prior to electrophoresis. Moreover, the inhibitor was added to all solutions used in the staining procedure. The experiments demonstrated that all proteolytically active bands disappeared in the presence of PMSF. In contrast, the addition of 3,4-DCI did not affect proteolytic activity; spontaneous regeneration of the active sites (Harper et al., 1985) may be the cause of this phenomenon.

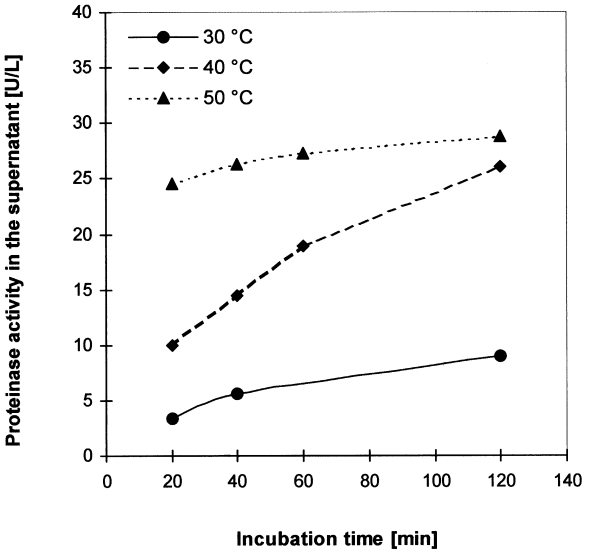

Fig. 7. Release of proteolytic activity from cells of *Brevibacterium linens* ATCC 9172.

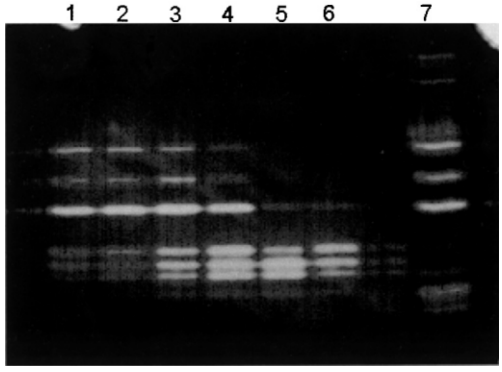

Fig. 8. Electrophoretic isoenzyme pattern of released extracellular proteases. Influence of temperature and incubation time **1**: 120 min at 30°C; **2**: 40 min at 40°C; **3**: 120 min at 40°C; **4**: 40 min at 50°C; **5**: 120 min at 50°C; **6**: 20 min at 60°C; **7**: culture filtrate.

Table 2
Specific protease inhibition[a]

Inhibitor	Protease activity (%)						
	0.01 μM	0.1 μM	1 μM	0.01 mM	0.1 mM	0.5 mM	1 mM
3,4-DCI	–	94	74	34	14	0	–
PMSF	–	–	99	98	69	23	4
Pefabloc SC	–	99	99	98	94	–	93
TPCK	–	–	96	100	98	96	85
EDTA	–	–	99	56	53	58	51
Phenanthrolin	–	–	96	98	83	54	52
E 64	–	100	99	99	97	–	96
Pepstatin A	98	100	95	98	–	–	–

[a] 3,4-DCI, 3,4-Dichloroisocoumarin; PMSF, Phenylmethylsulphonylfluoride; Pefabloc SC, 4-(2-Aminoethyl) benzolsulphonylfluoride-hydrochloride; TPCK, Tosylphenylalanylchlormethylketone; EDTA, Ethylendiamine tetraacetic acid; Phenanthroline, 1,10 Phenanthroline; E 64, L-*trans*-Epoxysuccinyl-leucylamid-(4-guanidin)-butan; Pepstatin A, Isovaleryl-Val-Val-(3S,4S)4-amino-3-hydroxy-6-methyl-heptane acid-Ala-(3S,4S)4-amino-3-hydroxy-6-methyl-heptane acid.

Inhibitors for other classes of proteases did not affect any band.

4. Discussion

According to Foissy (1973, 1974) the electrophoretic isoenzyme pattern of the proteases, formed by *B. linens*, is characteristic for each strain. This could also be demonstrated for the three strains tested in this work (ATCC 9172, ATCC 9174, Lactoflora 200). The culture filtrate of the strains ATCC 9172 and L 200 include several proteolytically active species, whereas strain ATCC 9174 forms only one protease. It could be clearly shown, that the multiplicity of proteases for strain ATCC 9172 is a result of aggregation from subunits and of autocatalytic degradation. Protease A (280 kDa), as well as proteases B1 and B2 (220 kDa each), are suggested to be aggregates of protease C (130 kDa), whereas protease D seems to be a fragment of proteolytic degradation of protease C. All proteases and proteolytically active fragments were identified as serine proteases. These results and the sequence of autodigestion may suggest that the multiple proteases of strain ATCC 9172 are the result of only one gene product. Protease C, the most stable of the high molecular weight proteases, was purified and characterised by Tomaschowá et al. (1998), having a molecular weight of 56 kDa by SDS-PAGE and 130 kDa by gelfiltration. The enzyme showed a pH optimum of 8.0, an optimal activity at 50°C and a stability in the pH range of 6.0 to 10.0; it was activated by Ca^{2+} and Mg^{2+}. Nearly identical properties were determined for the protease of *B. linens* ATCC 9174, which was purified and characterised by Rattray et al. (1995). The serine protease was a dimer (56 kDa by SDS-PAGE, 126 kDa by gel filtration) with a pH optimum at 8.5 and an optimal temperature of 50°C. It was stable between pH 6.0 and 10.0 and activated by Ca^{2+} and Mg^{2+} and NaCl.

Also, Hayashi et al. (1990a) found multiple forms of active protease for strain *B. linens* F. The five proteases which were purified and characterised were divided into two groups. The proteases in each group had almost identical properties. Moreover, the characteristics of the proteases of different groups were very similar. A similar autocatalytic behaviour to strain ATCC 9172 is possible.

Further investigations gave evidence for the presence of cell-associated proteolytic activity. The isoenzyme pattern of these enzymes, which could be extracted from the cells at elevated temperature (50°C), was identical to that of the soluble proteases in the cell-free culture medium. Ezzat, El-Soda, El-Shafei and Olson (1993; Ezzat et al., 1994) characterised a cell-associated protease of strain CNRZ 944, which could be solubilised by incubation of the cells in an appropriate buffer for 1 h at 30°C.

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