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Purification and characterization of extracellular proteinase produced by *Brevibacterium linens* ATCC 9172

Jarmila Tomaschová,[a]* Wolfgang Buchinger,[b] Werner Hampel[b] & Jaroslav Zemanovič[a]

[a]*Department of Milk, Fat and Food Hygiene, Faculty of Chemical Technology, Slovak University of Technology, 812 37 Bratislava, Slovak Republic*
[b]*Department of Biochemical Technology and Microbiology, University of Technology, 1060 Vienna, Austria*

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The micro-organism *Brevibacterium linens* ATCC 9172 produced five extracellular proteinases, as shown by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) with copolymerized gelatine. One of these was purified to homogeneity by ion-exchange chromatography and native preparative PAGE. The optimum pH and temperature for the proteinase were 8.0 and 50°C, respectively. The enzyme remained stable over a pH range from 6 to 10. The molecular weight estimated by SDS–PAGE was 56 kDa. Serine proteinase inhibitors 3,4-dichloroisocoumarin (3,4-DCI) and phenylmethylsulphonylfluoride (PMSF) inhibited, while Mg^{2+} and Ca^{2+} ions activated the proteinase.

INTRODUCTION

Brevibacterium linens is a coryneform bacterium with a typical orange-reddish colour. It is a well-known component of the bacterial smear of cheeses such as Tilsit, Limburger, Gubeen, etc. (Clancy and O'Sullivan, 1993). This bacterium produces esterases, amylases, lipases, peptidases and proteinases (Foissy, 1974). The growth of *B. linens* is important for the development of the characteristic aroma and flavour of surface-ripened cheeses.

Foissy (1978*a*,*b*) purified a leucinaminopeptidase from *B. linens* ATCC 9174 using gel filtration on Sephadex G-100 and preparative electrophoresis. The enzyme was stable between pH 3.0 and 11.5. The maximum activity of the purified enzyme was at pH 9.6 between 26 and 30°C. The molecular weight was estimated by thin-layer chromatography on Sephadex G-100 to be approximately 95 kDa and by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) to be 48 kDa. Rattray *et al.* (1995) purified an extracellular serine proteinase of *B. linens* ATCC 9174 by ion-exchange and hydrophobic interaction chromatography. The optimum values of pH and temperature of the proteinase were 8.5 and 50°C, respectively. They suggest that the native enzyme exists as a dimer. Juhász *et al.* (1990) purified an extracellular proteinase from *B. linens* with a molecular weight of about 52–55 kDa and optimum activity between pH 7.0 and 9.0 and at 45°C. Clancy and O'Sullivan (1993) partially purified and characterized a thermostable proteinase from *B. linens* IDM 376 which showed maximum activity at pH 7.5 and 67.5°C on azocasein. The enzyme had a molecular weight of 18.5 kDa. Hayashi *et al.* (1990) worked with *B. linens* F. Five serine proteinases were purified by a series of column chromatography. The molecular weights estimated by gel filtration were 37, 37, 44, 127 and 325, respectively.

This paper describes the purification and characterization of one of five extracellular proteinases produced by *B. linens* ATCC 9172.

MATERIALS AND METHODS

Bacterial strain and cultivation media

Brevibacterium linens ATCC 9172 was obtained from the American Type Culture Collection (Rockville, USA). The micro-organism was grown in Labor-Pilot-Bioreaktor (CF 2000 Fermentor, Chemap AG, Switzerland) at pH 7.0, 28°C, 23 h, 3.25 O_2 $dm^3\,min^{-1}$ and 800 rpm. The culture medium contained [g 10 $litres^{-1}$]: 4.1 g $MgSO_4.7H_2O$, 0.058 g $FeSO_4.7H_2O$, 0.064 g

*To whom correspondence should be addressed.

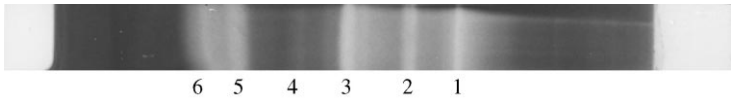

Fig. 1. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE; 12.4% T and 2.4% C gel) with copolymerized gelatine of the culture media.

$CaCl_2.2H_2O$, 2.7 g KH_2PO_4, 8.9 g $Na_2HPO_4.2H_2O$, 0.6 g NaCl, 1.2 g $(NH_4)_2SO_4$, 50 ml trace element solution (Wolin *et al*., 1963), 20 ml vitamin solution (Famelart *et al*., 1987) and 100 g Corn Steep Liquor (Austrian Agrarindustrie, Aschach, Austria) as the carbon source.

SDS–PAGE with copolymerized gelatine

A modification of the electrophoretic method of Heussen and Dowdle (1980) was used. The sample was prepared by mixing equal volumes of sample buffer (7.6 g litre^{-1} Tris, 30 g litre^{-1} SDS, 10% (v/v) glycerol and 50 mg litre^{-1} bromophenol blue, pH 6.8). The gels were stained with 0.1% Coomassie brilliant blue R-250 in ethanol:acetic acid:water (25:10:65), destained in ethanol:acetic acid:water (25:7:68) and evaluted by scanning on a TLC Scanner II (CAMAG, Switzerland).

Determination of enzyme activity

Proteolytic activity was determined by the method of Lin *et al*. (1969) using *N*,*N*-dimethylcasein as a substrate. The reaction was carried out at pH 7.5, 38°C for 1 h.

Purification of enzyme

Isolation of proteinase from culture media
After fermentation, the cells were removed on a Laborseparator (LWA 205, Westfalia Separator AG, Germany) at a flow rate of 6 litres h^{-1}. Proteins in the cell-free supernatant were concentrated by ultrafiltration on Filtron Minisette Cells (Filtron, USA) to 100 ml using 10 kDa cut-off membrane and spray-dried (Büchi 190, Flavil, Switzerland) on Celite 545.

Ion-exchange chromatography
The spray-dried powder was diluted in 0.01 M Tris–HCl buffer at pH 8.0, the Celite 545 separated by filtration and the filtrate dialysed against the 0.01 M Tris–HCl buffer at pH 8.0 overnight at 6°C. The sample was then applied to a column packed with DEAE–Sepharose CL-6B connected to a Bio-Rad Econo System (Bio-Rad Laboratories GmbH, Germany) and equilibrated with 0.01 M Tris–HCl buffer at pH 8.0. After eluting non-binding materials, the column was washed with a linear gradient of NaCl from 0 to 0.5 M in the 0.01 M Tris–HCl buffer. Fractions of 3 ml were collected and assayed for proteolytic activity and protein content by absorbance at 280 nm. The proteolytically active fractions were pooled, dialysed against 0.05 M Tris–HCl buffer at pH 8.0 and 6°C and concentrated to 1 ml using Centricon-10 (Amicon, USA).

Native preparative PAGE
Preparative electrophoresis was performed in Prep Cell 491 (Bio-Rad Laboratories, CA, USA). One millilitre of the sample (20 mg protein) was added to 3 ml sample buffer and loaded on to a gel (5 cm high 12% resolving and 2.5 cm high 4% stacking gel) for purification. Electrophoresis was performed at 12 W under cooling with ice-cooled anode buffer and operating the unit in a 6°C coldroom. The proteinase was collected by end elution with 50 mM Tris–HCl buffer at pH 8.0 at 1 ml min^{-1}. Fractions of 3 ml were collected.

Characterization of the enzyme

Effects of pH and temperature
The pH optimum of the enzyme was determined with dimethylcasein dissolved in buffers over the pH range 5.0–9.0. The pH stability was determined by incubating the enzyme with buffers (2 h, 20°C) between pH 3.0 and 12.0. The effect of temperature on enzyme activity was measured between 20 and 60°C.

Effect of inhibitors and ions on proteinase activity
The effects of ethylenediamine tetraacetic acide (EDTA), phenylmethylsulphonylfluoride (PMSF), 3,4-dichloroisocoumarin (3,4-DCI), SDS, Mg^{2+} and Ca^{2+} on proteinase activity were determined by adding the proteinase to a dimethylcasein solution containing the desired concentration of inhibitors and activators.

Molecular weight determination
The molecular weight of the proteinase was determined by SDS–PAGE (12.4% T, 2.4% C separating and 4% T, 2.4% C stacking polyacrylamide gel).* The sample was prepared by mixing equal volumes of sample buffer (7.6 g litre^{-1} Tris, 30 g litre^{-1} SDS, 10% (v/v) glycerol, 50 mg litre^{-1} bromophenol blue, 1% β-mercaptoethanol pH 6.8) and heating to 100°C for 3 min. The molecular weight marker SDS-6 (Sigma, USA), containing pepsin, trypsinogen, lyzozyme, β-lactoglobulin, egg and bovine albumins, was used.

*C, content of *N*,*N*-methylenebisacrylamide in mixture of acrylamide and *N*,*N*-methylenebisacrylamide; T, concentration (%) of mixture of acrylamide and *N*,*N*-methylenebisacrylamide in gel.

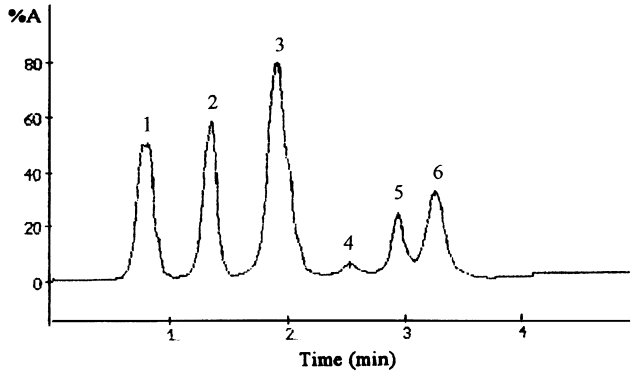

Fig. 2. Densitometry recording of sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) with copolymerized gelatine.

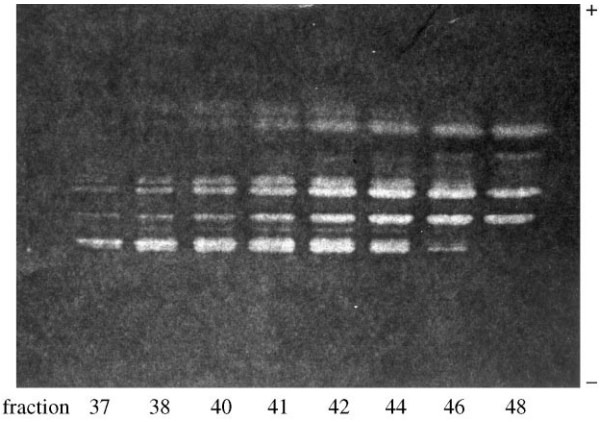

Fig. 4. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) with copolymerized gelatine of elution fractions with proteolytic activity from the ion-exchange chromatography.

RESULTS AND DISCUSSION

Purification of the proteinase

The culture media were treated as described in Materials and Methods. SDS–PAGE with copolymerized gelatine of the sample showed the presence of five proteolytic active bands (Fig. 1). Furthermore, the gel was evaluated by scanning on a TLC SCANNER II. The proteinase with the highest proteolytic activity (Fig. 2) was purified by ion-exchange chromatography and native preparative electrophoresis. The first step of purification was the ion-exchange chromatography. The dried powder was dissolved in 0.01 M Tris–HCl buffer at pH 8.0 and dialysed against 0.01 M Tris–HCl buffer at pH 8.0 and 6°C. Fifteen millilitres of this sample was applied to a column (3.5 × 15 cm) of DEAE–Sepharose CL-6B. Enzymes were eluted with a linear gradient of 0–0.5 M NaCl in 10 mM Tris–HCl buffer at pH 8.0. The assay of proteolytic activity indicated the presence of one proteolytically active peak (Fig. 3). The yield in this step was 83% of applied activity. This step resulted in a separation of contaminating material, but no isolation of proteinase was achieved (Fig. 4). The protease-rich fractions, 37–48 (Fig. 3), were pooled, concentrated using Amicon-10 (Amicon), mixed with sample buffer (1:3) and applied to a stacking gel for native preparative electrophoresis. The eluates were analysed by analytical SDS–PAGE with copolymerized gelatine (Fig. 5). As shown in Fig. 6, the third proteinase was purified to homogeneity. Native preparative electrophoresis is a good method for the isolation of heat-labile samples, as it allows the possibility of working at low temperatures, it is very fast and easy to process. Foissy (1978*a*) purified an aminopeptidase from *B. linens* ATCC 9174 by gel filtration and native preparative electrophoresis to homogeneity. Rattray *et al.* (1995) purified an extracellular proteinase by ion-exchange and hydrophobic interaction chromatography.

Characterization of the proteinase

Effect of pH and temperature on enzyme activity

The effect of pH on the purified proteinase was examined as described in Materials and Methods. The pH

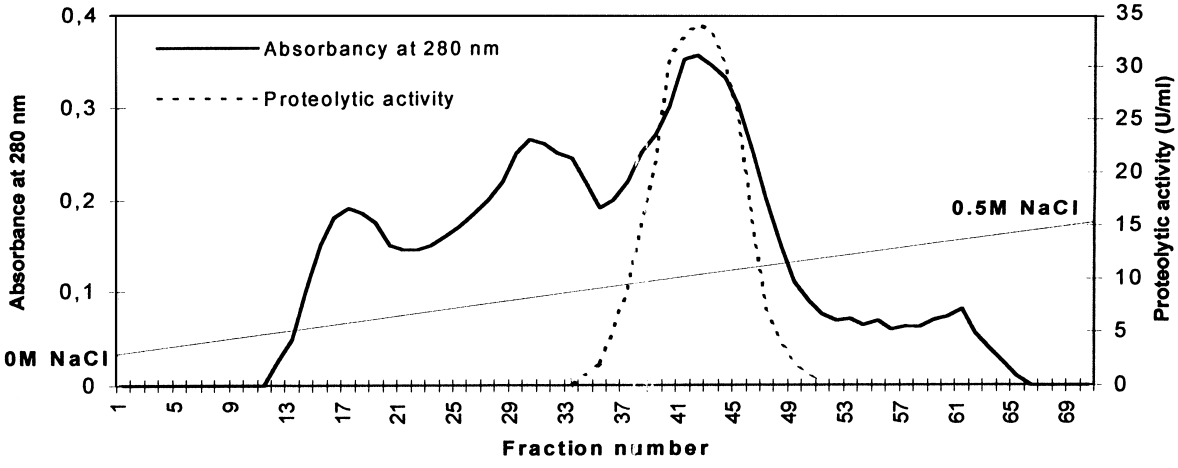

Fig. 3. Elution profile of DEAE–Sepharose CL-6B chromatography. The dialysed sample was applied to the column packed with DEAE–Sepharose CL-6B and separated with a linear gradient of 0–0.5 M NaCl in 10 mM Tris–HCl buffer at pH 8.0.

optimum was 8.0. At neutral pH, only 50% of enzyme activity remained (Fig. 7A). The enzyme was stable over the pH range 6.0–10.0 (Fig. 7C). The effect of temperature on enzyme activity is shown in Fig. 7B. The enzyme was most active at 50°C. Similar results were obtained by Rattray *et al.* (1995). The purified proteinase was stable in the pH range 6.0–10.0 and the optimum pH and temperature were 8.5 and 50°C, respectively. Juhász *et al.* (1990) have purified an extracellular proteinase *B. linens* with optimum activity from pH 7.0 to 9.0 and at 45°C. Březina *et al.* (1987) partially purified four serine proteinases produced by *B. linens*, with pH optima in the range 5.0–8.0 and with temperature optima at 50°C. Clancy and O'Sullivan (1993) purified a thermostable proteinase from *B. linens* IMD 376 with the temperature optimum at 67.5°C.

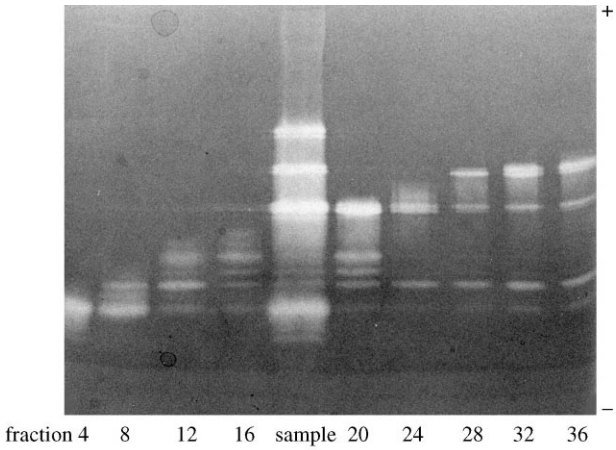

Fig. 5. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) with copolymerized gelatine of every fourth elution fraction with proteolytic activity from the native preparative electrophoresis.

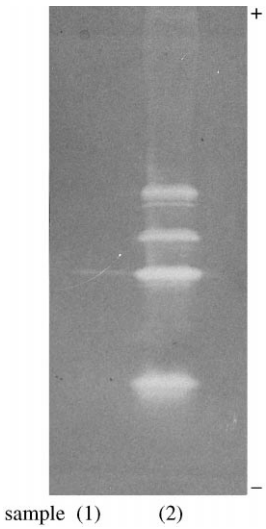

Fig. 6. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) electrophoresis with copolymerized gelatine (12.4% T and 2.4% C) of the purified proteinase (1) and non-purified sample (2).

Effect of some chemicals and ions on proteinase activity

EDTA at 1 mM inhibited the proteinase only to 40%. But a higher concentration of this metalloproteinase inhibitor, does not increase the percentage of inhibition. It is suggested that the proteinase was stabilized with some metal ions. The enzyme was strongly inhibited by PMFS and 3,4-DCI, indicating that this was a serine proteinase. Mg^{2+} and Ca^{2+} (10 mM) caused an increase of the proteolytic activity to about 140 and 128%, respectively; however, 0.5% SDS acted as an inhibitor. Only 23% of original activity remained. It has been

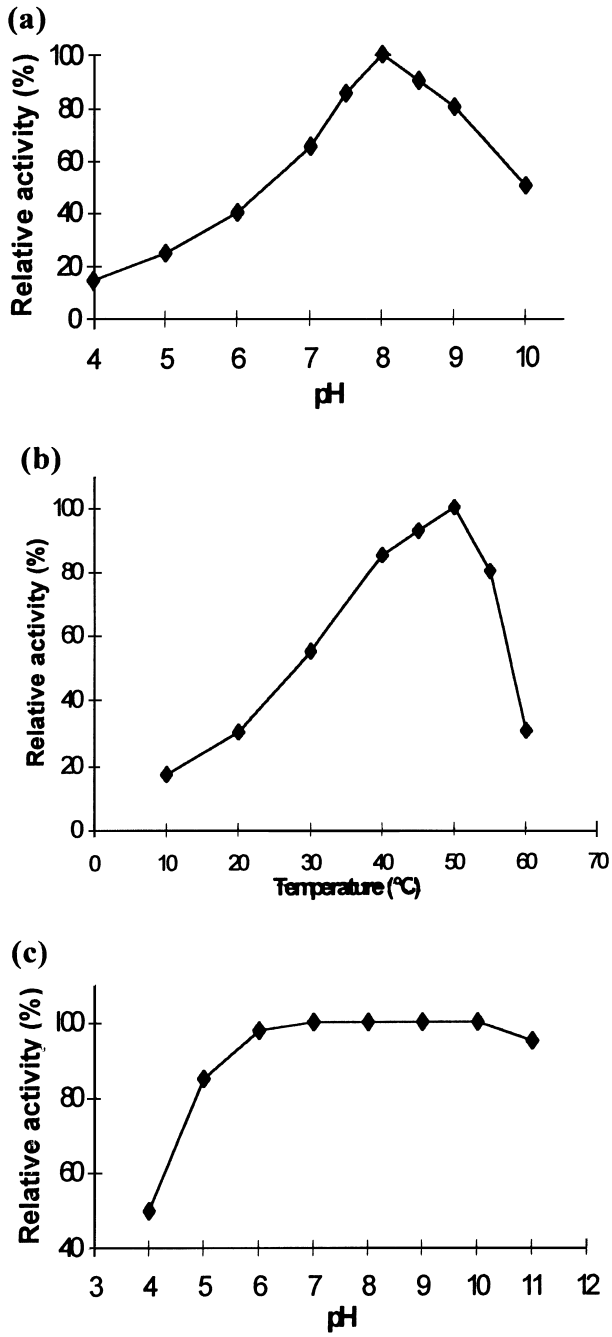

Fig. 7. Effect of pH (a) and temperature on proteolytic activity (b) and pH stability (c) of the proteinase produced by the micro-organism *Brevibacterium linens* ATCC 9172.

reported that *B. linens* produced serine (Hayashi *et al*., 1990; Juhász and Škárka, 1990; Rattray *et al*., 1995) and sulphydryl extracellular proteinases (Clancy and O'Sullivan, 1993).

Molecular weight determination

The molecular weight of the enzyme was determined to be approximately 56 kDa on 12.4% T and 2.4% C SDS–polyacrylamide gel. The molecular weight of native enzyme was not determined. Rattray *et al*. (1995) worked with *B. linens* ATCC 9172 and purified an extracellular proteinase with the same molecular weight determined by SDS–PAGE. By HPLC gel filtration, a single peak corresponding to a molecular weight of 126 kDa was detected. The authors suggested that the native enzyme exists as a dimer. Juhász *et al*. (1990) purified an extracellular proteinase from *B. linens* with a molecular weight of about 52–55 kDa.

The present results indicate that the purified proteinase from *B. linens* ATCC 9172 prepared in our laboratory had very similar properties to that produced by *B. linens* ATCC 9174 (Rattray *et al*., 1995). It is most likely that the proteinase from *B. linens* ATCC 9172 is the same as the proteinase from *B. linens* ATCC 9174. We believe that the presented characteristics of the enzyme will lead to a better understanding of the ripening process of surface-ripened cheeses.

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