Applied and Environmental Microbiology

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***Appl. Environ. Microbiol.* 1995, 61(9):3454.**

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Applied and Environmental Microbiology, Sept. 1995, p. 3454–3456 Vol. 61, No. 9
0099-2240/95/$04.00+0
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NOTES

Purification and Characterization of an Extracellular Proteinase from *Brevibacterium linens* ATCC 9174

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Received 21 February 1995/Accepted 21 June 1995

An extracellular serine proteinase from *Brevibacterium linens* ATCC 9174 was purified to homogeneity. pH and temperature optima were 8.5 and 50°C, respectively. The results for the molecular mass of the proteinase were 56 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 126 kDa by gel filtration, indicating that the native enzyme exists as a dimer. Mg^{2+} and Ca^{2+} activated the proteinase, as did NaCl; however, Hg^{2+}, Fe^{2+}, and Zn^{2+} caused strong inhibition. The sequence of the first 20 N-terminal amino acids was NH_2-Ala-Lys-Asn-Asp-Ala-Val-Gly-Gly-Met-Gly-Tyr-Leu-Ser-Met-Ile-Pro-Ser-Gln-Pro-Gly.

The surface microflora of various smear surface-ripened cheeses, such as Limburger, Gruyère, Münster, Brick, Appenzeller, and Tilsiter, includes yeasts, moulds, and bacteria (17, 19). Low-molecular-weight compounds produced at the surface through the action of various enzymes from the smear microflora diffuse into the interior of each cheese and contribute to the characteristic qualities of these cheeses; the extracellular enzymes are much too large to diffuse into the cheeses (20, 22). The growth of *Brevibacterium linens* on the surface is an essential prerequisite for the development of the characteristic flavors, colors, aromas, and textures of smear surface-ripened cheeses (1, 3, 15). Extracellular enzyme activities reported for *B. linens* include proteinase (7, 13, 16), aminopeptidase (7–11, 14), and esterase (7). The bacterium possesses intracellular peptidases (5, 7, 23, 26) and esterases (4, 7); cell-bound lipase and esterase activities have also been reported (24).

B. linens ATCC 9174 was obtained from the Microbiology Department, University College, Cork, Ireland. The organism was precultured in 250 ml of medium (1.0% trypticase peptone [wt/vol], 0.3% Bacto Peptone [wt/vol], 0.25% yeast extract [wt/vol], 0.5% glucose [wt/vol], 0.25% K_2HPO_4 [wt/vol], 0.02% $MgSO_4 \cdot 7H_2O$ [wt/vol]) in a 500-ml flask on an orbital shaker at 170 cycles per min and 23°C. After 48 h, 100 ml of preculture was used to inoculate 5 liters of the same medium in a 10-liter fermentor (B. Braun Diessel Biotech GmbH., Melsungen, Germany). The temperature was maintained at 23°C, with 10% O_2 saturation; the pH was not regulated.

After cultivation for 70 h, i.e., at late log phase, cells were removed by centrifugation at 14,500 × *g* for 25 min at 4°C. The cell-free supernatant was concentrated from 5 liters to 250 ml by ultrafiltration with a Sartocon Mini SM 17521 unit, which was fitted with 20-kDa cutoff membranes (Sartorius AG, Goettingen, Germany).

Proteinase activity was measured by the method described by Twinning et al. (27); 40 μl of fluorescein isothiocyanate-labelled casein (0.5% [wt/vol] in H_2O) was added to 40 μl of 100 mM Tris-HCl buffer (pH 8.5), and then 20 μl of enzyme solution was added. The reaction mixture was incubated at 50°C for 15 min, after which 240 μl of 5% (wt/vol) trichloroacetic acid was added, and the mixture was held for 1 h and then centrifuged at 9,000 × *g* at 4°C for 5 min. A sample (200 μl) of supernatant was added to 2 ml of 500 mM Tris-HCl (pH 8.5), and the fluorescence of the mixture was determined by using a Luminescence Spectrometer LS 50B Perkin-Elmer (Bodenseewerk GmbH, Überlingen, Germany) at excitation and emission wavelengths of 490 and 525 nm, respectively. One unit of proteinase activity was defined as the amount of enzyme which released 1% of the total initial fluorescence of the fluorescein isothiocyanate-labelled casein as trichloroacetic acid-soluble fluorescence after incubation for 15 min.

The concentrated cell-free supernatant was dialyzed against 10 mM sodium phosphate buffer (pH 7.0) and applied to a DEAE-Sepharose Fast Flow anion exchange column (Pharmacia Biotech GmbH, Freiburg, Germany) connected to a Bio-Rad Econo System (Bio-Rad Laboratories GmbH, München, Germany). The column was washed initially with 1 liter of 10 mM sodium phosphate buffer (pH 7.0) and then with a linear NaCl gradient (0 to 0.4 M) in the same buffer (2 liter); the NaCl concentration was then maintained at 0.4 M for 720 ml to elute the enzyme. Proteinase activity was eluted from the column at 0.4 M NaCl, with a 10-fold increase in specific activity. Active fractions were pooled, the NaCl concentration was adjusted to 2.0 M, and the fractions were applied to a Phenyl-Sepharose CL-4B hydrophobic interaction column. The column was washed with 50 ml of 10 mM sodium phosphate buffer (pH 7.0) containing 2.0 mol of NaCl per liter and then with a linear NaCl gradient from 2.0 to 0 M (180 ml) by using a Bio-Rad Econo System. The enzyme was eluted from the column at 1.0 M NaCl, and specific activity was increased 16-fold. Active fractions were dialyzed against 10 mM sodium phosphate buffer (pH 7.0) and then applied to a Mono-Q HR5/5 column (Pharmacia Biotech GmbH). The column was washed with 25 ml of 10 mM sodium phosphate buffer (pH 7.0) and then with a linear NaCl gradient (0 to 0.5 M) in the same buffer (60 ml) by using a high-performance liquid chromatography (HPLC) Gradient Pump 2249 (Pharmacia Biotech GmbH), and the activity was eluted at 0.4 M NaCl, with a 20-fold

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TABLE 1. Purification of the extracellular proteinase from *B. linens* ATCC 9174

Purification step	Amt of total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification factor	Activity yield (%)
Cell-free supernatant	182.7	20,100	110	1	100
Ultrafiltration retentate	89.8	11,400	127	1.2	57
DEAE-Sepharose	8.3	9,100	1,096	10.0	45
Phenyl-Sepharose	4.5	7,800	1,733	15.8	39
Mono-Q	2.3	4,900	2,130	19.4	24

increase in specific activity. The results of the purification protocol are summarized in Table 1.

The purity of the isolated enzyme was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18) and isoelectric focusing (pI of 4.1). By SDS-PAGE, a single band with a molecular mass of 56 kDa was observed, while by HPLC gel filtration on a TSK-gel G3000SW column (TosoHaas GmbH, Stuttgart, Germany) with 10 mM sodium phosphate buffer (pH 7.0) containing 0.4 mol of NaCl per liter, a single peak corresponding to a molecular mass of 126 kDa was detected, suggesting that the native enzyme exists as a dimer. This is largely in agreement with results reported by Juhász and Škárka (16), who suggested that their proteinase (not purified to homogeneity) in its native form is a dimer, with 52-to 55-kDa subunits. The five extracellular proteinases, A, B, C, D, and E, which were reported by Hayashi et al. (13), had molecular masses of 37, 37, 44, 127, and 325 kDa, respectively, by SDS-PAGE.

The optimum pH of the purified proteinase was 8.5 at 50°C with sodium acetate (pH 5 to 5.5), sodium phosphate (pH 6 to 7), Tris-HCl (pH 7.5 to 8.5), and sodium carbonate (pH 9 to 11) buffers (Fig. 1a). The optimum temperature was 50°C after incubation for 15 min in Tris-HCl (pH 8.5) (Fig. 1b). After incubation of the proteinase (pH 8.5) for 24 h, no activity was lost at 30°C; however, at 50°C only 10% initial activity remained. The proteinase was quite stable at 20°C in the pH range of 6 to 10, with maximum stability at its optimum pH.

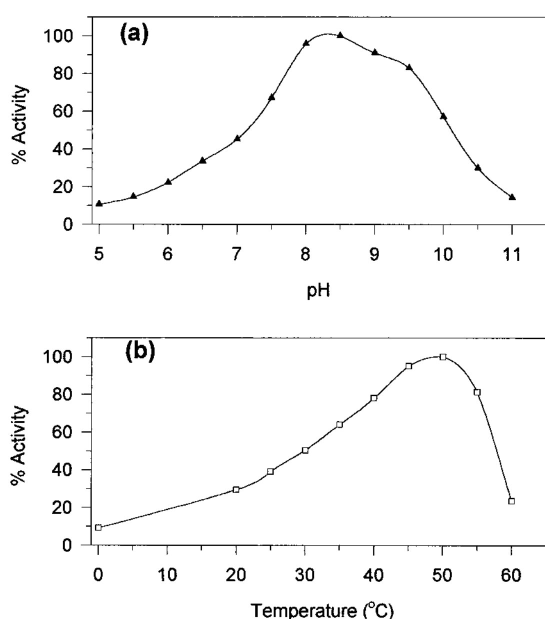

FIG. 1. Effects of pH at 50°C (a) and of temperature at pH 8.5 (b) on the activity of purified extracellular proteinase from *B. linens* ATCC 9174.

TABLE 2. Effect of chemical reagents and divalent cations on the activity of the extracellular proteinase from *B. linens* ATCC 9174

Reagent or cation[a]	Proteinase activity (%) 0.1 mM	Proteinase activity (%) 1.0 mM
Dithiothreitol	104	104
β-Mercaptoethanol	103	103
Cysteine	100	101
PCMB	77	26
Iodoacetic acid	94	62
Pefabloc SC	32	17
PMSF	69	44
EDTA	100	94
EGTA	99	85
1,10-Phenanthroline	107	83
Mg^{2+}	112	178
Ca^{2+}	109	117
Cu^{2+}	94	63
Mn^{2+}	92	49
Co^{2+}	96	41
Cd^{2+}	95	17
Ni^{2+}	90	25
Zn^{2+}	90	9
Fe^{2+}	97	0
Hg^{2+}	74	0

[a] PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethylsulfonyl fluoride; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid.

The effects of preincubating purified enzyme with activators or inhibitors for 15 min at 25°C are summarized in Table 2. Sulfhydryl-reducing agents or metal chelators had little effect on activity (Table 2). Iodoacetic acid and *p*-chloromercuribenzoate caused moderate inhibition, while the serine proteinase inhibitors phenylmethylsulfonyl fluoride and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefabloc SC; Merck, Darmstadt, Germany) caused extensive inhibition at 1.0 mM. Mg^{2+} and Ca^{2+} caused an increase in activity, while all the remaining metals caused reduction in activity, in the following order: $Hg^{2+} > Fe^{2+} > Zn^{2+} > Ni^{2+} > Cd^{2+} > Co^{2+} > Mn^{2+} > Cu^{2+}$ (Table 2). Proteinase activities were increased by 43, 76, and 63% in the presence of 0.14, 0.5, and 2.2 M NaCl, respectively.

The enzyme preparation was centrifuged through a ProSpin Sample Preparation Cartridge (Applied Biosystems Inc., Foster City, Calif.) onto a ProBlott polyvinylidene difluoride membrane. The N-terminal amino acid sequence was determined by Edman degradation on an automated pulsed liquid-phase protein-peptide sequencer (model 477A; Applied Biosystems Inc.). The sequence of the first 20 N-terminal amino acids was NH_2-Ala-Lys-Asn-Asp-Ala-Val-Gly-Gly-Met-Gly-Tyr-Leu-Ser-Met-Ile-Pro-Ser-Gln-Pro-Gly. Comparison of the N-terminal amino acid sequence against sequences from the Swiss-Prot release 28, PIR release 41, and GenBank release 84 data banks (DNASTAR Inc., Madison, Wis.) by the Lipman-Pearson protein alignment technique showed no significant homology. The purity of the enzyme was confirmed by no significant background interference during the N-terminal amino acid sequencing.

Although it is generally agreed that the extracellular proteinase of *B. linens* is a serine enzyme, considerable differences regarding its other properties have been reported elsewhere, perhaps because of wide interstrain variations (6, 21). The purified proteinase had pH and temperature optima of 8.5 and 50°C, respectively (Fig. 1a and b); pH and temperature optima

of 7.2 and 38°C, respectively, were reported (12) for the unpurified proteinase of *B. linens* 450, while the corresponding values from another *B. linens* strain were pH 7.0 and 25°C (25). Březina et al. (2) partially purified four extracellular serine proteinases from *B. linens*, with pH and temperature optima at 5.0 to 8.0 and 50°C, respectively. Hayashi et al. (13) reported that *B. linens* F produced five extracellular serine proteinases, i.e., A, B, C, D, and E, all with a pH optimum of 11; the temperature optimum for proteinases A and B was found to be 40°C, while proteinases C, D, and E were most active at 55°C. The pH and temperature optima of a partially purified extracellular serine proteinase from *B. linens* (isolated from the pure cheese culture Lactoflora 200; Laktos, Prague, Czech Republic) were reported (16) to be 7.0 to 8.5 and 45°C, respectively, which correspond closely with the values pH 8.5 and 50°C that were found in the present study for the proteinase of *B. linens* ATCC 9174. However, caution is required when comparing temperature optima which are not an intrinsic property of enzymes but which depend on incubation time and other factors; a short assay time yields a higher temperature optimum than a longer time does. The effects of pH and temperature on proteinase stability and activity, coupled with activation by NaCl, indicate that this enzyme should be able to contribute to proteolysis on the surfaces of smear-ripened cheeses.

We thank A. Healy, National Food Biotechnology Centre, University College, Cork, Ireland for the N-terminal amino acid sequencing. Financial assistance from a European Union COMETT grant is acknowledged.

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