EXTRACELLULAR PROTEINASE FROM *Brevibacterium linens*. PURIFICATION AND CHARACTERIZATION

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Brevibacterium linens produces proteolytic enzymes in cultivation broth. One of them was purified by application of a two step procedure involving ultrafiltration and molecular sieving. The isolated enzyme hydrolyzed natural substrates: casein, hemoglobin, ovalbumin and bovine serum albumin with ratios 22·2, 3·5, 1·6, 1·2 nkat mg^{-1}, respectively. For casein the apparent K_m 1·8 mg ml^{-1} was determined. The proteinase is fairly stable at pH 8·0 at 30°C. Short term incubation at 50°C denaturated the proteinase. SDS PAGE revealed an M_r of the proteolytic enzyme about 52 000 to 55 000.

There is ample evidence that proteolytic enzymes serve as key agents in many intra- and extracellular phenomena[1-3], as well as in industrial processes[4]. Purification schemes were reported for proteinases of various origin[5-12]. However, there is little knowledge about the biochemical properties of enzymes produced for food industry. *Brevibacterium linens*, widely used in manufacturing of surface ripening cheeses, manifested endo- and exoproteolytic activities[13-15]. An extracellular aminopeptidase synthesized by that species was studied by Foissy[16-17]. The aim of the present study is a biochemical characterization of an extracellular proteinase from cultivation broth of *B. linens*.

EXPERIMENTAL

Bacterial strain and cultivation: the strain of *B. linens* was originally obtained from Collection of Dairy Cultures Laktoflora Praha. The culture broth was routinely prepared from inorganic salts and proteins as inductors[18]. Prior to inoculation of bacteria the broth was sterilized, the culture was than kept at 28°C on a rotary shaker (180 rpm). Incubation was stopped when proteinase activity reached its maximum. The cells were harvested by centrifugation (15 000 × g. 10 min) and the supernatant was used for purification of an extracellular proteinase.

Purification: 500 ml of the culture broth was concentrated by ultrafiltration through YM 30 filter (Amicon) down to 10 ml. The concentrate was used for subsequent gel chromatography or was stored frozen. The concentrate (2 ml) was applied to a column of Sephacryl S 200 (1·6 × × 90 cm) equilibrated with 0·002 mol l^{-1} phosphate buffer pH 8·0. After elution at a flow rate

7·5 ml cm^{-2} h^{-1} with the same buffer the active fractions were pooled, ultrafiltrated and used for further experiments or stored frozen at −18°C.

Enzyme assay: proteolytic activity was determined by the method of Anson[19] with the following modifications: the substrate was casein (Difco Laboratories) dissolved in 0·25 mol l^{-1} NaCl buffered with 0·3 mol l^{-1} phosphate pH 8·0. The reaction was carried out in 2 ml 0·5% casein solution with an appropriate amount of enzyme at 45°C for 20 min. After addition of 3 ml 5% TCA, (w v^{-1}), the samples were centrifuged. To 1 ml of TCA soluble digestion product 2 ml of 0·6 mol l^{-1} NaOH and 0·6 ml Folin–Ciocalteu phenol reagent (MEDIKA Bratislava) diluted with water 1 : 1, were added. Zero time blank was included and absorbancy at 620 nm was measured. One unit of proteolytic activity was defined as the amount of the enzyme that liberated low molecular weight digestion products corresponding to 1 μmol tyrosine s^{-1}.

The proteolytic activity with hemoglobin (SERVA), ovalbumin (Difco Laboratories) and bovine serum albumin (MEDIKA) were measured at the same condition as casein hydrolysis.

Kinetic measurement: kinetics were measured at pH 8·0 with casein as substrate at concentrations between 0·5 and 10 mg ml^{-1}. The enzyme concentration was held constant approximately 1 mg ml^{-1}. Data were plotted according to Lineweaver and Burk.

Heat stability was tested by incubation of the enzyme solution in phosphate buffer pH 8·0 at temperatures of 40°C to 60°C for up to 4 hours. Residual activities were determined at indicated time intervals as mentioned before.

Protein concentration was determined by the method of Lowry et al.[20], using bovine serum albumin as a standard.

Molecular weight of the purified enzyme was determined by discontinuous gel electrophoresis in 10% separating gel, with SDS according to Laemmli[21] on a vertical slab at constant voltage 100 V. Molecular weight standard (LMW calibration kit — Pharmacia), was prepared as a sample and used for calibration of the gel. Proteins were stained with ammoniacal silver stain method of Oakley et al.[22].

RESULTS

The purification of an extracellular proteinase was carried out using a combination of ultrafiltration and gel filtration. The concentrate after YM 30 dialysis contained the majority of proteinase activity. The sample was applied to a column of Sephacryl S 200. Proteinase activity was eluted in a single peak (Fig. 1, Table I). The final preparation has about 15 fold higher specific activity compared to centrifugated fermentation broth. Upon examination of a purified sample on SDS polyacrylamide gel, it revealed two high molecular weight species 52 000 and 55 000 and one 20 000 polypeptide (Fig. 2). Taking into account the result of ultrafiltration the high molecular weight proteins have proteolytic activity.

The activity of isolated proteinase was tested on natural polypeptides. Each substrate was incubated in phosphate buffer with an equal amount of the enzyme at 45°C for 15 min. The rates of hydrolysis of casein, hemoglobin, BSA and ovalbumin were 22·2, 3·5, 1·6 and 1·2, respectively. This corresponds to relative specific activities for these substrates 100 : 16 : 7 : 5.

Kinetic properties of *B. linens* proteinase have been tested with casein as a substrate at pH 8·0 and temperature 45°C. When plotted according to Lineweaver and Burk a linear function was obtained with an x-intercept indicating an apparent K_m in the range of 1·8 mg ml^{-1} (Fig. 3).

Upon incubation at a constant pH 8·0 at various temperature and time intervals the course of denaturation of proteinase was monitored (Fig. 4). The results indicated an increased thermal sensitivity above 40°C. Short term incubation above 45°C virtually destroyed all activity. At 30°C a negligible loss of initial activity was noticed as compared to 90% at 50°C after 60 min incubation.

TABLE I
Purification of proteinase from *B. linens*

Step	Volume ml	Total activity nkat	Total protein mg	Specific activity nkat mg^{-1}	Recovery %
Supernatant	500	18 900	735	25·7	—
YM 30	10	9 968	146	68·3	52·6
Sephacryl S 200	1	260	0·64	406·0	1·4

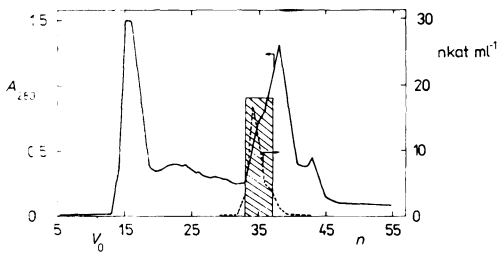

FIG. 1
Elution profile of the extracellular proteolytic enzyme of *B. linens* from Sephacryl S 200 column. A_{280} (———) absorption at 280 nm, nkat ml^{-1} (— — —) proteinase activity, *n* fraction number

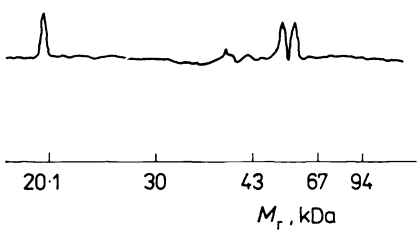

FIG. 2
Densitograph of SDS PAGE profile of purified *B. linens* proteinase. Calibration standard was LMW Calibration kit. M_r molecular weights of standard proteins: 20·1 trypsin inhibitor, 30 carbonic anhydrase, 43 ovalbumin, 67 bovine serum albumin, 94 phosphorylase b

DISCUSSION

This study demonstrates the presence of an extracellular proteolytic enzyme in the cultivation broth of *B. linens* and reports a simple method for its partial purification and characterization. Ultrafiltration, the initial step, yielded a product with modestly enhanced specific activity. A portion of that was further separated chromatographically on a molecular sieving column. This step resulted in a separation from contaminating material. The obtained fraction with proteolytic activity represented only about 1·2% of the proteins applied to the column. At this stage the final preparation has been purified about 15 fold with approximately 1·4% recovery of activity.

Unlike most proteolytic enzymes of microbial origin *B. linens* proteinase exhibits a narrow substrate specificity[11,23]. Under the conditions used only casein was hydrolyzed effectively. For casein at optimal reaction conditions the apparent K_m value 1·8 mg ml^{-1} was found. In this respect the proteinase of *B. linens* resembles the enzyme isolated from *P. aeruginosa* (ref.[24]).

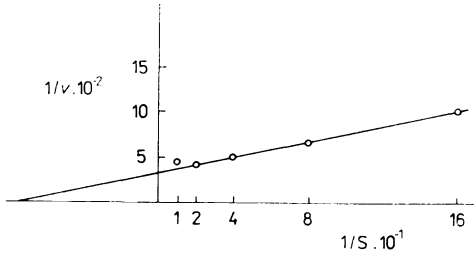

FIG. 3

Kinetics of caseinolysis by *B. linens* extracellular proteinase. Reaction conditions were: temperature 45°C, pH of the substrate 8·0. v reaction velocity (nkat ml^{-1}), S substrate concentration (mg ml^{-1})

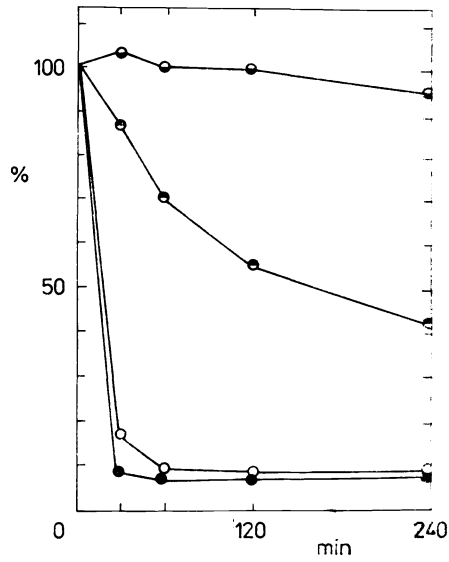

FIG. 4

Thermal stability of extracellular proteinase of *B. linens*. At pH 8·0 and at 30°C ◒, 40°C ◓ 50°C ○, 55°C ● the samples were kept up to four hours. Then the residual activity on casein was measured. The control value was 57·6 nkat ml^{-1} (100%)

The isolated enzyme was found to be stable at 30°C up to 4 hours at alkaline pH. Short term incubation over optimal temperature of caseinolysis resulted in a loss of proteolytic activity. This observation may indicate the stabilizing effect of the substrate protein[24,25].

Electrophoresis of the inactivated enzyme in the presence of SDS revealed protein bands corresponding to M_r 52 000 and 55 000. These values are in good agreament with those reported by Foissy[18,19] for aminopeptidase of *B. linens*.

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