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Thermostable alkaline protease produced by *Bacillus thermoruber* — a new species of *Bacillus*

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Summary. The proteolytic activity produced by a new species of *Bacillus* isolated in our laboratory was investigated. This enzyme was purified to homogeneity from cell-free culture liquids of *B. thermoruber*. The purification procedure included ion-exchange chromatography on DEAE-Sephadex A-50 and α-casein agarose affinity chromatography. The protease consists of one polypeptide chain with a molecular weight of 39000 ± 800. The isoelectric point was 5.3; the optimum pH and temperature for proteolytic activity (on casein) was found to be pH 9 and 45°C respectively. Enzyme activity was inhibited by PMSF and EDTA. The stability was considerably increased by addition of Ca^{2+}, and the protease exhibited a relatively high thermal stability. The alkaline protease shows a preference for leucine in the carboxylic side of the peptide bond of the substrate. The K_m value for benzyloxycarbonyl-Ala-Ala-Leu-*p*-nitroanilide was 2.5 mM.

Introduction

During a screening on thermophilic microorganisms we isolated a spore-forming bacterium that, for its characteristics, represents a new species of *Bacillus*. This new species, designated *Bacillus thermoruber* has been described in a previous paper (Manachini et al. 1985). Additional studies have shown that *B. thermoruber* possesses high proteolytic activity that can be readily detected in a variety of media. This activity, found in the culture liquids, is due to the presence of a thermostable, alkaline protease.

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This enzyme has never been characterized and since the thermostability and the activity at high pH values of proteolytic enzymes are properties that can have many practical uses (Cheetham 1985; Ward 1983), we report in this paper the purification of the extracellular protease and provide information on its properties and characteristics.

Materials and methods

Bacterial strain. The strain of *B. thermoruber* used in this study was the type strain BT_2^T (MIM. 30.8.38) deposited in the collection of the Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Sezione di Microbiologia Industriale of Milan (Italy).

Culture conditions. The strain BT_2^T was cultivated in liquid medium (GYE) containing (g l^{-1} of tap water): glucose (sterilized separately), 15.0; yeast extract, 5.0; $CaCl_2 \cdot 2H_2O$, 0.2; pH 7. Incubation was made on alternating shaker (100 4-cm strokes min^{-1}) at 45°C. For the study of protease secretion versus growth of organism, 100 ml Erlenmeyer flasks containing 15 ml of the above medium were inoculated with 10^6 cells ml^{-1}. Every 8 to 12 h, the content of one of the flasks was employed to assess the growth by optical density measurement at 650 nm ($A_{650\,nm}$; 1-cm cuvette). A value of $A_{650} = 1.00$ corresponded to 6.5×10^8 microorganisms per ml. The supernatant from each sampling was then assayed for proteolytic activity (see below) using a casein solution as the substrate.

Enzyme assays. All enzyme assays were performed at 45°C unless stated otherwise.

The proteolytic activity was normally assayed in 50 mM Tris-HCl-2 mM $CaCl_2$ buffer (pH 9) with 0.625% casein (Hammarsten, Merck, Darmstadt, Germany). 4 ml of casein solution were incubated with 1 ml of enzyme preparation. After 30 min the reaction was stopped by the addition of 5 ml of 5% (wt/vol) trichloroacetic acid (TCA). The insoluble part of the mixture was removed by filtration on paper (Whatman n° 5) and the absorbance of the supernatant measured at 275 nm. One unity of proteolytic activity was defined as the amount of enzyme that liberated 1 µmol of tyrosine from casein per min under the above conditions.

The peptidase activity was determined by measuring *p*-nitroaniline liberation from the substrates benzoyl-Tyr-*p*-nitroanilide (Benzoyl-Tyr-pNA), benzyloxycarbonyl-Phe-*p*-nitroanilide (Z-Phe-pNA), succinyl-Gly-Gly-Phe-*p*-nitroanilide (Suc-Gly-Gly-Phe-pNA), benzyloxycarbonyl-Ala-Ala-Leu-*p*-nitroanilide (Z-Ala-Ala-Leu-pNA), benzyloxycarbonyl-Gly-Gly-Leu-*p*-nitroanilide (Z-Gly-Gly-Leu-pNA), pyroglutamyl-Gly-Arg-*p*-nitroanilide (Pyr-Gly-Arg-pNA) and benzyloxycarbonyl-Arg-*p*-nitroanilide (Z-Arg-pNA) (Novabiochem, Laufelfingen, Switzerland). The standard assay mixture contained 5 to 100 µl of the enzyme solution, 50 mM Tris-HCl-2 mM $CaCl_2$ buffer (pH 9) up to 1.25 ml and 25 µl of the substrate from the stock solution of 5 mg ml^{-1} in dimethylformamide. After 5 to 15 min of incubation, the reaction was stopped with 0.25 ml of 2 M sodium citrate buffer (pH 5) and the released *p*-nitroaniline was assayed spectrophotometrically at 410 nm (Strongin et al. 1978). The molar absorbance of *p*-nitroaniline at 410 nm was equal to 8900 M^{-1} cm^{-1}. One unit of activity is equal to the amount of the enzyme that hydrolyzes 1 µmol of the substrate per min, under the standard conditions.

The esterolytic activity was determined with benzoyl-L-arginine methyl ester (BAME) and acetyl-L-tyrosine ethyl ester (ATEE) as substrates. The hydrolysis of the two esters was performed as described by Strongin et al. (1978), by measuring the optical density at 254 and 237 nm respectively. The activity was expressed in arbitrary units (OD per min per mg of enzyme) not comparable with those for chromogenic peptide substrates.

Protein determination. Protein concentrations were determined by the Folin phenol method (Lowry et al. 1951). Bovine serum albumin was used as the standard.

Enzyme purification. At the end of the fermentation period, bacterial cells were removed by centrifugation and the cell-free supernatant was concentrated and dialyzed against 20 mM Tris-HCl-2 mM $CaCl_2$ buffer (pH 8.5) (buffer A) using Diaflo hollow fiber cartridges (cut-off 10000 MW) (Amicon Corp., Lexington, Mass.). The dialyzed material was applied to a DEAE-Sephadex A-50 column (2.5 × 20 cm) (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with the buffer A and eluted with a linear gradient of 0.0 to 1.0 M NaCl in the starting buffer. Fractions of 3 ml were collected; the flow rate was 25 ml h^{-1}. The active fractions were pooled, concentrated and dialyzed by ultrafiltration and chromatographed on an α-casein agarose column (1.6 × 12 cm) (Sigma, St. Louis, Mo.) equilibrated with the buffer A. Contaminating proteins were removed from the column by washing with the same buffer, then by additional washing with buffer A containing 1 M NaCl (buffer B). The protease fraction was eluted with buffer B containing 25% isopropanol (Strongin et al. 1978). Active fractions were pooled, dialyzed against 50 mM Tris-HCl-2 mM $CaCl_2$ (pH 9) and then stored in small aliquots at −20° C.

Electrophoresis. Homogeneity of the protease fraction was examined by the polyacrylamide gel electrophoresis method described by Moore et al. (1980).

Molecular weight determination. The molecular weight of the purified enzyme was estimated by the method of Andrews (1964) using a Sephadex G-100 column (1.8 × 60 cm) (Pharmacia). Chromatography was run a 8 ml h^{-1} using the buffer A plus 0.1 M NaCl. Calibration standards (Pharmacia) included bovine serum albumin, ovoalbumin, chymotrypsinogen A and ribonuclease A.

SDS-PAGE was performed with a vertical slab gel according to the method of Laemmli (1970). Phosphorylase b, bovine serum albumin, ovoalbumin, carbonic anhydrase, soybean trypsin inhibitor, α-lactalbumin were used as molecular weight standards (Pharmacia).

Isoelectric focusing. This was performed on a LKB 2117 Multiphor apparatus and LKB 2103 power supply (LKB Instruments, Bromma, Sweden). 5.7% polyacrylamide gel containing 0.1% carrier Ampholines (LKB) of pH range 3.5—10 was used. Gels were focused at 10° C at a constant power of 5 W per gel for 3 h. The pH gradient was determined by slicing the gel in 10 by 5 mm portions and eluting the slices in KCl 0.1 M overnight. The pH of the eluates was measured with a Radiometer pHmeter.

Effect of metal ions and protease inhibitors. The effects were tested at pH optimum of the protease. Solutions of $CaCl_2$, $MnCl_2$, $ZnCl_2$, $FeSO_4$, $CuSO_4$ and $MgSO_4$ were prepared in the concentration 4 mM. Solutions of phenylmethane-sulfonyl fluoride (PMSF) (Merck), 4-hydroxy-mercuribenzoic acid (pCMB) (Sigma), *o*-phenantroline (Sigma), EDTA (Baker chemicals B.V., Deventer, Holland), iodoacetic acid (Sigma), 2-mercaptoethanol (Merck), dithiothreitol (Bio Rad), soybean trypsin inhibitor (Sigma) were prepared at a concentration of 2 mM and 6 mM. Enzyme solution was mixed with metal ion solutions or inhibitor solutions in the ratio 1:1 and preincubated for 15 min at 25° C. Then protease activity was determined as described above. Results were recorded as the percent residual activity calculated with reference to activity of controls incubated in the absence of the substances used in this study.

Reactivation of EDTA-inactivated protease. The protease was inactivated with 1 mM EDTA by incubation at 25° C for 15 min. To this inactivated enzyme solution, the metal ions noted above were added to yield a final concentration of 2 mM. Each of these mixtures was further incubated at 25° C for 15 min and the restored enzyme activity was measured.

Results

Growth and enzyme production

The growth curve of *B. thermoruber* in GYE medium is shown in Fig. 1. Data for total enzymatic activity in the supernatants indicate that proteolytic activity appears after 18 h of incubation and peaks when the culture has reached stationary phase of growth (ca. 28 h).

Purification of the extracellular protease

All purification steps were carried out at 4° C. A summary of the purification procedure is given in Table 1. Most of the colored material in the crude enzyme solution, as well as 80% of the applied proteins to a column of DEAE-Sephadex were removed; the proteolytic component was eluted at 0.3 M NaCl. The active fractions were concen-

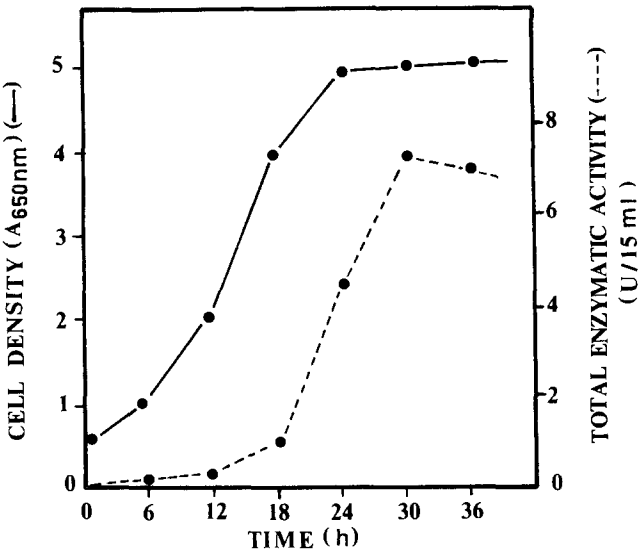

Fig. 1. Time course of culture of *B. thermoruber* strain BT_2^T and production profile of protease activity

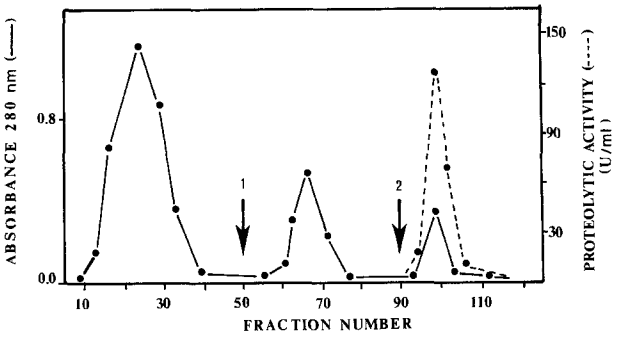

Fig. 2. Affinity chromatography of *B. thermoruber* protease on α-casein agarose. The flow rate was 8 ml h^{-1} and fractions of 1.5 ml were collected. *First arrow:* application of 20 mM Tris-HCl-2 mM $CaCl_2$-1 M NaCl (pH 8.5). *Second arrow:* addition of 25% isopropanol. See text for details

trated by ultrafiltration and applied to an α-casein agarose column. This final step of purification, that yielded a high increase in the specific activity of the protease, produced the elution profile shown in Fig. 2.

Homogeneity of the peak eluted was confirmed by polyacrylamide gel electrophoresis. Half of the gel was stained for proteins, the other half was assayed for protease activity. For this assay the gel was placed in a plate of agar-casein (1% agar and 1% casein dissolved in 50 mM Tris-HCl-2 mM $CaCl_2$ buffer (pH 9); after incubation at 45° C the gel was removed and TCA 5% added. A zone of clearing indicated the presence of protease activity. The staining revealed a single protein band with a R_f of 0.53 against the marker dye bromophenol blue; this band exhibited proteolytic activity on the agar-casein plates.

Molecular weight determination

The molecular weight of the purified enzyme was estimated to be 39000 ± 1000 by gel filtration on Sephadex G-100. When the molecular weight of the protease was determined by SDS-PAGE, only one stained band was obtained, which corresponded to a molecular weight of 39000 ± 500 indicating that the enzyme did not consist of more than one peptide chain.

Isoelectric focusing

The isoelectric point for the *B. thermoruber* protease was estimated to be 5.3.

Effect of temperature and pH on protease activity

The enzyme exhibited its activity predominantly in the higher pH range (pH 8.5 to 11), with an optimum pH of activity of 9. At pH 7.5 the activity was only 20% of that at pH 9. The optimum temperature for enzyme activity was 45° C.

Enzyme stability

The enzyme retained full activity after incubation in the pH range of 9—11 at 10° C for 24 h and lost 60% of its activity at pH 6 under the same conditions. When kept at 30° C for 24 h the protease

Table 1. Purification steps and recovery of *B. thermoruber* protease

Purification step	Protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield %	Purification factor
Culture liquids	5800	1750	0.3	100	1.0
Ultrafiltrate	996	1487	1.5	85	5.0
DEAE-Sephadex A-50	190	1115	5.9	64	19.5
α-Casein agarose	0.32	669	2090.0	38	6968.0

appeared stable only at pH 9. In the presence of 2 mM Ca^{2+} no loss of activity was detected in 60 min at 60° C; the protease retained still 60% of its activity after heating for 30 min at 70° C and 40% after treatment at 80° C for 5 min. Without Ca^{2+} the enzyme activity was only 40% of full activity after 60 min at 60° C.

Effect of inhibitors and metal ions

Metal ions markedly affected protease activity. Ca^{2+} was particularly effective in stimulating activity. The other metal ions tested, at a concentration of 2 mM, activated the protease two to three fold, while Cu^{2+} at the same concentration showed a less positive effect. PMSF, inhibitor of serine proteases, at a concentration of 1 mM inactivated the enzyme completely, while soybean trypsin inhibitor was not inhibitor for *B. thermoruber* protease. Sulphydryl inhibitors such as iodoacetic acid and pCMB did not affect the protease. The enzyme was not inactivated by *o*-phenantroline, but almost completely by 1 mM EDTA. This could suggest a Ca^{2+} requirement for stability of this enzyme. The absence of this ion during any stage of the prufication process, resulted in a loss of activity.

Effect of metal ions on EDTA-inhibited enzyme

Inhibition by EDTA was reversible. The inhibitory effect in presence of EDTA could be reversed completely by Fe^{2+} at a concentration of 2 mM. At the same concentration Cu^{2+} restored the activity of the enzyme to almost 70%, while the other metal ions reactivated the enzyme to a lesser degree. $CaCl_2$ had a significant effect at a concentration of 10 mM. The inability of Ca^{2+} to completely reactivate EDTA-inhibited enzyme was probably due to interaction of the casein, used as substrate, with the metal ion. The ability of Ca^{2+} to reactivate EDTA-inhibited enzyme was emphasized by using a different substrate. When the protease sample (preincubated with 1 mM EDTA, further preincubated in presence of 2 mM $CaCl_2$) was assayed with hemoglobin, the activity was restored to the extent of about 90% of that of uninhibited enzyme.

Substrate specificity

Specificity of the protease towards various synthetic substrates was examined (Table 2). The best substrate of those tested was Z-Ala-Ala-Leu-pNa, a good substrate for subtilisin (Morihara 1974). Z-Gly-Gly-Leu-pNA and Suc-Gly-Gly-Phe-pNA were also hydrolyzed. The *B. thermoruber* protease failed to hydrolyze the substrates benzoyl-Tyr-pNA, Pyr-Gly-Arg-pNA and Z-Arg-pNA.

Table 2. Peptidase activity of *B. thermoruber* protease

Substrate	Specific activity (U/mg protein)
Z-Ala-Ala-Leu-pNA	2.01
Z-Gly-Gly-Leu-pNA	0.74
Z-Phe-pNA	0.033
Suc-Gly-Gly-Phe-pNA	0.002
Benzoyl-Tyr-pNA	—
Pyr-Gly-Arg-pNA	—
Z-Arg-pNA	—

The protease shows a rather low esterolytic activity towards ATEE (0.12 OD per min per mg of enzyme) and does not hydrolyze BAME.

Enzyme kinetic

The rates of hydrolysis of Z-Ala-Ala-Leu-pNA by the protease were determined. With this substrate Michaelis-Menten kinetic seemed to be followed. The values obtained for V_{max} and K_m from Lineweaver-Burk double-reciprocal plots were 2.4 μmol of *p*-nitroaniline released per min per mg of enzyme and 2.5 mM, respectively.

Discussion

The protease described in the present paper was produced by *Bacillus thermoruber* during the post-exponential phase of growth, in according to most extracellular proteases secreted by *Bacillus* sp. (Debabov 1982). The procedure described for purification of the proteolytic enzyme resulted in a 7000-fold purification with a final yield of 38%. A relatively low pI value permitted removal of most of the contaminating proteins by ion-exchange chromatography, whereas the affinity chromatography on α-casein-agarose gave practically pure protease. The recovery of active protein from this last step was somewhat lower than expected, due to autolysis.

The protease belongs to the class of serine proteases, as shown by its optimum pH for activity (pH 9) and by its full inactivation by PMSF, a common inhibitor of these enzymes (Morihara

1974; Ward 1983). Furthermore, as other *Bacillus* serine proteases, this enzyme appears to be activated and stabilized by Ca^{2+} (Kelly and Fogarty 1976; Strongin et al. 1979; Ward 1983) and shows a high degree of thermal stability in the presence of 2 mM $CaCl_2$. EDTA at a concentration of 1 mM led to 90% inhibition of the enzyme. The reversibility of EDTA inhibition confirms that the metal ions have a role in maintaining an active conformation of the protease. Generally, the serine proteases are not inhibited by metal chelating agents; however there are examples of serine proteases that are affected by EDTA (Gnosspelius 1978; Izotova et al. 1983; Kato et al. 1974; Strongin et al. 1979).

Since the specific trypsin inhibitor did not inhibit the protease, nor were the trypsin substrates Z-Arg-pNA and BAME hydrolyzed by the enzyme, the characteristics of the enzyme are not those of a trypsin-type protease. The specificity of the enzyme against synthetic substrates agree fairly well with the alkaline-serine proteases shown by Morihara (1974): in fact the protease exhibits specificities towards aromatic or hydrophobic amino acid residues such as phenylalanine and leucine at the carboxyl side of the splitting point.

In conclusion, we isolated pure extracellular, thermostable protease from *B. thermoruber,* a new species of *Bacillus*. The characteristics of the *B. thermoruber* protease that have been discussed are consistent with those established for alkaline-serine proteases.

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