NEUTRAL PROTEASES WITH DIFFERENT THERMOSTABILITIES FROM A FACULTATIVE STRAIN OF *BACILLUS STEAROTHERMOPHILUS* GROWN AT 40° AND AT 50°

W. SIDLER* and H. ZUBER

Institut für Molekularbiologie und Biophysik, Eidgen. Technische Hochschule, Zürich-Hönggerberg, 8049 Zürich, Switzerland

Received 26 June 1972

1. Introduction

Extracellular proteases from microorganisms have been investigated for more than 20 years [1]. They have been classified into three groups by their optimum pH for hydrolysis i.e. acid, neutral and alkaline proteases. The neutral proteases have been well differentiated from the alkaline proteases [2–4].

During our work on enzymes from thermophilic microorganisms we have found a neutral protease in the culture medium of *Bacillus stearothermophilus*. Neutral protease activity has also been observed by Endo in this organism [5].

Many strains of *B.stearothermophilus* are capable of growing in two different temperature ranges: in the mesophilic range (30°–45°) and in the thermophilic range (50°–65°). They are therefore facultative thermophilic organisms [6].

In this paper we demonstrate that different neutral proteases are produced in the two different temperature ranges. Some of the properties of the pure enzymes and their comparison with other well known bacterial proteases produced by *B.Thermoproteolyticus* and *B.subtilis* are described [7–10].

The neutral protease produced at 50° exhibits a greater thermostability than that produced during growth at 40°.

2. Materials and methods

Thermolysin and the proteases produced by *B.subtilis* as well as all the reagents are commercially available products.

The proteolytic activity was determined with the insoluble substrate Hide Powder Azure (Cal Biochem) by the method of H.Rinderknecht et al. [11]. For standard tests 25 mg hide powder azure were suspended in a 5 ml solution of the enzyme in 0.05 M Tris/HCl buffer, pH 7.5, containing 0.01 M $CaCl_2$. The test tubes were closed with parafilm, incubated for 15 min at 40° and gently agitated every minute during this period. At the end of incubation the test

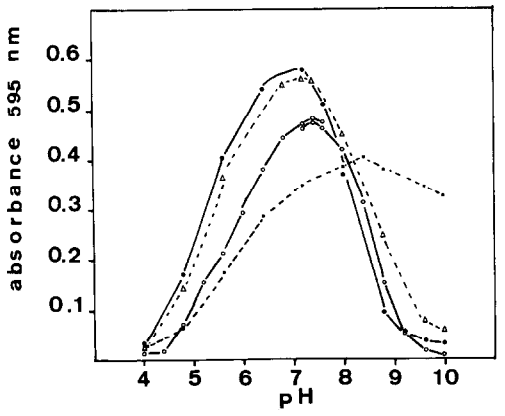

Fig. 1. The dependence of proteolytic activity on pH. *B.stearothermophilus* neutral protease M (mesophilic) ●–●–● and TH (thermophilic) ○–○–○; Thermolysin △–△–△; Subtilisin A Novo ■–■–■. The assays were carried out under standard conditions (see Materials and methods) in 0.05 M Tris/maleate buffer containing 0.01 M $CaCl_2$ pH 4.0–7.6 and in 0.05 M Tris/HCl buffer containing 0.01 M $CaCl_2$ pH 7.2–10.0.

* This work forms part of the doctoral thesis of W.Sidler, E.T.H., Zurich.

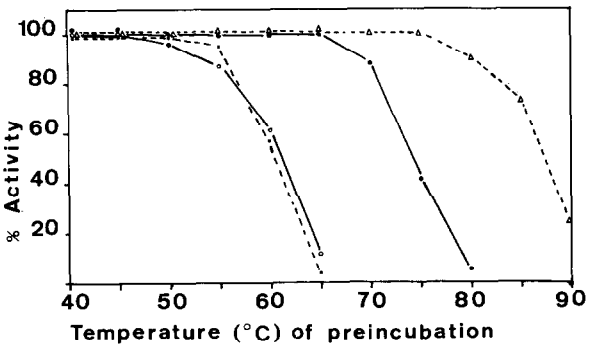

Fig. 2. Thermostability of the different neutral proteases. 6 ml samples without substrate were preincubated at different temperatures for 15 min. The mixture was cooled in ice water and 5 ml were assayed under standard conditions. *B.stearothermophilus* neutral protease M(o–o–o); *B.stearothermophilus* neutral protease TH(•–•–•), Thermolysin (△--△--△) and *B.subtilis* neutral protease (□- - -□- - -□).

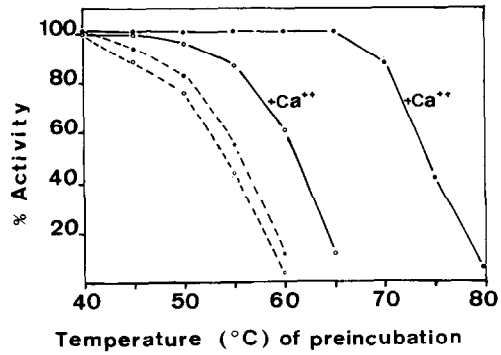

Fig. 3. Effect of Ca^{2+}-ions on the heat stability. The experiments were carried out as described under fig.2. *B.stearothermophilus* neutral protease M, with or without Ca^{2+}, resp.: (o–o–o), (o--o--o); *B.stearothermophilus* neutral protease TH with or without Ca^{2+}, resp.: (•–•–•), (•--•--•).

tubes were cooled in ice water and the suspension filtered. The liberated blue dye was measured in a spectrophotometer at 595 nm.

Hydrolytic activity of the enzyme on synthetic peptides was determined by incubating 1 ml of 1 mM peptide solution in the same buffer described above at 40° for 30 min with the various proteases. After incubation a suitable portion of the assay mixture was chromatographed on Merck cellulose thin-layer chromatography plates in the solvent system n-butanol – acetic acid – water, 4:4:1 (by volume) and developed with ninhydrin.

In these studies *B.stearothermophilus* strain NCIB 8924 was used. For enzyme production under mesophilic conditions (40°) cells were grown in a New Brunswick Microferm in Lab Lemco medium (2 g Bacto Trypton Difco, 3 g Lab Lemco Beef Extract Oxoid, 2 g Cerelose, 3 g K_2HPO_4, 1 g KH_2PO_4 per litre deionised water, giving pH 7.1).

A 10-litre batch of this medium was sterilized at 120° for 40 min. After cooling to 40°, a 1 litre inoculum of *B.stearothermophilus* was added and incubated at 40° under forced aeration (1 l/min per litre medium) and stirring (200 rpm). 1 ml of antifoam (A-Emulsion, Dow-Corning, USA) was added. Growth was followed by measuring turbidity at 546 nm. The proteolytic activity in the medium reached a maximum after the exponential phase of growth, usually 4–5 hr after inoculation. The culture was cooled to 40° and the cells removed by centrifugation.

The enzyme produced under thermophilic conditions (50° cultivation temperature) in Lab Lemco medium was kindly provided as an ammonium sulphate precipitate of the culture medium (3000 l batch) by CIBA-Geigy Ltd, Basle, Switzerland.

The two extracellular proteases were precipitated from the cell free culture medium by ammonium sulphate precipitation (90% saturation). The collected precipitate was further purified similarly to the method of Keay and Circulis for the neutral protease of *B.subtilis* [12]. The precipitate was dissolved in 2% calcium acetate and the insoluble material removed by centrifugation. Purification continued by acetone fractionation and treatment with DEAE-Sephadex. CM-cellulose (Bio-Rad) was, however, substituted for hydroxylapatite in the last chromatography step [7]. The column was equilibrated with the starting buffer (0.001 M Tris-maleate, 0.002 M calcium acetate pH 6.4) and the protein mixture was chromatographed with a linear gradient from 0.0–0.2 M NaCl in the starting buffer. The purified enzyme showed one band in polyacrylamide disc electrophoresis at pH 8.9 (Ornstein [13] and Davis [14]).

3. Results and discussion

Inhibition experiments with EDTA (0.001 M) and DFP (0.005 M) were carried out in the standard assay mixture. Both proteases produced by *B.stearothermophilus* are inhibited strongly by EDTA but DFP has no significant effect exactly as observed with Thermolysin and *B.subtilis* neutral protease.

As shown in fig.1 the pH-optimum of the two proteases is close to pH 7.4 in Tris/HCl buffer and Tris-maleate buffer. The pH of the buffers were adjusted at room temp. (22°). A difference in pH optimum between these two proteases was not observed by this assay method. It should be remembered that the pH of Tris buffer shifts to lower pH when warmed to 40°.

B.stearothermophilus proteases, Thermolysin and *B.subtilis* neutral protease hydrolysed Z–Gly–Leu–Tyr into Z–Gly and Leu–Tyr. On the other hand Z–Gly–Gly–Gly and Z–Leu–Gly–Gly were not hydrolysed. This indicates that *B.stearothermophilus* proteases cleave on the amino side of leucine and show a specificity similar to Thermolysin and *B.subtilis* neutral protease.

As both proteases produced by *B.stearothermophilus* are inhibited by EDTA and have a pH optimum close to pH 7.4 they may be classed as neutral proteases in the scheme of Hagihara [1].

The two neutral proteases from *B.stearothermophilus* differ in thermostability (fig.2). *B.stearothermophilus* neutral protease M (grown under mesophilic conditions) is denatured by heat between 55° and 60° at a rate similar to *B.subtilis* neutral protease [7]. On the other hand *B.stearothermophilus* neutral protease TH (grown under thermophilic conditions) is stable up to 65–70°, although not as stable as Thermolysin which survives up to 75–80°. These studies were made with 10 mM Ca^{2+} ions present during preincubation at the various temperatures.

The thermostabilizing effect of Ca^{2+} ions on Thermolysin and *B.subtilis* neutral protease can also be demonstrated with the *B.stearothermophilus* neutral proteases (fig.3). In contrast to the results obtained in the presence of calcium, after heating at 40° for 15 min without Ca^{2+} ions all four proteases are partially inactivated by heat but are completely destroyed after 15 min of preincubation at 60°.

Studies on the enzyme structures will show whether the two neutral proteases are two different forms of a protein coded by one gene or if they originate from two separate cistrons and may represent an interesting system to study factors involved in producing thermostability.

* DFP: diisopropylfluorophosphate.

Acknowledgements

The authors gratefully acknowledge the help of Drs. J.Gruner and E.Voser of CIBA-Geigy Ltd., Basle, in preparing large cultures of bacteria. Our cordial thanks are expressed to Miss Christine Beck for her skilful technical assistance in microbiological experiments and Peter Bützer for preparing the peptide Z–Gly–Gly–Gly. This work was supported by the Schweizerische Nationalfonds (Project No. 3.379.70).

References

[1] B.Hagihara, The Enzymes, Vol. 4, eds. P.D.Boyer, H.Lardy and K.Myrbäck (Academic Press, Inc., New York, 1958) p.193.
[2] K.Morihara, Biochem.Biophys.Res.Commun.26 (1967) 656.
[3] K.Morihara, H.Tsuzuki and T.Oka, Arch.Biochem. Biophys.123 (1968) 572.
[4] B.S.Hartley, Ann.Rev.Biochem.29 (1960) 45.
[5] S.Endo, Hakko Kogaku Zasshi 40 (1962) 346.
[6] H.Zuber, Naturwiss.Rundschau 22 (1969) 16.
[7] J.D.McConn, D.Tsuru and K.T.Yasunobu, J.Biol.Chem. 239 (1964) 3706.
[8] Y.Otha, J.Biol.Chem.242 (1967) 509.
[9] J.D.McConn, D.Tsuru and K.T.Yasunobu, J.Biol. Chem.240 (1965) 2415.
[10] R.Morihara, T.Oka and H.Tsuzuki, Arch.Biochem. Biophys.138 (1970) 515.
[11] H.Rinderknecht, M-C.Geokas, P.Silverman and B.J. Haverback, Clin.Chim.Acta 21 (1968) 197.
[12] L.Keay and N.Cirulis, Prep.Biochemistry 1 (1971) 177.
[13] L.Ornstein, Ann.N.Y.Acad.Sci.121 (1964) 321.
[14] B.J.Davis, Ann.N.Y.Acad.Sci.121 (1964) 404.