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CALCIUM REQUIREMENTS OF RESIDUAL PROTEASE IN BACILLUS SUBTILIS DB104

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Summary

Bacillus subtilis DB104, a double mutant which does not synthesize neutral or alkaline proteases, was shown to exhibit some residual proteolytic activity when grown in both batch and continuous cultures. A major protein component responsible for about 70% of extracellular residual protease activity was reversibly deactivated by removal of calcium.

Introduction

Escherichia coli and *Bacillus subtilis* are the most widely used bacteria for hosting recombinant DNA vectors primarily due to the availability of a large mass of information regarding the genetics and physiology of these organisms. Appropriate vectors for cloning foreign genes as well as the procedures for their introduction into these host systems are well developed. The genetic characteristics of *B. subtilis* have been the most studied among the gram positive bacteria. *B. subtilis,* as well as other gram positive bacteria, has a number of advantages over the gram negative type. The cells are capable of growth on either minimal or complex media and the nature of its single cell wall allows for many foreign proteins to be excreted into the surrounding medium much more efficiently than from *E. coli.* Also characteristic of the single walled cell envelope is the absence of a toxic lipopolysaccharide which is present in *E. coli* (Gryczan, 1982). The genes for a number of proteins including TEM ß-lactamase (Wong *et al.*,1986), interferons (Sibakov *et al.*, 1984), and rat proinsulin (Mosbach *et al.*, 1983) have been cloned successfully on *B. subtilis* secretion vectors and the protein products were excreted into the culture fluid. However, in each case the secreted protein was quickly degraded by proteolytic action. These extracellular proteolytic enzymes comprise a major limitation to the effective utilization of *B. subtilis* as a host organism.

The predominant extracellular proteases excreted by *B. subtilis* have been well characterized and belong in one of three classes; alkaline (serine active site) (Stepanov *et al.*, 1977), neutral (Mezes and Lampen, 1985), and to a lesser extent acidic proteases or esterases.

A double mutant of *B. subtilis* which is deficient in neutral and alkaline proteases has been constructed (Kawamura and Doi, 1984) and was shown to exhibit 4% of the proteolytic activity found in wild type cells. We have recently reported on the repression of protease activity in continuous cultures of *B. subtilis* (Strohm *et al*). Approximately 50-fold reduction of protease activity for wild type cells and 7-fold reduction for the low protease mutant DB104 was observed. In this study we report a calcium dependence of catalytic activity of a protein component responsible for about 70% of residual protease activity in cultures of DB104. This low level of residual protease activity is substantial enough to degrade some of the more sensitive proteins such as growth hormones and other eucaryotic proteins.

Materials and Methods

Organism: *Bacillus subtilis* DB104, a double mutant strain deficient in extracellular neutral and serine proteases, was kindly supplied by Dr. Roy H. Doi (University of California, Biochemistry and Molecular Biology Department).

Medium Composition: The complex growth medium used throughout was formulated as follows; Difco Nutrient Broth (0.5 g/l), casein hydrolyzate (0.5 g/l), tryptophan (0.02 g/l), KCl (0.5 g/l), $MgSO_4 \cdot 7H_2O$ (0.0625 g/l), $MnCl_2$ (0.005 g/l), $CaCl_2$ (0.515 mM), $FeSO_4$ (0.0005 mM), $ZnCl_2$ (0.05 mM), and glucose (2 g/l). The pH was adjusted to a final value of 7.1 ± 0.1 by addition of NaOH.

Culture Apparatus: Batch cultures were achieved in shake flasks. Sterile media was inoculated with a single colony of cells and incubated at 37°C in a G24 Environmental Incubator Shaker (New Brunswick Scientific Co.). Continuous cultivation was performed in a Multigen model F-1000 bioreactor (New Brunswick Scientific Co.) with a 330 ml working volume reaction vessel. Oxygen was supplied by aeration and the inlet air stream was passed through sterile distilled water in a packed column before entering the reaction vessel. Sterilization of the growth medium, reactor vessel and all components in direct contact with the reactor contents, feed and waste streams was accomplished by autoclaving at 121°C for 15 minutes. The temperature

within the reactor was maintained at 37°C and the rate of agitation was held constant at 200 rpm.

Cell growth was monitored by measuring the optical density at 660 nm. Inoculum was prepared in the same manner as the batch culture and was added at a volume approximately 7% (v/v) of the fermentor volume.

Cells were first grown in batch until late exponential phase of growth at which point the reactor was put into a continuous mode at a dilution rate of 0.4 hr^{-1}. Samples were taken at steady state which was assumed when optical density and pH were constant over at least two residence times.

Protease Assay: Protease activity was monitored indirectly by following the degradation of ß-lactamase by a method similar to that of Citri (Citri *et al.*, 1960). Samples were filtered through a 0.2 µm filter and 990 µl of supernatant was preincubated at 37°C for 1 hour at which time 10 µl of ß-lactamase was added. Following the desired incubation period, 25 µl of sample was added to 2.5ml of penicillin G suspended in a 0.05 M phosphate buffer and absorbance at 240 nm was measured vs. time. Ethylendiamine tetraacetic acid (EDTA), a strong chelator of divalent metal ions, or ethylenglycol-bis-(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), a chelator specific for calcium, was added to some samples to a concentration of 1 mM and these samples were preincubated for 1 hour prior to the addition of ß-lactamase.

Results and Discussion

Figure 1 shows the rates of degradation of ß-lactamase in samples from both batch and continuous culture. The optical densities of the samples were 3.7 and 3.8 respectively and pH was measured to be 5.8 in batch and 5.4 in continuous. The batch culture sample was taken after growth had entered stationary phase and therefore the greater protease activity, as is apparent by the greater rate of degradation of ß-lactamase, is as expected. The fraction of ß-lactamase remaining in the sample is reported as the ratio of the ß-lactamase activity at incubation time (t) to that at 2 hours of incubation due to small fluctuations of the data during the initiation of the reaction.

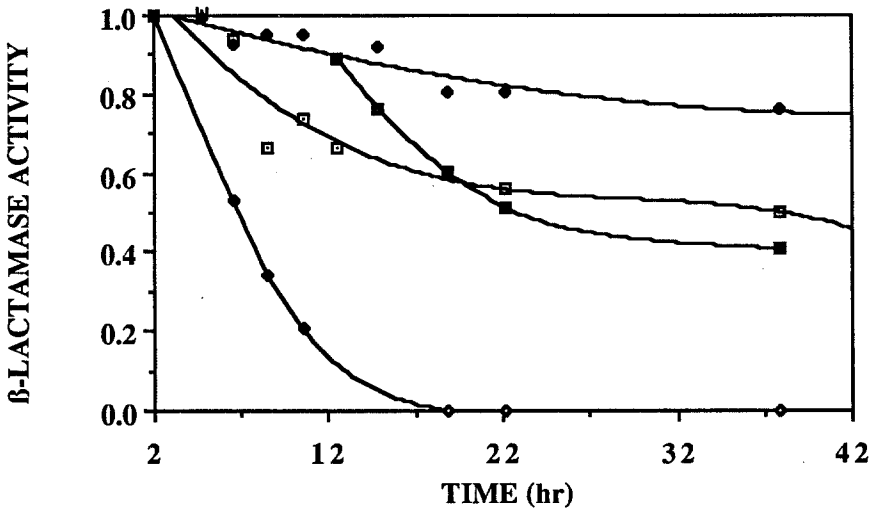

Figure 1 Degradation of ß-lactamase in incubation mixtures containing the supernatant from glucose-limited batch (◇) and chemostat (□, ■, ◆) culture. The incubation mixture contained either EGTA (■, ◆) or no chelator (◇, □). A reactivation of protease activity was observed upon addition of $CaCl_2$ to sample fraction preincubated with EGTA (■).

Our continuous culture experiments showed that calcium limitation of growth of *B. subtilis* DB104 resulted in a substantially reduced level of protease activity. It was inconclusive, however, whether the effect of the divalent ion was at the level of proteolytic enzyme synthesis or activity.

A sample was taken from a glucose-limited chemostat culture of *B. subtilis* DB104 and a portion of the supernatant was divided into two aliquots. One aliquot was preincubated with EGTA and the degradation profiles of ß-lactamase for both aliquots are shown in Figure 1. Following 12 hours of incubation, the sample containing EGTA was divided into two equal fractions and $CaCl_2$ was added to one fraction to a concentration of 20 mM. Upon addition of the calcium ions, a rapid degradation of ß-lactamase was observed, indicating a reactivation of the residual protease due to the calcium. A control sample consisting of growth medium, ß-lactamase and $CaCl_2$ was tested to insure that the observed degradation of protein was not due solely to the presence of $CaCl_2$. These results indicate that calcium ions are required for catalytic activity rather than stability or synthesis of the residual proteases.

A fraction of the supernatant from the continuous culture sample was preincubated with EDTA and tested for degradation of ß-lactamase. The results of each

of the three fractions from the continuous culture are given in Figure 2a. It was observed that in all cases the activity of ß-lactamase decays in an exponential manner and the data is replotted on semi-logarithmic coordinates in Figure 2b. It is therefore reasonable to compare the rates of decay in terms of half life where half life is defined as the period of incubation required for the activity of ß-lactamase to reach 1/2 its original value. The half life values for the batch and continuous culture samples were 4.25 and 23 hours respectively. With addition of EGTA , a half life value of 80 hours was obtained. From these values it is apparent that about 70% of the residual protease activity found in the continuous culture sample can be deactivated by the addition of EGTA. The response of the sample to EDTA is similar to that observed with EGTA, indicating no significant dependence of residual protease activity on divalent metal ions other than calcium.

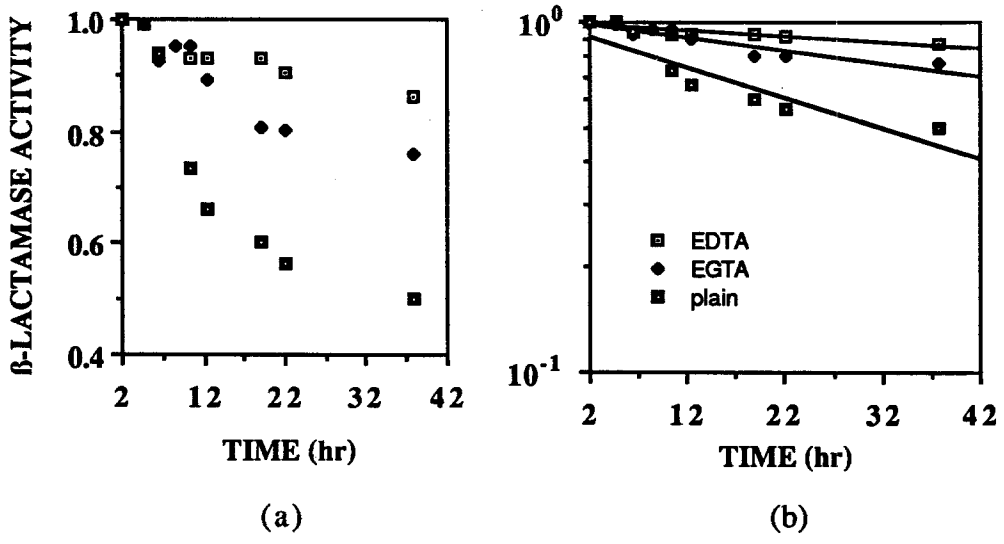

Figure 2 a) Rate of degradation of ß-lactamase in incubation mixtures containing supernatant from glucose-limited chemostat culture. The incubation mixture contained either EDTA (□), EGTA (◆) or no chelator (■).

b) Same data shown in Fig. 2a plotted on semi-logarithmic coordinates.

An intracellular serine protease isolated from *B. subtilis* A-50 (Stepanov *et al.*, 1977) exhibited an irreversible inactivation of the enzyme in the absence of calcium ions. The reactivation of *B. subtilis* DB104 residual protease therefore disallows the possibility of the protease activity being due to intracellular enzymes released due to cell lysis.

In conclusion, these experiments demonstrate that most of the residual protease activity in cultures of the low protease mutant *B. subtilis* DB104 can be reversibly deactivated by removal of calcium ions from the extracellular environment by the action of EGTA and is completely restored by reintroduction of the same metal ions. We have begun work toward purification of this protease by use of calcium chelate affinity chromatography. Once isolated, the protein can be used as a probe for the identification and subsequent deactivation of the chromosomal gene coding for this protease.

References

Citri, N., Garber, N., and Sela, M. (1960). *J. Biol. Chem.*, **235**, 3454.

Gryczan, T.J., (1982). Molecular Cloning in Bacillus subtilis. In: *The Molecular Biology of the Bacilli*, D. Dubnau, ed. vol. 1, p. 307, New York: Academic Press, Inc.

Kawamura, F., and Doi, R.H.(1984). *J. Bacteriol.*, **160**, 442-444.

Mezes, P.S., and Lampen, J.O. (1985). In: *The Molecular Biology of the Bacilli*, D. Dubnau, ed. vol. 2, p. 151, New York: Academic Press, Inc.

Mosbach, K., Birnbaum, S., Hardy, K., Davis, J., and Bulow, J. (1983). *Nature*, **302**, 543-545.

Sibakov, M., Lehtovarra, P., Petterson, R., Lundstrom, K., Kalkkinen, N.,Ulmanen, I., Takkinen, K., Kaariainen, L., Palva, I., and Sarvas, M. (1984). In: *Genetics and Biotechnology of Bacilli*, A.T. Ganesan and J.A. Hoch, eds., p. 153, New York: Academic Press, Inc.

Stepanov, V.M., Strongin, A.Y., Izotova, L.S., Abronov, Z.T., Lyublinskay, L.A., Ermakova, L.M., Baratova, L.A., and Belyanova, L.P. (1977). *Biochem. Biophys. Res. Commun.*, **77**, 298-305.

Strohm, W., Jeong, J.W., Longo, A., and Ataai, M.M. to be submitted. *Biotech. Bioeng.*

Wong, S., Kawamura, F., and Doi, R.H. (1986). *J. Bacteriol.*, **168**, 1005-1009.