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A mutant of Bacillus cereus T, strain 876, is inhibited in protease activity and as a consequence is also inhibited in sporulation and germination (Aronson, Angelo and Holt, 1971, J. Bacteriol. 106: 1016-1025). Protease activity in this mutant is heat sensitive so cells must be grown at 30°, rather than 37°, to prevent selection for revertants. Alex Keynan and Harlyn Halverson et al. found that a peptide extracted from autodigested Pronase compensated for the mutation during germination of permeablized spores. Before publishing their results the organism was lost and so had to be replaced. It is thought this mutant may be a model organism for the study of reactions linking heat activation to breakdown of the spore cortex.

Materials and Methods

Isolation, growth and harvesting of Bacillus cereus T 876 was best achieved on modified G agar (Gollakota and Halverson, 1960, J. Bacteriol. 79: 1-8.) at 30°C after 5-7 days. Spores were washed off the plates with 2 ml of distilled water and a glass spreader. After washing twice with 0.05% Tween 80, spores were separated from vegetative cells by floating a sample on a solution of 30% Renografin. Samples were centrifuged at 15,000 rpm for 40 min. The pellet was washed three times in 0.05% Tween 80 and resuspended in distilled water. Spores were always more than 99.9% free of vegetative cells by direct microscopic count.

Spores were permeablized by placing spores in 4M urea and 7% v/v B-mercaptoethanol. Time of permeablization was varied between 3 and 10 hours. Spores were then washed twice and resuspended in 0.05% Tween 80. No difference was found in germination experiments between permeablized spores that had been heat shocked (65°, 40 min) and those that had not.

Germination medium contains 1.0 mg/ml L-alanine and 0.5 mg/ml adenosine. At first a buffer of 0.5M KH_2PO_4 and K_2HPO_4, pH 7.5 was used, but in later experiments 0.6 mg/ml NaCl, 0.1 mg/ml Na_2SO_4, 0.07 mg/ml KH_2PO_4, pH 7 was used. Potential compensators for inhibited germination in Bacillus cereus T 876 (e.g., protease autodigests) were always used in 1.0 mg/ml concentrations. Germinations were carried out at 30°C and measured as percentage decrease in $O.D._{600}$. A rapid method for screening substances for mutant compensation was developed using a Beckman DU-7 Spectrometer and thin plastic cuvettes. This method allowed germination of as many as 12 determinations, each using as little as 0.6 ml of spore suspension, to be measured with high accuracy.

Protease autodigests were prepared by incubating 10.0 mg/ml of the desired protease in a 37°C water bath for 30 minutes, and then denaturing in a 100°C bath for 15 minutes.

centrifugation. Because results with standards were not consistent, trichloroacetic acid precipitation was later omitted from the procedure.) The supernatent was then added to 0.5 ml of distilled water. To this, 1 ml of 0.5% $K_4Fe(CN)_6$ and 1 ml of a 0.53% Na_2CO_3 and 0.065% KCN solution was added. Samples were placed in a 100°C bath for 15 min and then cooled. Five ml of a solution of 0.15% $NH_4Fe(SO_4)_2$ and 0.1% sodium dodecyl sulfate were then added. $O.D._{690}$ was measured after 15 minutes and compared to a standard generated with measured amounts of N-acetyl glucosamine, ranging from 0 to 10 ug/ml.

Results

A new culture of Bacillus cereus T 876 was supplied by Arthur I. Aronson. Germination charactoristics were then determined and compared to the wild type strain (fig.1). Germination curves were similar to those previously described for Bacillus cereus T 876.

Hexosamine release was measured and found to be inhibited by the mutation to a degree comparable to inhibition of germination. Measuring of hexosamine release in the presence of potential compensators was found to be impossible by this method because all of the potential compensators gave strong positive hexosamine reactions. Trichloroacetic acid precipitation before centrifugation of the spores may remove the cross reacting peptides but this experiment was never tried.

The compensatory pronase effect itself could not be duplicated with consistency in this lab. Besides the permeabilizing and buffer changes mentioned earlier, the following substances were assayed for compensation of the mutation: casein hydrolysate (ICN), Bactopeptone, Bacto casamino acids, trypticase peptone extract, Sigma protease from Streptomyces griseus (not Pronase), Sigma protease from Bacillus subtilis and Boehringer's nonspecific protease from Streptomyces griseus. Sigma has changed its product and Pronase itself is no longer available in the form used by Keynan et al.

Conclusion

Because the compensatory effect seen by Keynan, Halverson et al. could not be reproduced by this lab, the project was dropped. However some success was made by the development of a rapid, material-conserving, simple method for assaying substances for the ability to compensate for the mutation in Bacillus cereus T 876. by Sigma) is used.

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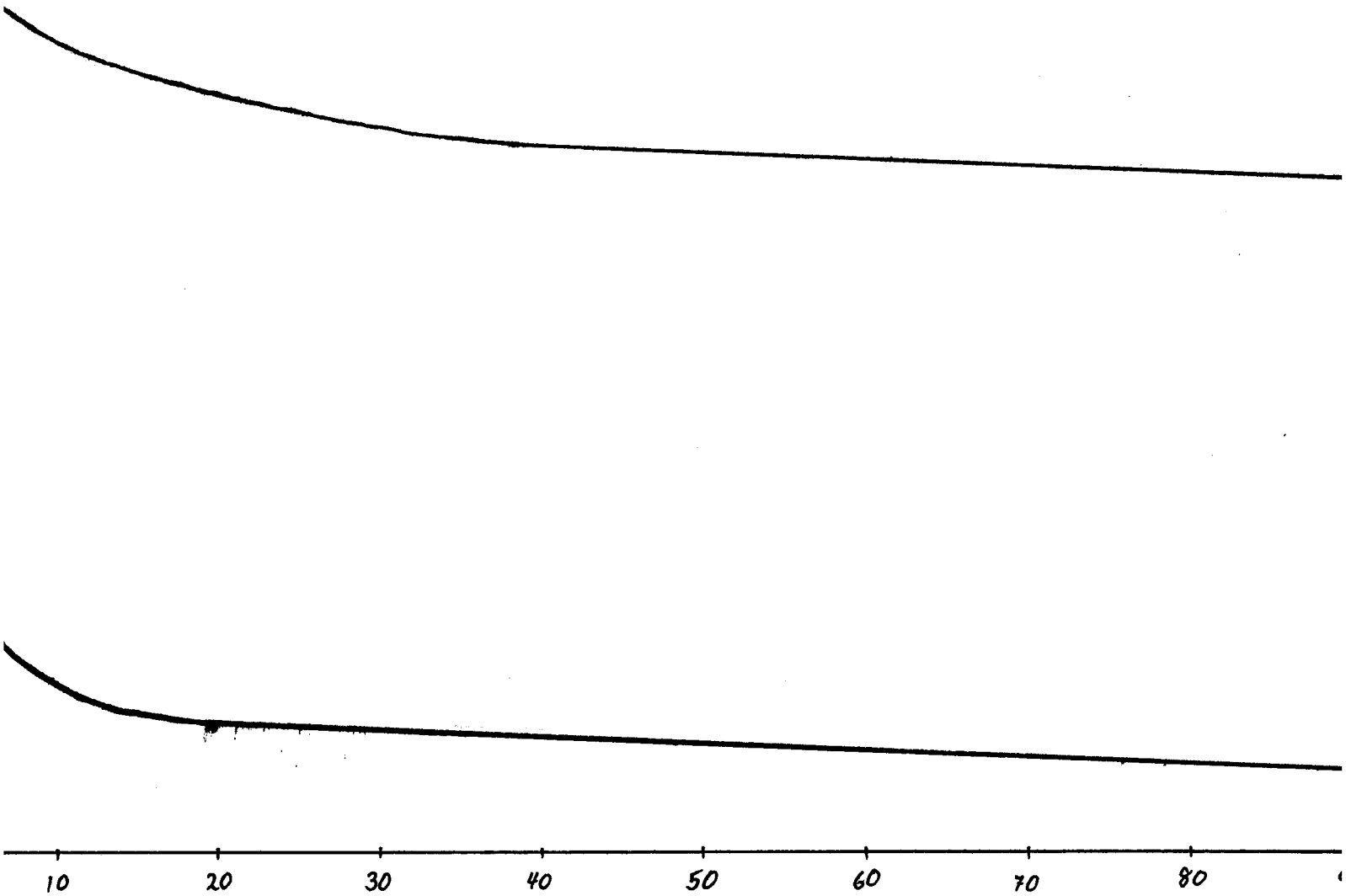

— = BACILLUS CEREUS T 876 TIME (MIN)

— = BACILLUS CEREUS T WILD TYPE

FIG. 1 - GERMINATION CURVE