OFFICE OF NAVAL RESEARCH

Final Report

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Contractor: University of Illinois, Urbana

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Title of Project: "Enzymatic Studies on Thermophilic Bacteria"

Objectives:

(1) To prepare crystalline α-amylase from thermophilic bacteria and to study the physical and chemical properties of the preparations in order to obtain data relative to the problem of thermal stability.

(2) To study the biosynthesis of α-amylase by Bacillus stearothermophilus.

(3) To study conditions of induction of a lysogenic system in B. stearothermophilus.

(4) To isolate and purify enzymes from thermophilic bacteria related to amino acid and carbohydrate metabolism.

(5) To study the physical and chemical properties of the above enzymes.

Summary of Results:

A. Studies on α-amylase of Bacillus stearothermophilus.

1. Sequence studies

Studies were initiated on the sequence of the α-amylase of B. stearothermophilus by using chains A and B which are formed by performic acid oxidation. Conditions for the isolation of peptides were examined and efforts were made to obtain sufficient material to study the sequence of the two chains.

B. Biosynthesis of α-amylase by B. stearothermophilus

1. Effect of carbon sources

A chemically defined medium was devised for use in α-amylase induction studies. The addition of 0.1% casein hydrolysate to the chemically defined medium permitted growth on fructose and with glucose, sucrose,maltose, starch and glycerol it shortened the lag period and increased both the growth rate and the total enzyme produced. Growth did not occur when gluconate, acetate or succinate were used as carbon sources. α-Amylase was produced during the logarithmic phase of growth; the amount produced was inversely proportional to the rate of growth. The poorer the carbon source for growth (glycerol, k = 0.24; glucose, k = 0.26; sucrose, k = 0.42), the higher was the amount of enzyme produced (glycerol, 109 units/ml; glucose, 103 units/ml; sucrose, 0.45 units/ml). Cells grown on technical-grade maltose (k = 0.26) or starch (k = 0.42) did not conform to this relationship in

that unusually large amounts of α-amylase were produced (362 and 225 units/ml, respectively). Cells grown on fructose or sucrose had the same growth rate (k = 0.42), but smaller amounts of α-amylase were produced on fructose (fructose, 0 to 4 units/ml; sucrose, 45 units/ml). An intracellular α-amylase was not detected in this organism.

2. Induction of α-amylase by maltodextrins

Technical grade maltose contained 3.5% glucose, 0.5% maltotriose, and 2.5% of the higher molecular weight maltodextrins. The first five homologues (maltose being the first in the series) of the maltodextrin series were isolated and purified. Each member of the series was found to be chromatographically pure. The physical and chemical properties were determined. It was shown that the contaminating maltodextrins found in technical grade maltose were linear (1-4 linked) polymers of glucose, ranging from maltotriose to maltohexaose. The addition of maltose, maltotriose, maltohexaose, maltopentaose and maltotetraose (all at 10^{-4} M) to cultures growing in a chemically defined medium resulted in a stimulation in the differential rate of α-amylase synthesis by 1.2, 1.6, 1.9, 2.3 and 3.0 times that of the sucrose control, while glucose had no effect. The induction data suggested that

the B. stearothermophilus strain studied is a partial constitutive strain with respect to α-amylase synthesis.

3. Induced biosynthesis of α-amylase by growing cultures

The maximal differential rate (K) α-amylase synthesis was usually two to three times that of the sucrose control culture, over an inducer concentration range of 5×10^{-4} to 1×10^{-3} M. With maltotetraose, higher concentrations decreased the K value, whereas higher concentrations of maltose were needed to obtain maximal K values. Glucose, in concentrations from 10^{-5} to 10^{-2} M, had no effect on the differential rate of enzyme synthesis. Cultures growing on maltotriose, maltotetraose, maltopentaose, and maltohexaose exhibited the same growth rate (k) and differential rate of α-amylase synthesis over a concentration range of 2.92×10^{-4} to 1.46×10^{-2} M. Growth of cultures in various concentrations of pure maltose revealed that with concentrations of maltose ranging from 2.92×10^{-3} to 1.46×10^{-2} M the K value for α-amylase production increased 18-fold. The amount of maltose utilized, during the growth period, at each concentration of maltose, was constant. Diauxic type growth was observed when maltose was used in addition to another carbon source (i.e., glucose, glycerol, fructose or sucrose). Maltose was not utilized until the other carbon source had been metabolized. Phenyl-, methyl- and ethyl-α-D-glucoside and methyl-β-D-maltoside were good inducers of

α-amylase and would not serve as a carbon source in a chemically defined medium supplemented with 0.1% casein hydrolysate. These compounds were therefore gratuitous inducers of α-amylase. Isomaltose, panose, butyl-α-D-glucoside, and methyl-α-D-maltotetraoside were not effective as inducers of α-amylase. Fructose had an inhibitory effect on constitutive (41%) and inducible (55%) α-amylase formation; glucose had no effect.

4. De novo synthesis of α-amylase

The pH optimum for the synthesis of α-amylase by washed cell suspensions of B. stearothermophilus 1503-4R was 6.7. α-Amylase synthesis began soon after the addition of the inducer (maltose, methyl-β-D-maltoside, or phenyl-α-D-glucoside, at 10^{-3} M), proceeded at a linear rate for 60 min, and then leveled off. Cell suspensions without inducer produced small amounts of α-amylase. The addition of glucose (2×10^{-3} M), sucrose (10^{-3} M), or glycerol (4×10^{-3} M) to washed cell suspensions failed to stimulate the production of α-amylase. Nitrogen starvation of washed cells for 60 min with fructose as a carbon source or by induction with pure maltose showed that the ability to produce α-amylase was lost. Examination of the amino acid pool at this time showed a general depletion of amino acids and the complete disappearance of tyrosine,

phenylalanine, proline, and valine. Replenishment of the amino acid pool with casein hydrolysate (0.5%) restored the ability of the cells to produce α-amylase. Chloramphenicol and 8-azaguanine were shown to inhibit α-amylase synthesis. Inhibition was observed immediately upon the addition of chloramphenicol to cell suspensions preinduced for varying periods of time. Actinomycin D and mitomycin C also inhibited α-amylase synthesis when added to washed-cell suspensions. The amino acid analogues, norvaline, norleucine, and ethionine inhibited α-amylase formation by 72, 53, and 38%, respectively. p-Fluorophenylalanine inhibited the synthesis of active α-amylase by 92% and the incorporation of proline-C^{14} into α-amylase and cellular proteins by 95 and 74% respectively.

5. Preferential synthesis of α-amylase

Washed-cell suspensions of B. stearothermophilus 1503-4R induced with pure maltose preferentially synthesized α-amylase in the presence of 5-methyl-tryptophan (5-MT). 5-MT did not inhibit the formation of active α-amylase or the incorporation of proline-C^{14} into the enzyme. In contrast, p-fluorophenylalanine inhibited the formation of active α-amylase by 92% and the incorporation of proline-C^{14} into the enzyme by 95%. p-Fluorophenylalanine and 5-MT

inhibited cellular protein synthesis, as measured by proline-C^{14} incorporation, by 74 and 72%, respectively.

C. Cytochrome pigments of B. stearothermophilus

A number of different methods were tried in an attempt to solubilize the cytochrome of this organism without success. The cytochrome is quite tightly bound to a lipid-cell granule.

D. Studies on a lysogenic system in Bacillus stearothermophilus

1. Induction and properties of a temperate phage

B. stearothermophilus 1503-4R growing at 55 C was induced to lyse when either 0.05 µg/ml of mitomycin C was added or when exposed to UV for 30 sec. Lysis of the induced cultures occurred 45 to 60 min after induction. Phage were assayed on B. stearothermophilus 4S giving turbid plaques 0.05 to 0.3 cm in diameter. Non-induced cultures of 1503-4R spontaneously produced one phage per 2.8×10^6 bacterial cells. The optimum temperature for phage production and assay was found to be 55 C. B. stearothermophilus 1503-4R was immune to the isolated temperate phage TP-1 and to a clear-plaque mutant phage TP-1C even when tested at phage multiplicities of 100. TP-1 and TP-1C phage were identical morphologically having a head 65 mµ in diameter and a tail 240 mµ long and 12 mµ wide. TP-1C phage deoxyribonucleic acid (DNA) had an $s_{20,w}$ value of 24.1 and a calculated molecular

weight of 1.21×10^7. DNA base compositions of TP-1 and TP-1C phage were identical (42% guanine + cytosine) but differed significantly from those of the lysogenic or indicator strains of B. stearothermophilus (50% guanine + cytosine). No unusual bases were detected in either the bacterial or phage DNA.

2. Synthesis of phage DNA in induced cultures

The synthesis of DNA, in mitomycin C (MC) induced cultures of B. stearothermophilus 1503-4R (TP-1), occurs in two stages. The rate of DNA synthesis (number of doublings of DNA/hr) in the first stage is 1.08 and lasts for 30 min. In the second stage, the rate of DNA synthesis is 5.5 and continues until the culture lyses. The difference in GC composition of B. stearothermophilus DNA (50%) and TP-1 phage DNA (42%) makes it possible to separate these two DNA's on methylated albumin kieselguhr columns and to determine in what stage the phage DNA is synthesized. Partially purified DNA was prepared from samples of a culture grown in the presence of thymidine-2-C^{14} and exposed to MC for varying periods of time. The radioactivity of the bacterial and phage DNA was determined and the ratio of bacterial DNA to phage DNA was plotted against time after MC induction. The synthesis of phage DNA occurs rapidly during the first stage and is complete

within 15 to 20 min after induction, while bacterial DNA is either degraded or its rate of synthesis is decreased. In the second stage only bacterial DNA is synthesized. In support of this is the finding that the synthesis of mature phage is complete 30 min after induction.

E. Properties and In vivo Transcription of a Lytic Thermophilic Bacteriophage

A bacteriophage (TP-84) will lyse certain strains of B. stearothermophilus over the temperature range of 43-70 C. TP-84 has a head 45 mμ in diameter and a tail 160 mμ in length; it has an $s_{20,w}$ of 400 and bands at a density of ρ = 1.508 g/cc in CsCl, pH 8.5. Phenol extracted TP-84 DNA has an $s_{20,w}$ of 30 and a base composition of 42% GC as determined by CsCl density gradient centrifugation (ρ = 1.704 g/cc); 43% GC from Tm measurements (87.2 C with a 41% hyperchromic shift); and 45% GC from analyses of phosphodiesterase digests.

TP-84 DNA denatured by heat or alkali forms two bands in a CsCl density gradient, ρ_L = 1.717 and ρ_H = 1.727. The L and H strands were isolated by stepwise chromatography on a methylated albumin kieselguhr column. Hybridization of the isolated strands with P^{32}-pulse labeled RNA from TP-84 infected cells (3-21 min after infection) showed RNAase resistant binding of RNA in the ratio of about 20H to 1L. It appears

that the H strand is primarily responsible for TP-84 induced RNA synthesis.

F. Isolation of strains of B. stearothermophilus with Altered Requirements for Spore Germination

It was found that exposure at 121 C for 12 min induced heritable changes in spores of Bacillus stearothermophilus 1518-S. The mutants were shown to have altered requirements for spore germination.

G. Reports and Publications

Abstracts

Welker, N. E. and L. L. Campbell 1962 Inducers of the α-amylase of Bacillus stearothermophilus. Bacteriol. Proc. 1962, 106.

Welker, N. E. and L. L. Campbell 1963 Evidence for the de novo synthesis of α-amylase by Bacillus stearothermophilus. Bacteriol. Proc. 1963, 114.

Welker, N. E. and L. L. Campbell 1964 Induction of a bacteriophage in Bacillus stearothermophilus. Bacteriol. Proc. 1964, 136.

Saunders, G. F. and L. L. Campbell 1964 Properties and in vivo transcription of a thermophilic bacteriophage. Abstract 148th Meeting American Chemical Society, Section C, p. 36.

Publications

Welker, N. E., and L. L. Campbell 1963 Effect of carbon sources on formation of α-amylase by Bacillus stearothermophilus. J. Bacteriol. 86:681-686.

Welker, N. E., and L. L. Campbell 1963 Induction of α-amylase of Bacillus stearothermophilus by maltodextrins. J. Bacteriol. 86:687-691.

Welker, N. E., and L. L. Campbell 1963 Induced biosynthesis of α-amylase by growing cultures of Bacillus stearothermophilus J. Bacteriol. 86:1196-1201.

Welker, N. E., and L. L. Campbell 1963 De novo synthesis of α-amylase by Bacillus stearothermophilus. J. Bacteriol. 86: 1202-1210.

Welker, N. E., and L. L. Campbell 1964 Preferential synthesis of α-amylase by Bacillus stearothermophilus in the presence of 5-methyl-tryptophan. J. Bacteriol. 87:828-831.

Welker, N. E., and L. L. Campbell 1965 Induction and properties of a temperate bacteriophage from Bacillus stearothermophilus. J. Bacteriol. 89:in press.

Campbell, L. L., C. M. Richards, and E. E. Sniff 1965 Isolation of strains of Bacillus stearothermophilus with altered requirements for spore germination. In Spores III, L. L. Campbell and H. O. Halvorson (eds). In press.