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by Richard N Ushijima

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Master of Science in Bacteriology at Montana State College
Montana State University

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The experiments also revealed the presence of a phosphoglueomutase, which converts glucose-l-phosphate to glucose-6-phosphate, a product uti-lizable through the glycolytic pathways.

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ABSTRACT

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Large quantities of cells were grown on a starch medium, and the cells were harvested by centrifugation. The cell-free enzyme extract was prepared by grinding the cells with glass beads in a micro-Waring blendor.

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INTRODUCTION

By virtue of radiant energy from the sun, plants containing chlorophyll are able to utilize carbon dioxide and synthesize complex carbohydrates. Carbohydrates are stored by all plants as reserve food, and starch is by far the most abundant and widely distributed.

Investigation of starch began in the early 1800's. According to Whistler and Smart (1953), in 1811 Kirchoff treated an aqueous solution of starch with sulfuric acid and obtained crystalline glucose. In the latter years of the 19th century, Emil Fischer concluded that starch consisted of pyranose forms of glucose.

The empirical formula of starch is written as $(C_6H_{10}O_5)_x$. However, starch consists of either amylose (straight chain glucose molecules) or amylopectin (branched chain glucose molecules). Some of the properties of starch are listed on Table I.

There are many microorganisms possessing enzymes which catalyze the hydrolysis of starch. These enzymes, known as amylases, enable the microorganisms to utilize starch as the primary source of carbohydrate. The enzymatic hydrolysis of starch by the alpha and the beta amylases was demonstrated in 1833 by Payen and Perez (Whistler and Smart, 1953). Both of these enzymes will readily hydrolyze the 1-4 linkages of the starch molecules but will not hydrolyze the 1-6 linkages, which occur in branching chains.

Alpha amylase, known as the liquefying enzyme, hydrolyzes amylose into glucose, maltose, and maltotriose. The enzyme hydrolyzes amylopectin into glucose, maltose, isomaltose, maltotriose, and a variety of small molecular weight dextrins containing alpha, 1-6 linkages. Beta amylase,

TABLE I

Properties of starch

AMYLOSE	AMYLOPECTIN
1) high digestibility with alpha and beta amylase (100 per cent)	low digestibility with beta amylase (50 per cent)
2) relatively low molecular weight--molecule contains 220 glucose residues with only one non-reducing end group per molecule	high molecular weight--molecule contains over 1,000 glucose residues with one non-reducing end group for every 20 to 30 glucose residues
3) deep blue with iodine	purple or reddish color with iodine
4) adsorption of large amounts of iodine isopotentially in potentiometer titration	does not adsorb iodine isopotentially
5) forms good films	forms poor films
6) poor pasting characteristics	makes strong pastes

known as the saccharifying enzyme, hydrolyzes amylose from the non-reducing end of the starch fraction to maltose. Amylopectin is similarly hydrolyzed, but further hydrolysis by beta amylase is blocked when a 1-6 linkage is encountered, leaving limit dextrins.

Some plants and animals possess the enzymes phosphorylases, which catalyze the breakdown or synthesis of starch-like materials. Cori et al (1938) were the first to discover this enzyme in the muscle tissues of animals. Hanes (1940) found starch phosphorylase in pea seeds and in potato tubers.

Hobson and MacPhearson (1952) claimed to have detected a trace of starch phosphorylase in a streptococcus that was isolated from the rumen

of sheep, but no definite evidence was given to substantiate their conclusions. Furthermore, it is questionable as to whether the phosphorolysis was due to a maltose phosphorylase or a starch phosphorylase in a system where starch is hydrolyzed to maltose in the presence of an amylase, since maltose phosphorylase has been shown to exist in microorganisms (Fitting and Doudoroff, 1952). There is also the possibility that the two enzymes mentioned are identical.

The reactions which are believed to occur in starch phosphorolysis and maltose phosphorolysis are as follows:

$$(\text{glucose})_n + H_3PO_4 \longrightarrow \text{glucose-1-phosphate} + (\text{glucose})_{n-1}$$

$$\text{maltose} + H_3PO_4 \longrightarrow \text{glucose-1- phosphate} + \text{glucose}$$

Like the hydrolysis of starch by amylase, phosphorolysis occurs at the 1-4 linkages but is blocked by the 1-6 linkages. In the presence of inorganic phosphate, the phosphorolysis of starch proceeds from the non-reducing end of the starch molecule releasing glucose-1-phosphate. Phosphoglucomutase converts the glucose-1-phosphate to glucose-6-phosphate, which can then enter into the glycolytic scheme through the action of established enzyme systems.

The purpose of this investigation was to search into the possibility of a starch phosphorylase being present in a bacterium. Streptococcus bovis was chosen as the experimental bacterium because of its rapid growth on starch. Furthermore, being a lactic acid bacterium which lives anaerobically in the rumen of cattle, Streptococcus bovis, through hydrolysis, is able to obtain only a small portion of the energy available from starch. Therefore, this organism might be expected to utilize starch in another manner that would yield more energy. One method whereby this could be done would be to break down starch by phosphorolysis.

Streptococcus bovis and other microorganisms living in the rumen of cattle break down the complex starch molecule by enzymatic action into simple compounds. The enzymatic degradation of complex carbohydrates is especially beneficial to the animal, since it does not have a ptyalin in the saliva or an amylase to secrete into the rumen. Hence, the animal relies on microorganisms or plant enzymes for rumen digestion.

Streptococcus bovis was first isolated by Winslow and Palmer (1910) but was later described and named by Orla-Jensen (Sherman, 1937).

Some characteristics of Streptococcus bovis, as defined by Bergey's Manual of Determinative Bacteriology (Breed, et al, 1948) are as follows: gram positive cocci appearing in short chains; acid in milk at 3 to 5 days, followed by reduction of litmus; tolerant of 2 per cent NaCl but not 4 per cent NaCl; non-hemolytic, facultatively anaerobic, nitrites not produced from nitrates, starch hydrolyzed.

METHODS AND MATERIALS

CULTURE

A culture of Streptococcus bovis was isolated from the rumen fluid of an Angus bull. Serial dilutions of fresh rumen fluid were made in tubes of a sterilized culture medium consisting of 1.0 per cent commercial grade starch, 1.0 per cent yeast extract, 10 per cent by volume of the rumen fluid, 1.5 per cent agar, and tap water. The roll tube technique as suggested by Hungate (1950) was used. The tubes were incubated at 35 C for 20 hours.

Starch hydrolyzing colonies were surrounded by clear zones. These were picked with sterile pasteur pipettes and reinoculated into other sterilized culture media. Further serial dilutions were made to insure the isolation of a pure colony. A pure culture having most of the characteristics of Streptococcus bovis, as defined by the Bergey's Manual of Determinative Bacteriology, was transferred into stock culture agar containing 1.0 per cent added soluble starch. Stock cultures of Streptococcus bovis were viable for over three months on this medium.

PREPARATION OF LARGE CULTURES

Several grams of bacterial cells were required to prepare cell-free enzyme extracts of Streptococcus bovis. In order to obtain a sufficient quantity of bacterial cells, a large volume of culture fluid was necessary.

A 10 ml culture, which was inoculated with the organism picked from the stock culture agar medium, served as the inoculum for a two liter culture.

Two liters of medium consisting of 0.8 per cent commercial starch, 0.5 per cent yeast extract, 0.2 per cent peptone, 0.05 per cent K_2HPO_4,

0.05 per cent KH_2PO_4, 0.002 per cent $(NH_4)_2SO_4$, 0.002 per cent NaCl, 0.0005 per cent $MgSO_4$, 0.0001 per cent $CaCl_2$, 0.0001 per cent sodium thioglycollate, and 0.5 per cent $NaHCO_3$ yielded about 20 grams of wet cells. This quantity was sufficient for several experiments.

Since anaerobic conditions favored the growth of the organism, oxygen was removed from the medium after inoculation by flushing with carbon dioxide. The carbon dioxide in combination with the $NaHCO_3$ also served as a buffering system at about pH 7.0.

Occasionally, more than 20 grams of cells were needed. Instead of growing several two liter cultures, a twenty liter culture in a carboy was used. Cultures of this size required continuous mixing for the most rapid growth. This was done with a magnetic stirrer.

All of the cultures were incubated at 35 C. The average incubation time was thirty hours, at which time the culture was ready for harvesting. The cells were harvested in a Sharples super centrifuge at a relative centrifugal force of 62,000 G. The cell paste obtained was used for the preparation of enzyme extracts.

PREPARATION OF CELL-FREE BACTERIAL EXTRACTS

The cell-free enzyme extract was prepared by grinding the cell paste with glass beads. The method of Lamanna and Mallette (1954) was used. A micro-Waring blendor surrounded with an ice jacket was used for grinding the cells. Five ml of distilled water were mixed with 20 grams of wet cells for one minute. Forty-five grams of glass beads were then slowly added while continuously mixing. After 20 minutes, 20 ml of 0.1 M pH 7.5 Tris buffer (tris hydroxy aminomethane) were added, and the mixing was continued

for an additional five minutes. The mixture was centrifuged at 8-10,000 G for one and a half hours under refrigeration. The supernatant fluid contained the enzymes active on starch.

A protein determination on the cell-free enzyme preparation was done as suggested by Levin and Braur (1951) using the Biuret reagent, which was standardized by the kjeldahl method. The enzyme extract usually contained about 12 mg of protein per ml.

PHOSPHORUS DETERMINATION

Inorganic phosphate concentration was determined by the method of Lowry and Lopez (1946) without protein precipitation. Two normal HCl was used to stop the reaction instead of trichloroacetic acid. A blank containing the same concentration of enzyme preparation and the reagents, as in the other tubes but without added inorganic phosphate, served as a control for the presence of inorganic phosphate in the enzyme preparation. The blank also contained glucose instead of soluble starch. All readings were made with the Klett-Summerson colorimeter using filter 66 (red).

To assay for acid labile phosphate esters, such as glucose-1-phosphate, an equal volume of 2 N HCl was added to an aliquot of the reaction mixture, which was then boiled for seven minutes. A quantitative determination of the alkaline labile phosphate esters, as glucose-6-phosphate, was made by adding an equal volume of 2 N NaOH to the reaction mixture, which was then boiled for 30 minutes.

The phosphate released from the esters was measurable colorimetrically. Therefore, the increase in concentration of inorganic phosphate following acid or alkaline hydrolysis determined the concentration of organic phosphate esters formed in the reaction mixtures.

CHROMATOGRAPHY

The ascending paper chromatographic technique was used to identify the phosphate esters. Schleicher and Schuell No. 589 paper was utilized with the solvents of Bandurski and Axelrod (1951); 60 parts of methanol, 15 parts of 88 per cent formic acid, and 5 parts of water. The chromatograms were run at 9 C in glass battery jars sealed with scotch tape.

After approximately 24 hours, the chromatograms were dried and were then dipped through the indicator of Burrows, Grylls and Harrison (1952); 1 g ammonium molybdate, 8 ml water, 3 ml 12 N hydrochloric acid, 3 ml perchloric acid, and 86 ml acetone. After redrying completely, the chromatograms were placed under an ultraviolet lamp for 5 minutes to develop the blue color of the phosphomolybdic complexes formed.

Reducing sugars were chromatogrammed by the descending paper chromatographic technique. The solvent of Jeanes et al (1951), butanol 5 parts, pyridine 3 parts, water 1.5 parts and the indicator of Baar (1955), 99 ml 85 per cent isopropanol, 1.66 g phthalic acid, and 1 ml aniline, were used. The chromatograms were developed at 100 C for ten minutes. Reducing sugars produced brown colored spots on the paper.

GLUCOSE DETERMINATION

In several instances it was necessary to measure glucose in the presence of other reducing sugars. This can be done manometrically by using glucose oxidase (Keilin and Hartree, 1948). The source of glucose oxidase was a commercial enzyme, Takamine Dee-O.

Glucose oxidase oxidizes glucose to gluconic acid and hydrogen peroxide as follows:

$$C_6H_{12}O_6 + H_2O + O_2 \text{ ---------- } C_6H_{12}O_7 + H_2O_2$$

In the presence of catalase, present in the commercial glucose oxidase, and added ethyl alcohol, the hydrogen peroxide is decomposed to water and oxygen with the simultaneous coupled oxidation of the alcohol. The oxygen uptake can be measured manometrically in a Warburg respirometer and is proportional to the amount of glucose oxidized. This method is highly specific for glucose.

EXPERIMENTAL RESULTS

PHOSPHORYLASE

The possibility of a starch phosphorylase being part of the enzyme system of Streptococcus bovis was investigated. The phosphorylase activity was measured by a reduction of inorganic phosphate in the reaction mixtures containing a cell-free enzyme preparation, inorganic phosphate, and either maltose or soluble starch (Table II).

TABLE II

Test for phosphorylase activity

TUBE NUMBER	1	2	3	4	5	6
Tris buffer pH 7.5	1.0	1.0	1.0	1.0	1.0	1.0
Enzyme	.5	.5	.5*	.5*	.5	.5
$MgSO_4$ (.1 M)	.05	.05	.05	.05	.05	.05
NaF (.5 M)	.2	.2	.2	.2	.2	.2
K_2HPO_4 (.1 M)		.2	.2	.2	.2	.2
Soluble starch (.1 M)				.5		.5
Maltose (.1 M)			.5		.5	
Glucose (.1 M)	.5	.5				
Distilled water	.25	.05	.05	.05	.05	.05
Total volume in ml	2.5	2.5	2.5	2.5	2.5	2.5

* Boiled enzyme

The $MgSO_4$ was added to activate any phosphorylase present and the NaF was added to inhibit any phosphatase activity which might interfere with the

accumulation of organic phosphate esters. The reaction tubes containing glucose served as a blank and a control. The temperature of the reaction mixture was maintained at 37 C throughout the experiment. The concentration of the inorganic phosphate and the acid labile phosphate esters were determined at 0, 30, 60, and 120 minute intervals.

As shown in figure 1 and figure 2, approximately 1.6 micromoles/ml of inorganic phosphate was converted to organic phosphate esters at 120 minutes with both maltose and soluble starch as substrates. The determination of acid labile phosphate esters indicated that 1.2 micromoles/ml of the inorganic phosphate had been incorporated into glucose-1-phosphate. Although no determination of the alkaline labile phosphate ester concentration was made, it was assumed that 0.4 micromole/ml of the phosphate was in the form of glucose-6-phosphate.

An aliquot of the reaction mixture chromatogrammed for reducing sugars showed that maltose was being formed as a result of amylase action on soluble starch. Consequently, no assumption can be made as to whether the phosphorylase was acting on maltose or starch.

Therefore, to demonstrate unequivocally the presence of a starch phosphorylase, it was necessary that the alpha amylase activity be inhibited without inhibiting the phosphorylase activity. This required further investigations of amylase activity.

AMYLASE

The majority of the starch hydrolyzing microorganisms found in the rumen have been shown to have an alpha amylase (Hobson and MacPherson, 1952). A preliminary experiment was made to determine whether Streptococcus bovis had an alpha or a beta amylase. This was done with the cell-free enzyme

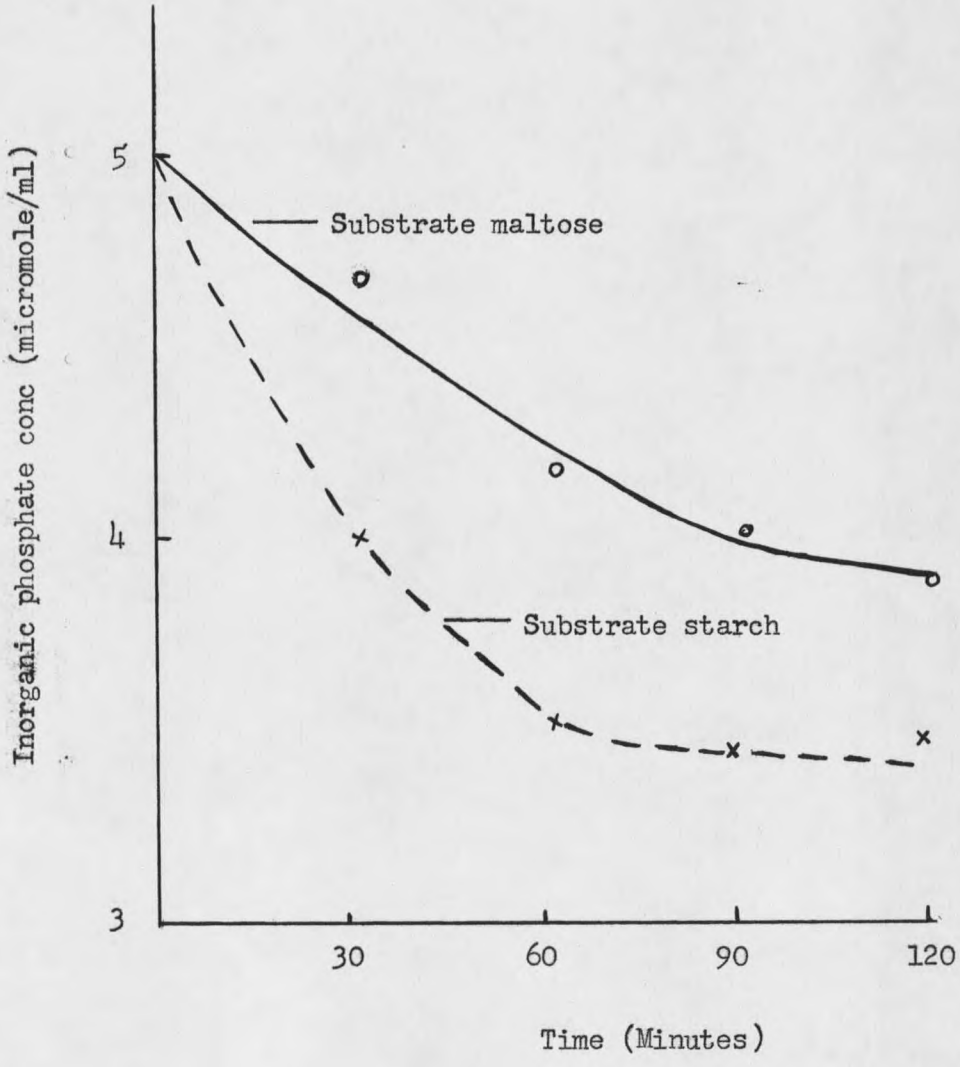

Figure 1 - Disappearance of inorganic phosphate during phosphorolysis of starch and maltose.

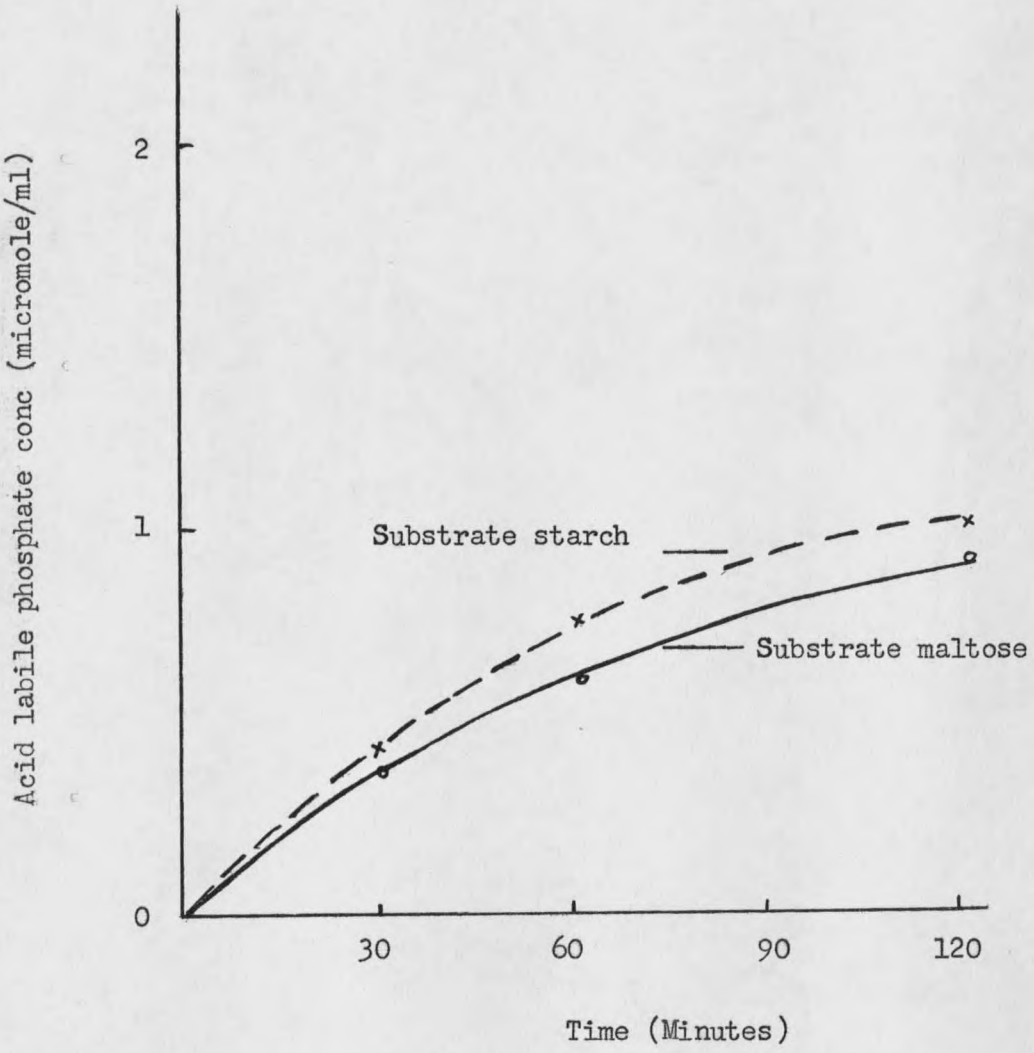

Figure 2 - Formation of acid labile phosphate esters during phosphorolysis of starch and maltose

preparation and also with the cell-free supernatant of the culture medium in which the bacteria had been grown.

The reaction mixtures consisted of 0.5 ml of the enzyme preparation or the supernatant, 0.5 ml of 0.1 M amylopectin, and 1.0 ml of 0.05 M tris buffer pH 7.5 and were incubated at 37 C. A drop of iodine was added to an aliquot of each of the reaction mixtures at four minute intervals to determine the time required for hydrolysis of all of the starch.

Alpha amylase will completely hydrolyze amylopectin, destroying its ability to form a blue colored complex with iodine. Beta amylase, however, cannot hydrolyze amylopectin completely. When iodine is added at the end point of hydrolysis, a bluish-red color, which is characteristic of a beta amylase-amylopectin reaction, develops.

The reaction mixtures containing either the cell extract or the supernatant showed that the amylopectin was completely hydrolyzed within twenty minutes, demonstrating that starch hydrolysis was due to alpha amylase.

In order to demonstrate the presence of a starch phosphorylase in the presence of amylase, it was necessary that the amylase activity be inhibited without inhibiting the phosphorylase activity. Otherwise, no definite conclusion could be drawn as to whether phosphorylation was due to a maltose phosphorylase or a starch phosphorylase.

No published methods of separating amylase from phosphorylase were found. Therefore, the most practical methods for obtaining separate phosphorolytic activity appeared to be through the use of inhibitory chemicals and the influence of pH.