POLYSACCHARIDE SYNTHESIS ON IMMOBILIZED PHOSPHORYLASE

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ABSTRACT

Immobilized potato phosphorylase has been prepared both by adsorption onto colloidal silica and also by attachment to porous glass. Enzyme loading on the silica derivative was greater than with porous glass, but the glass derivative had superior physical properties for flow-through operations. Each of the immobilized preparations could synthesize polysaccharide over a wide molecular-weight range up to the limit of solubility of the polysaccharide product. The porous-glass derivative was used in continuous operation at 45° and had an observed half-life of 28 days.

INTRODUCTION

Immobilized enzymes, such as trypsin[1] and chymotrypsin[2], which act on macromolecular substrates, have been widely studied. However, relatively little is known regarding the use of immobilized enzymes to produce a macromolecular product. We have examined a system for producing polysaccharide from D-glucose by using immobilized enzymes. The key enzyme in this system is phosphorylase, which catalyzes the reversible formation or degradation of polysaccharide as shown.

$$x \text{ D-glucosyl phosphate} + (\text{D-glucose})_n \longrightarrow (\text{D-glucose})_{n+x} + xP_i$$

The equilibrium position, although pH-dependent, can be adjusted in favor of polysaccharide synthesis. This report describes the immobilization of phosphorylase by two different techniques and its use in the synthesis of polysaccharide.

EXPERIMENTAL

Purification and assay of potato phosphorylase. — Phosphorylase was purified from potatoes according to the procedure of Lee[3]. The assay was based on the appearance of phosphate, which was determined by a modified Fiske–Subbarow method[4]. The enzyme solution to be assayed was added to 0.8 ml of a citrate–amylopectin solution (1% amylopectin in 0.125M citrate, pH 6.3) and enough water to make a total volume of 0.9 ml. After a 5-min incubation period at 30°, 0.1 ml of D-glucosyl phosphate (0.1M) was added and the reaction was timed for 5 min. At the end of 5 min,

the phosphate that had formed was determined by adding 0.1 ml of the assay mixture to 2 ml of 7.2% perchloric acid. Ammonium molybdate (0.3 ml, 3.32%, *w/v*) was then added followed by water (0.3 ml) and 0.3 ml of amidol reagent (0.4%, *w/v* 2,4-diaminophenol dihydrochloride in 8%, *w/v* $NaHSO_3$). Color was allowed to develop for 20 min and was then measured at 680 nm. For a blank, 0.1 ml of water was used in place of the assay-mixture aliquot. To correct for phosphate present in the D-glucosyl phosphate and for any hydrolysis caused by the perchloric acid, 0.1 ml of D-glucosyl phosphate was added to 0.8 ml of citrate–amylopectin solution and water (0.1 ml) in place of the enzyme. A 0.1-ml aliquot of this mixture was then used for phosphate analysis and the value obtained used to correct the amount of phosphate determined for the enzyme-catalyzed reaction.

The initial concentration of D-glucosyl phosphate was determined by heating the tube containing the 0.1-ml aliquot in 2 ml of perchloric acid for 15 min in a boiling water bath. The increased amount of phosphate required dilution before subjection to the phosphate analysis.

Enzyme units were defined as $K \times 1000$, where K is the first-order velocity constant ($\log_{10}$, min^{-1}). K was calculated by the following equation:

$$K = \frac{1}{t} \log \frac{X_e}{X_e - X}$$

where X_e is the percentage of D-glucosyl phosphate which would be converted into polysaccharide at equilibrium at certain pH, and X the percent converted at time t (in min) under the assay conditions. At pH 6.3, the value of X_e was 85.

Preparation of phosphorylase–silica–cellulose product. — A solution of purified potato phosphorylase (2800 units, an excess of enzyme relative to the binding capacity of the added support) in 4 ml of 0.5M, citrate buffer (pH 6.3) was treated with 10 mg of poly(ethyleneimine)-coated silica as a suspension in citrate buffer. (The silica used was fumed silica, 0.012 μm particle diameter, obtained from Sigma Chemical Co.) The poly(ethyleneimine)-silica[5–7] was necessary, as untreated silica was not so effective in binding the enzyme in the neutral pH range. After stirring for 30 min glutaraldehyde (10 μl; 2.5% aqueous solution) was added and the mixture was stirred for an additional 15 min. The enzyme–silica envelope was collected by centrifugation and the pellet washed several times with citrate buffer. After the last wash, the solids were suspended in pH 7,4, 0.1M Tris buffer, treated with 10 ml "active-ester" cellulose[7], and stirred at 4° overnight. The product was filtered on a medium-porosity fritted-glass funnel and washed with pH 6.3 citrate buffer.

Preparation of immobilized phosphorylase by using porous, alkylamine glass. — A sample of alkylamine glass, 550 A, 40–80 mesh was kindly supplied by Corning Glass Works. To attach the enzyme to the glass, two methods were used. In each case, the amount of enzyme added was in excess of the binding capacity for the amount of support used.

Method I. Glutaraldehyde coupling. The alkylamine glass (100 mg) was suspended in 2 ml of pH 7.4 Tris buffer (0.1M) and treated with 1 ml of 25% glutaralde-

hyde. The contents were gently mixed for about 15 min, during which time the glass became light yellow. The derivatized glass was washed by decantation with water until the odor of glutaraldehyde could no longer be detected (about 8–10 washes). A phosphorylase solution (1050 units in 2 ml) in pH 7.4 Tris buffer was treated portionwise with the glutaraldehyde–glass, mixing gently during the additions. After 30 min, the supernatant was removed and assayed. The glass-bound enzyme was washed thoroughly with Tris buffer and water, and then assayed. To obtain reproducible aliquots of the glass, the standard measurement adopted was 1 cm of packed glass in a 50-μl disposable pipette.

Method II. 2-Amino-4,6-dichloro-s-triazine coupling. The alkylamine glass (100 mg) was mixed with 1 ml of a solution made by dissolving 2-amino-4,6-dichloro-*s*-triazine[8] (0.1 g) in acetone (2.5 ml) and water (2.5 ml) at 50°. After 5 min at 50°, 0.4 ml of a second solution (0.15 g of sodium carbonate in 1 ml of water +0.6 ml of 12M HCl) was added and kept for 5 min at 50°. The pH was lowered to below 7 with 6M HCl and the glass washed well with acetone–water and finally with water. To couple the protein, the entire amount of derivatized glass was mixed with 1 ml of phosphorylase solution (1050 units) and 1 ml of 0.1M borate buffer, pH 8.5. After 2 h at room temperature, the extent of coupling was determined by assaying the supernatant and the washed glass.

Effect of primer concentration on the properties of the product synthesized by phosphorylase. — A 4% solution of degraded amylopectin, for use as a primer, was prepared by adding 1.0 g of amylopectin to 10 ml of water and 1 ml of M HCl and heating the mixture in a boiling water bath until aliquots gave only a weak starch–iodine reaction with a KI–I_2 reagent (2% and 0.2%). Sodium citrate (0.725 g) was then added and the pH adjusted to 6.3. A small amount of insoluble material was removed by centrifugation, and the supernatant was made up a total volume of 25 ml. For experiments in which the molecular weight of the product was to be determined, the degraded amylopectin, after neutralization, was subjected to chromatography on Sephadex G-50. The fraction staining a light-violet color with the starch–iodine reagent, appearing before maltose and D-glucose, was collected and lyophilized.

Substrate solutions were made from the 4% stock solution of degraded amylopectin to give various dilutions. To each diluted primer was added 0.2 ml of immobilized phosphorylase (200 units, adsorbed on colloidal silica) and 1.0 ml of D-glucosyl phosphate (0.3M, pH 6.3) to give a final volume of 3.0 ml. Aliquots were removed, after reaction overnight, for phosphate analysis and for determining the absorption maximum of the starch–iodine complex. The remainder of each tube was treated with M KOH (3 ml) and the viscosity measured at 30° by using a No. 50 Cannon viscometer and sample volume of 6 ml.

Determination of K_m. — The initial reaction velocity (μmoles per min) was determined for a series of well-stirred solutions in which the concentration of D-glucosyl phosphate was varied over the range 0.5–10 mM. Each solution contained 0.8 ml of primer solution (1% amylopectin in 0.125M citrate, pH 6.3), 0.1 ml enzyme (soluble or immobilized), and 0.1 ml of D-glucosyl phosphate. The reaction was

stopped at 5 min and assayed for appearance of phosphate. The initial reaction-velocity was calculated by $K \times 2.3 \times [S]$, where $[S]$ is the intial concentration of D-glucosyl phosphate. K_m values were then calculated from Lineweaver–Burk plots.

RESULTS

The two methods used for making immobilized phosphorylase differ widely in terms of binding capacity of the support material and physical properties of the product. The amount of enzyme attached per gram of porous glass is lower than the amount per gram on a silica–cellulose support (Table I), but the physical properties of the glass product are superior for flow-through operations.

TABLE I

ACTIVITY OF IMMOBILIZED PHOSPHORYLASE

Support	*Coupling method*	*Activity (units/g support)*
Silica–cellulose	Adsorption followed by cross-linking and covalent attachment	12,000–15,000
Porous glass	Glutaraldehyde	2500–3200
Porous glass	2-Amino-4,6-dichloro-*s*-triazine	1850

The apparent K_m for D-glucosyl phosphate was determined by investigating the effect of substrate concentration on the reaction rate for both soluble phosphorylase and that immobilized on colloidal silica. The apparent K_m at 30° was found to be 2.50 mM for the soluble enzyme. By using well-stirred suspensions of phosphorylase on colloidal silica, the apparent K_m for D-glucosyl phosphate was found to be 2.53 mM. These values are lower than a previously reported value (3.5 mM)[3], but our measurements were made with a higher concentration of primer.

During initial experiments with both the soluble and immobilized enzyme, precipitation of the polysaccharide product was observed as the reaction proceeded. The precipitation of the product is related to the growing amylose-like chains, which become insoluble when a certain size is reached. This problem can be avoided by using a ratio of D-glucosyl phosphate to primer that allows chain elongation to remain below this critical limit. It is also helpful to begin with a degraded primer of smaller molecular-weight and to operate the reaction at higher temperatures. The effect of primer concentration on the type of product formed is shown in Table II. The data in Table II were taken from experiments where phosphorylase–silica derivatives were used. However, similar results were obtained with the phosphorylase–glass derivatives.

Small columns containing 300–400 mg of the immobilized phosphorylase–glass derivative were examined for their stability upon continuous operation, and for the effect of flow-rate on activity. The effect of flow-rate on the fraction of D-glucosyl phosphate converted is shown in Fig. 1. The column was operated at 30° with varying initial substrate concentrations, as indicated. The pH of the solution was 6.3, which

TABLE II

EFFECT OF PRIMER CONCENTRATION ON THE PROPERTIES OF THE POLYSACCHARIDE PRODUCED BY USING PHOSPHORYLASE IMMOBILIZED ON COLLOIDAL SILICA

Initial concentration of primer[a] (%)	*Intrinsic viscosity*	*Estimated molecular weight of polysaccharide*[b]
0.106	0.74	71,800
0.466	0.314	30,500
1.0	0.198	19,200

[a]Primer used was an acid-degraded amylopectin (fractionated on Sephadex G-50) having an approximate molecular weight of 5000. [b]Calculated according to the equation[12] $[\eta] = 1.67 \times 10^{-3}$ degree of polymerization.

means that the equilibrium position for the reaction lies at 85% conversion. The primer used in all of the column studies was an acid-degraded amylopectin, which stained light violet with the starch–iodide reagent, and had an estimated molecular weight of 5000, as determined by viscosity measurements.

The stability of this immobilized phosphorylase derivative was examined by operating two small columns at 45° with a continuous feed of substrate solution. The primer concentration was 0.8% for one column and 0.05% for the other, with the D-glucosyl phosphate concentration being 0.01M for each. The solutions were made

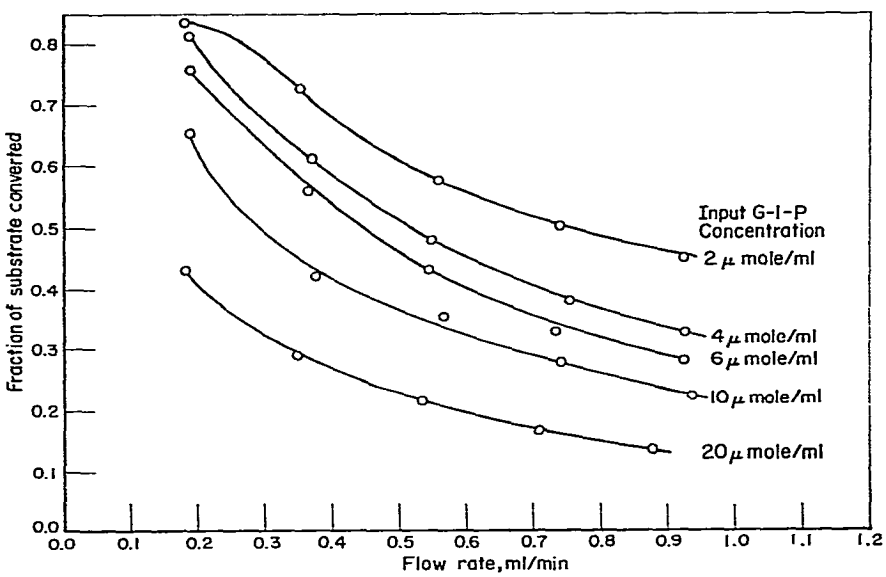

Fig. 1. Effect of flow-rate on the activity of a phosphorylase column. The column was packed with phosphorylase–glass (235 units) prepared via alkylamine glass and glutaraldehyde as described in the text. Initial substrate solutions (G-1-P = D-glucosyl phosphate) were made with 0.1M citrate buffer (pH 6.3) and with an acid-degraded amylopectin as primer (0.8% primer concentration). The column was maintained at 30°.

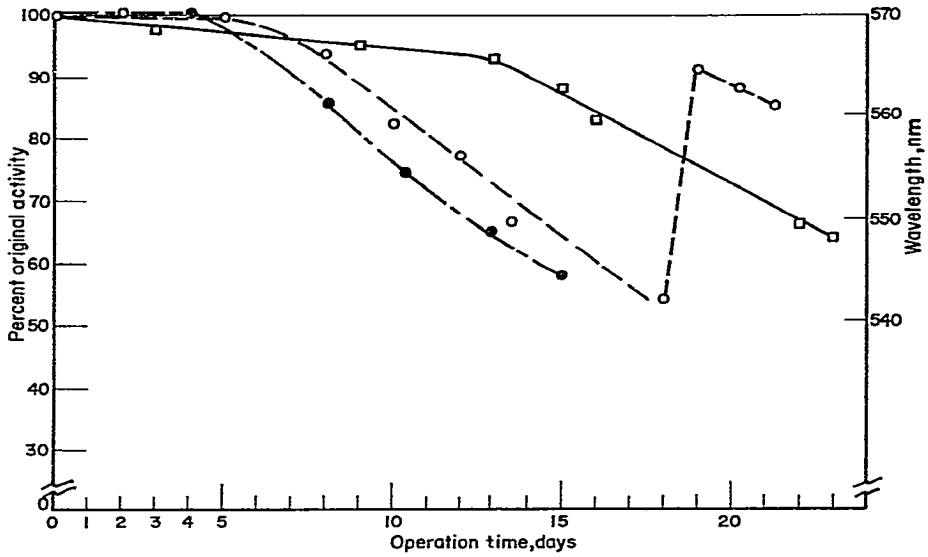

Fig. 2. Stability of a phosphorylase column during continuous operation. Two columns were packed with phosphorylase–glass (900 units in each) prepared via alkylamine glass and glutaraldehyde as described in the text. Each column was maintained at 45° with a continuous feed of substrate solution, which consisted of 0.01M D-glucosyl phosphate in 0.1M citrate (pH 6.3) and the primer concentration (acid-degraded amylopectin) indicated. The flow-rate in each case was 0.14 ml/min. After 18 days of operation, each column was treated with a glucoamylase solution for 1 h, washed with citrate buffer, and the substrate solution started again. □ primer concentration = 0.8%; ○ primer concentration = 0.05%; ◕ wavelength of the absorption maximum of the polysaccharide–iodine complex observed with the product isolated from the column effluent with 0.05% primer.

with 0.1M citrate buffer, pH 6.3. The results of this experiment are shown in Fig. 2. The two primer concentrations were selected to allow products of different molecular weight to be formed. With the porous-glass support, products of larger molecular weight might have a tendency to plug-up the support and shield the enzyme from further access to the substrate solution. The high concentration of primer was selected to permit formation of low molecular-weight product, so that any observed loss of activity could be attributed to factors other than column blockage. With this column, the half-life was observed to be approximately 28 days. After about 13 days of operation, the rate of loss of activity began to increase for some unexplained reason. The column having a lower concentration of primer did produce a higher molecular-weight product, as could be seen by the blue color of the starch–iodine complex compared with the light-violet color produced with the starting material. This column lost activity more rapidly than the column producing a low molecular-weight product. Product isolated from the column effluent at various times showed a decrease in the wavelength of maximal adsorption of the polysaccharide–iodine complex that paralled the decrease in percent of conversion of D-glucosyl phosphate (Fig. 2). It has been shown previously that the chain-length of linear amylose-like oligomers is related to the wavelength of the absorption maximum for the iodine complex[9,10].

After several days of operation, the glass, which was brown initially, became white. A sample of the glass was removed from the column and stained with the KI–I_2 reagent. The entire glass particle became dark blue, suggesting that it was coated with polysaccharide. A second sample of the glass was allowed to remain in contact with glucoamylase for an hour in an effort to digest this coating enzymically. After this treatment, very little blue-staining material could be observed. Therefore, when the activity of the column had decreased to near 50% of the starting activity, a solution of glucoamylase was passed through the column for 1 h. After thorough washing with citrate buffer, the substrate solution was again passed through the column. As seen in Fig. 2, the activity was restored almost to the starting value by this treatment. A similar treatment with glucoamylase had little effect on the other column. The longer half-life of the column that produced higher-molecular-weight product may be due to a decrease in the solubility of the glass as a result of the polysaccharide coating that forms on the glass surface. Solubility of the glass support during continuous operation has been cited as one of the major factors leading to a decrease in activity of enzymes immobilized on porous glass[11]. Modified glass supports have recently been prepared[11] that have improved solubility characteristics and correspondingly longer half-lives. It will be of interest to examine the phosphorylase system with these newer supports.

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REFERENCES

1 A. BAR-ELI AND E. KATCHALSKI, *J. Biol. Chem.*, 238 (1963) 1690.
2 R. P. PATEL, D. V. LOPIEKES, S. P. BROWN, AND S. PRICE, *Biopolymers*, 5 (1967) 577.
3 Y. P. LEE, *Biochim. Biophys. Acta*, 43 (1960) 18 and 25.
4 R. J. L. ALLEN, *Biochem. J.*, 34 (1940) 858.
5 R. HAYNES AND K. A. WALSH, *Biochem. Biophys. Res. Commun.*, 36 (1969) 235.
6 K. A. WALSH, M. PANGBURN, AND R. HAYNES, *Abstr. Papers Amer. Chem. Soc. Meeting*, 162 (1971) ORPL 008.
7 D. L. MARSHALL, J. L. WALTER, AND R. D. FALB, *Biotech. Bioeng.*, in press.
8 G. G. KAY AND M. D. LILLY, *Biochim. Biophys. Acta*, 198 (1970) 276.
9 W. BANKS, C. T. GREENWOOD, AND K. M. KHAN, *Staerke*, 22 (1970) 292.
10 B. PFANNEMULLER, H. MAYERHOFER, AND R. C. SCHULZ, *Makromol. Chem.*, 121 (1969) 147.
11 H. H. WEETALL AND N. B. HAVEWALA, *Biotech. Bioeng.*, in press.
12 O. A. MOE, in *Starch, Its Sources, Production and Uses*, by C. A. BRAUTLECHT, Reinhold Publishing Corp., 1953, New York, Chap. 21, p. 365.