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Effects of Immobilization on the Kinetics of Enzyme-Catalyzed Reactions. I. Glucose Oxidase in a Recirculation Reactor System

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Summary

Glucose oxidase from *Aspergillus niger* was immobilized on nonporous glass beads by covalent bonding and its kinetics were studied in a packed-column recycle reactor. The optimum pH of the immobilized enzyme was the same as that of soluble enzyme; however, immobilized glucose oxidase showed a sharper pH-activity profile than that of the soluble enzyme. The kinetic behavior of immobilized glucose oxidase at optimum pH and 25°C was similar to that of the soluble enzyme, but the immobilized material showed increased temperature sensitivity. Immobilized glucose oxidase showed no loss in activity on storage at 4°C for nearly ten weeks. On continuous use for 60 hr, the immobilized enzyme showed about a 40% loss in activity but no change in the kinetic constant.

INTRODUCTION

With the recent development of immobilization techniques, interesting potential applications of enzymes as catalysts have been proposed in fields as diverse as medicine, sewage treatment, and industrial processing. Since enzymes depend upon specific three-dimensional conformation of their molecules for activity, any physical influence of the matrix or chemical modification of the enzyme might alter its properties. Indeed, a number of recent publications[1–6] have reported altered properties of enzymes after immobilization. However, it has not always been taken into consideration that the apparent change in the chemical properties are not entirely due to the physical influence of the matrix or chemical modification of the enzyme. External and internal diffusion effects can considerably alter the Michaelis-Menten constant, the activity, and the thermal sensitivity. For engineering purposes, better understanding is needed of immobilized enzyme kinetics and the factors that influence

the rate of the reaction. In this study glucose oxidase was immobilized on nonporous glass beads by covalent bonding and its kinetics studied under well-defined reactor geometry and flow conditions. Glass was chosen as the support material because of its strength and incompressibility.

BACKGROUND

The kinetics of the homogeneous glucose oxidase reaction have been widely studied[7–12] with β-D glucose, at 25°C, and pH = 5.5. The mechanism is generally given as

$$E_0 + G \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E_0 - G \xrightarrow{k_2} E_r + P \tag{1}$$

$$E_r + O_2 \xrightarrow{k_4} E_0 + H_2O_2 \tag{2}$$

where E_0, E_r, and $E_0 - G$ stand for the oxidized and reduced forms of the enzyme and the enzyme complex, respectively. Based upon this mechanism the reaction rate at steady state can be expressed as

$$v = \frac{k_{cat}E_T[O_2][G]}{[O_2][G] + \frac{k_{cat}}{k_{red}}[O_2] + \frac{k_{cat}}{k_{ox}}[G]} \tag{3}$$

where E_T is the enzyme concentration, $k_{cat} = k_2$, $k_{ox} = k_4$ and $k_{red} = (k_1k_2/k_{-1} + k_2)$ or (k_1k_2/k_{-1}), if the assumption is made of rapid equilibrium.

For a given amount of enzyme and at fixed glucose concentration eq. (3) can be reduced to

$$v = \frac{V_{max}[O_2]}{[O_2] + K_M} \tag{4}$$

where

$$K_M = \frac{k_{cat}k_{red}[G]}{k_{ox}k_{red}[G] + k_{cat}k_{ox}} \tag{5}$$

and

$$V_{max} = \frac{k_{cat}k_{red}E_T[G]}{k_{red}[G] + k_{cat}} \tag{6}$$

Previous kinetic studies of glucose oxidase, immobilized by various techniques, have indicated that immobilization can affect the properties of the enzyme. For example, Weibel and Bright[13] immobilized

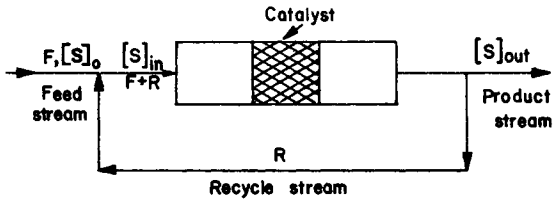

Fig. 1. Flowsheet for a recycle reactor system.

glucose oxidase on porous glass beads by covalent bonding and found that the apparent bimolecular constant was increased by a factor of 14. For gel entrapped glucose oxidase, Hinberg et al.[14] observed an increase in the value of the bimolecular constant by a factor of 2, but Miyamura and Suzuki[15] found that the values of the kinetic constants approached those of soluble enzyme as the particle size was decreased. Glucose oxidase crosslinked on a cellophane membrane[16] and covalently coupled on a nickel oxide screen[17] showed no change in the value of kinetic constants. In the same studies the optimum pH of the enzyme also did not change, however, in both cases the sensitivity of the enzyme to changes in pH was increased. Immobilized glucose oxidase showed decreased temperature sensitivity[17] and increased storage stability.[18]

The chemical engineering literature[19] describes the advantages of a packed-bed reactor linked to an external recirculation system. In such a continuous flow recirculation reactor system part of the effluent stream is returned and mixed with the feed stream, as schematically shown in Figure 1. A mass balance on the substrate at the mixing point gives:

$$[S]_{in} = \frac{F[S]_0 + R[S]_{out}}{F + R} \tag{7}$$

As the (R/F) ratio is increased sufficiently, the concentration changes within the reactor decrease to the point where the reactor is called "differential," i.e., the reaction may be considered to occur at a constant average concentration level. The overall conversion must however be significant enough to be detectable by the available measurement techniques. Under these circumstances the reaction rate can be calculated from

$$v = \frac{F([S]_0 - [S]_{out})}{W} \tag{8}$$

where W is the catalyst weight. Equation (8) can also be written in the modified form

$$v = \frac{F[O_2]_S(1 - X)}{W} \tag{9}$$

where $[O_2]_S$ is the saturated oxygen concentration. Values for different temperatures are reported in the literature.[20]

Since the glucose concentrations used in this study were very much higher than those of oxygen, they can be assumed to be constant throughout the reactor and equal to the inlet value. From eq. (7) the inlet oxygen concentration to the reactor can be written as

$$[O_2]_{in} = \frac{[O_2](F + RX)}{F + R} \tag{10}$$

and the average oxygen concentration is given by

$$[O_2] = \frac{[O_2]_{in} + X[O_2]_S}{2} \tag{11}$$

Equations (9)–(11) provide the numerical values needed in the evaluation of the rate expression eq. (3). Kinetic constants in this rate expression were calculated by using Rosenbrock's search technique.[21]

EXPERIMENTAL

Materials

Nonporous glass beads (40–60 mesh) used for covalent coupling of glucose oxidase were obtained from Ana Laboratories Incorporated, New Haven, Connecticut. The enzyme preparation (analytical grade from *Aspergillus niger*) used for immobilization was obtained from Sigma Chemical Company, St. Louis, Missouri and further purified. D-Glucose solutions of different concentrations were prepared by using "Baker analyzed" reagents purchased from J. J. Baker Chemical Company, Phillipsburg, N. J. The buffer solution used was 0.1M sodium acetate and the pH was adjusted to the desired level by adding acetic acid. Since the enzyme preparation contained trace amounts of catalase, 0.1mM KCN was added to suppress its activity. EDTA in the amount of 0.5mM was also added to the glucose solution to protect the enzyme from metal ions which may deactivate it. Sodium acetate, KCN and EDTA used were analytical grade materials (J. J. Baker). Compressed air used

for saturating the glucose solution was obtained from Linde Gas Company. All solutions were prepared from distilled–deionized water.

Methods

The nonporous glass beads were prepared by first adding 100 ml of water to 50 g of beads and then slowly adding 100 ml of 50% hydrofluoric acid, allowing the contents of the beaker to cool between additions. The mixture was allowed to react for 1 hr and then 10*N* NaOH was added, enough to cover the beads. The slurry was heated to 80°C for 1 hr, washed with distilled water, and dried overnight in an oven at 80°C. The dry beads were immersed in a 2% solution of 3-aminopropyltriethoxy silane in acetone. Excess liquid was decanted and the beads were allowed to stand in an oven at 45°C for 24 hr. The alkylamine glass was refluxed for 24 hr in 200 ml of chloroform containing 10 ml of triethylamine and 20 g of nitrobenzoyl chloride. The beads were washed with chloroform and ethyl alcohol and dried in an oven at 60°C for 12 hr. The arylamine glass was reduced by refluxing in 200 ml of 5% (w/v) sodium dithionite in water for 1 hr. The beads were washed with water and benzene and dried at 60°C.

For diazotization and coupling the glass was slurried in 50 ml of 2*N* HCl and placed in an ice bath in a dessicator connected to a vacuum source. When cool, 2.5 g of sodium nitrate was added to the slurry; the reaction was allowed to proceed under vacuum for 20 min. The beads were then quickly but thoroughly washed with ice-cold 1% (w/v) sulfamic acid, until no more bubbling was seen. A 0.1*M* Tris–Cl solution (pH = 8.7) was used for a last rinse. Excess liquid from the top of the beads was removed by decantation to prevent dilution of the enzyme solution. Glucose oxidase that had been column purified and concentrated was diluted by 1:10 and 10 ml was added to the glass beads. The reaction was allowed to proceed for 1 hr. The beads were then washed with Tris–Cl buffer thoroughly to remove the loosely bound enzyme and the supernatant containing unreacted enzyme was saved. Glucose oxidase beads were stored at pH = 6.5 in the cold.

Recycle Reactor

The essential features of the recycle system consist of the reactor, the feed preparation and product analysis parts, and the measurement and control devices. Glucose feed solution is maintained within ±0.2°C by a Blue M Electric Co. Model MR-3240A-1 con-

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