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Characteristics and Applications of Immobilized Glucoamylase

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고정화 글루코아밀라제의 성질과 응용

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요 약

Glucoamylase를 ZrO_2로 피복된 96% porous glass에 azo-linkage를 형성시켜 결합하게 한후, 2.5% glutaraldehyde로 처리하여 효소를 고정화시켰다. 효소기질로는 용해도가 높고 점도가 낮은 30% enzyme thinned cornstarch (dextrose equivalent 값 : 24)를 사용하여 plug flow-column reactor에서 연속반응시켰다. 반응 최적 pH는 수용성효소의 5.0보다 alkaline 쪽으로 기울어져 7.0으로 나타났고, 고정화반응에 따라 열안정성이 높아지고 40~60°C에서 최적 온도범위를 가리키며, Km값은 수용성 효소의 1.25mM보다 낮은 1.04 mM값을 보여 주었다. 따라서, pH 7.0, 45°C에서 160시간 동안 corn starch를 기질로 효소반응을 시켜 glucose 90.3%, maltose 8.0%인 DE값 94.0인 전분당분해산물을 획득할 수 있었다.

Introduction

The immobilized enzymes may be used in a continuous flow-through reactor, yielding effluents of high product content. Immobilized enzymes have been prepared by various methods, including polymerization onto organic polymer lattices and attachment to polymers of amino acids, coupling to cellulose and polystyrene derivatives, and immobilization in starch and acrylamide gels.

Aside from such organic polymers as mentioned, many investigators have been successful in attaching enzymes to a porous glass support. Weetall *et al.*[1~5] were the first to report the attachment of enzymes to porous glass supports, indicating that enzymes attached to inorganic carriers offer several potential advantages over enzymes attached to organic polymers.

After then, the physicochemical action of several silane coupling agents on porous silica glass has been investigated. The silanization

process wasfound to be a reaction between the glass surface hydroxyls and the amino-functional silane coupling agent. In addition, the potential application of the aminosilane derivatives of a porous glass has been evident to numerous enzymes[6~11].

Marsh *et al.*[12] also reported on glucoamylase immobilized on porous glass. They used 10% γ-aminopropyltriethoxysilane both solid and porous glass. In that paper, such reaction conditions as the time required for complete silanization was unknown. This study was undertaken to investigate the activity and the stability of insoluble enzyme derivative of glucoamylase coupled to porous glass and to determine problems encountered in its application to the hydrolysis of cornstarch.

Materials and Methods

Materials

Glucoamylase prepared from *Rhizopus* spp. was obtained from Sigma Chemical Co. (St. Louis, U.S.A.). This enzyme contained 7,250 units of glucoamylase activity against 30% enzyme-thinned cornstarch as a substrate per gm solid. One unit represents the production of 5 mmole of glucose in a minute at 50°C. The enzyme was bound through azo-linkages to an arylamine derivative of ZrO_2 porous glass. The glass derivative (pore size: 550Å, 40/80 mesh) was provided by Corning Glass Works, Corning N.Y. in U.S.A. Analytical grade reagents and double-distilled water were used throughout this work. Substrate for glucoamylase was an enzyme thinned 30% cornstarch solution (Dextrose Equivalent, DE, 24; A.E. Staley Company, Illinois, U.S.A).

Methods

Preparation of Immobilized Glucoamylase

The diazotization and coupling procedures as described by Okos[13] and Weetall[14] were followed with modifications in reactant concentrations and evacuation procedures. Twenty five grams of arylamine derivative (Pierce Chemical Co., Illinois, U.S.A.) were added to a trypsinizing flask containing 300m*l* of 0.7N HCl and 0.7g of $NaNO_2$. The flask, partially submerged in an ice bath, was connected to a rotary evaporator, rotated, and after 20 min a vacuum was drawn. After 30 min the diazotized glass was filter-washed with cold 3% sulfamic acid and cold distilled water. A sufficient volume of 2.5% glutaraldehyde in 0.1M phosphate buffer at pH 7.0 was added to the bound support so that the derivatized support material was covered. The reaction was performed at room temperture for 1hr, which included 40 min in a vacuum desiccator with occaional stirring. The carrier was then washed with 3l of distilled water. For immobilization, 100mg of purified enzyme was offered per gram of derivatized support material. The enzyme was dissolved in cold 0.1M phosphate buffer at pH 7 and added to the support material in an ice bath with occasional stirring. The solution pH was maintained at 6.8~7.0, and the reaction was continued for 2hr. The immobilized enzyme was washed with 3l of distilled water and stored at 4°C as a wet cake.

Reactor

A glucoamylase-glass derivative prepared by azo-linkage was continuously assayed over a period of 160hrs with the substrate of 30% cornstarch in the apparatus designed in Fig.1.

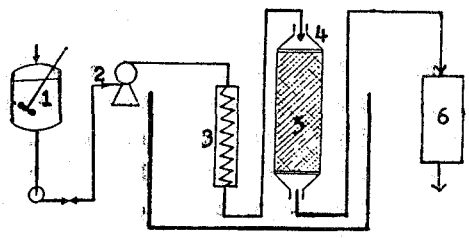

Fig. 1. Plug flow-column reactor containing immobilized glucoamylase covalently coupled to ZrO_2 coated porous glass.
1. Feed tank 2. Pump
3. Preheater 4. Column reactor
5. Bound enzyme 6. Cooler

Flow rates through the column were maintained up to 2.5ml/min by LKB Miniflow Precision Micropump. A peristaltic pump provided a steady flow of substrate to the preheater, where the solution was heated to 70°C and passed through a jacketed column containing a fixed bed of glass beads to eliminate dissolved gas. The substrate was then cooled to the desired temperature and passed through the column reactor. The reactor, in which the enzymatically active support was used, consisted of a 50cm×2.0cm i.d. jacketed glass column. The temperature was adjusted by forcing water from a controlled temperature bath through the column jacket and column temperature was monitored by a thermocouple located below the enzyme bed. The column effluent was collected in a fraction collector, precipitated with trichloroacetic acid, and examined spectrophotometrically at 280nm. On the other hand, the same procedure for soluble glucoamylase was used, but the substrate was allowed to react in the enzyme batch reactor.

Enzyme activity assays

Initial soluble and immobilized glucoamylase activity were assayed at 60°C for 1hr at pH 5.0 and 7.0, respectively. Substrate was 50ml of 30% cornstarch, prepared from 24 Dextrose Equivalent (DE) spray-dried enzyme thinned cornstarch, to which a known quantity of soluble and immobilized enzyme were added. Total glucose was determined with a Glucostat Special Kit (Worthington Biochemical Corp., Freehold, special New Jersey).

Determination of Half-life

Half-life determinations and regression analysis plots were performed on column data by assuming exponential decay of activity with the following equations[5].

$$K=\frac{2.303}{t}\log\frac{E_0}{E}$$

$$T=\frac{0.693}{K}$$

where K : enzyme decay constant

t : operation time of reactor

E_0 : initial enzyme activity

T : half-life

Apparant Km

The apparent Michaelis-Menten constants (Km) were determined by plotting S_0X vs ln X from the following equation according to the method of Havewala & Pitcher[15].

$$S_0X=Km\ ln\ (1-X)+Vm\ L/Vs,$$

where S_0 is the intial substrate concentration,

Km is the apparent Michaelis-Menten constant,

X is the mole fraction of conversion $X=(S_0-S)/S_0$,

Vm is the apparent maximum reaction rate,

Vs is the superficial velocity in colume reactor,

and L is the length of column reactor.

Results and Disscusion

Immobilization of an enzyme on the surface of a carrier changes the characteristic behavior of that enzyme. The change can be attributed to the character and the electrostatic charge on the surface of the carrier in most cases. In this work some kinetic parameters of the immobilized glucoamylase preparation were determined. Comparative studies were also carried out with the soluble enzyme.

Free and immobilized enzymes showed different pH optima.

Fig. 2 shows that this optimum shifted when glucoamylase was immobilized. This seems to be due to the change of the surface of the inorganic carrier. The soluble enzyme was the most stable at pH 5.0, while the immobilized one showed optimum pH at 7.0 and represented much broader pH stability than the soluble one. Goldstein[16]in a detailed study found that the shift in pH optimun if enzyme immobilized on water-insoluble carriers would

be the result of an unequal distribution of hydrogen ions, hydroxyl ions, hydroxyl ions and charged substrates. The local hydrogen ion concentration in the domain of the charged enzyme carrier is dependent upon the carrier charge. Thus, the pH at the surface where the enzyme is located would be higher or lower than the external bulk solution, creating an apparent shift in pH profile[17].

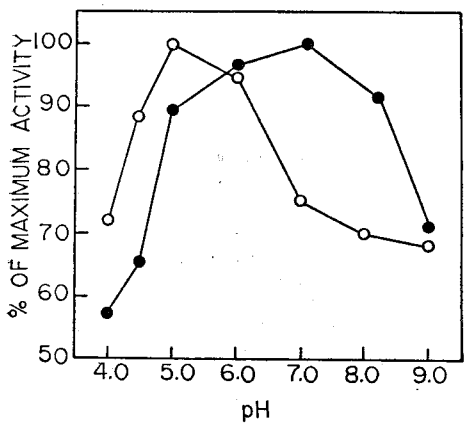

Fig. 2. Effect of increasing pH on the stabillty of enzymes.
○——○ : Soluble glucoamylase
●——● : Immobilized glucoamylase

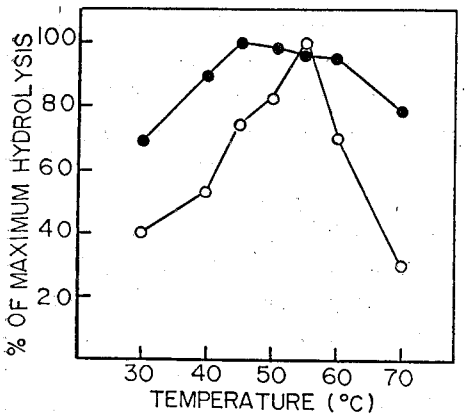

Fig. 3. Stability of free and immobilized glucoamylase treated at different temperature for 30 min.
○——○ : Soluble glucoamylase
●——● : Immobilized glucoamylase

Fig. 3 shows the remained activities of the soluble and immobilized glucoamylase treated at indicated temperatures for 30 minutes and then assayed at 25°C.

The results showed that the reaction velocity of soluble enzyme increased linearly with the temperature rising up to 55°C and declined rapidly above that, while immobilized glucoamylase showed greater stability at higher temperatures than soluble enzyme. The immoblized enzyme showed much broader thermal stability than the soluble one. Fig. 2 and Fig. 3 represent that glucoamylase has increased pH and thermal stabilities on immobilization.

Km value of immobilized glucoamylase for 30% enzyme thinned cornstarch as a substrate is compared with that of soluble enzyme in Table 1.

Table 1. Relative Km values of soluble and immobilized glucoamylase

Enzyme	Km(mM)
Soluble glucoamylase	1.25
Immobilized glucoamylase	1.04

Half-life of immobilized glucoamylase preparations stored at different temperatures are summarized in Table 2.

Table 2. Effect of temperature on half life for immobilized glucoamylase activity

Temperature(°C)	Half life(days)
40	875
45	750
50	250
55	75
60	15

Half-life can be increased substantially by operating at lower temperatures without greatly affecting reaction rates. Thus, by operating at 40°C to 45°C, the half-life is extended over a period of two to three years with only a 70 to 80% decrease in reaction

rates compared with operation at 60°C. If a column were operated at 45°C over a period of three half-lives, that column could be used 5.3 years, by assuming there was no unexplained catastrophic loss of activity resulting from bacterial contamination or incorrect handling. This system would be small, compact, inexpensive and could be completely automated from the solubilization of the starch to the storage of the final product.

Glucoamylase studies on porous glass columns with freshly prepared enzyme thinned cornstarch have given DE values in excess of 90 in the past[14]. In addition, columns must be used with substrates of relatively low viscosity and high solubility to prevent clogging or extremely high pressure drops across the length of the reactor. Therefore, in this study 30% enzyme thinned cornstarch(DE 24) was used as a substrate for glucoamylase. The operational conditions of column reactor for immobilized glucoamylase are summarized in Table 3.

Table 3. Summary of operational studies on immobilized glucoamylase covalently coupled to ZrO_2 coated porous glass

pH	7.0
Temperature, °C	45
Operation time, hr	160
Volumn of substrate, L	25
Decay constant, hr^{-1},	2.5×10^{-5}

Reactions were conducted with 30% star syrup prepared from spray-dried enzyme thinned cornstarch and added to the immobilized glucoamylase column reactor. The thinned starch was run through a continuous column resulting in DE 94.0 of sugar product from which 90.3% glucose and 8.0% maltose could be tobtained (Table 4). Glucose levels in the immobilized system of this study are similar to those of the soluble systems[14]. Weetall *et al.*[14),17)] reported that by starting with activated charcoal, and running through the immobilized enzyme column in a continuous, 93~94% glucose in the product could be obtained with little difficulty. Considering these results, a higher glucose yield seems to be possible by using freshly prepared substrate under optimized conditions.

Table 4. Saccharide compositions of enzyme-converted cornstarch

Conversion	Immobilized glucoamylase
DE	94.0
DP1, Glucose %	90.3
DP2 %	8.0
Higher saccharides %	1.7

This investigation demonstrates the potential application of immobilized glucoamylase to the hydrolysis fo starch in cereals. Many of the problems encountered in this study can be corrected by the proper choice of reaction conditions; catalyst support, reactor design, and reactor sanitation or catalyst sanitizing. Further investigation to define the partial irreversible inhibitor present in cereals would be desirable, but not essential, to commercial application.

Abstract

Glucoamylases catalyze a stepwise hydrolysis of starch with the production of glucose. In order to make an efficient conversion of starch into glucose, glucoamylases prepared from Rhizopus spp.(Sigma Co.) were attached to a porous glass and immobilized by glutaraldehyde-induced crosslinking. The porous glass used in this study was ZrO_2 coated, 40~80 mesh, 550 Å pore diameter. Using the forgoing glass, we could couple as much as 50 mg of protein per gram of carrier. Substrate for the glucoamylase was an enzyme-modified thin-boiling 30% cornstarch solution used where greater solubility and low viscosity are desired. Immobilized glucoamylase had an

optimum pH 7.0 to the alkaline side of soluble enzyme. Km values of immobilized and soluble enzyme were 1.04 mM and 1.25mM, respectively. The thermal stability of glucoamylase was increased by immobilization and the immobilized enzyme showed an optimum temperature at 40~60°C. The continuous conversion of cornstarch to glucose by use of immobilized glucoamylase resulted in the production of a more than 90 DE product.

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