Hydrolysis of Sucrose by Invertase Entrapped in Calcium Alginate Gel

Tai-Boong Uhm, Jai-Sik Hong and Si-Myung Byun[1]

Department of Food Science and Technology
Jeonbug National University
Jeonju, Korea

[1]Department of Biological Science and Engineering
Korea Advanced Institute of Science and Technology
Seoul, Korea

칼슘 알지네이트 젤에 고정화시킨 Invertase에 의한 설탕의 가수분해

엄 태 붕 · 홍 재 식 · 변 시명[1]
전북대학교 농과대학 식품가공학과
[1]한국과학기술원 생물공학과

초 록

설탕 가수분해효소인 invertase를 칼슘 알지네이트젤에 고정화시켰다. 고정화된 입자는 균일한 크기와 모양을 가졌으며 컬럼 충진에 사용 할수 있는 좋은 기계적 강도를 보였다. 최적 효소 활성을 얻기 위하여 알지네이트 젤의 농도, 염화칼슘의 농도, 그리고 염화칼슘 입자의 반응 시간에 따른 영향 등을 조사하였다. 그 결과 2% 알지네이트 젤을 0.2 'M 염화 칼슘에 2시간 반응시켰을 때 가장 좋은 활성을 보였으며 이때 68%의 효소 수율을 나타냈다. 최적화된 조건에 의해 제조한 고정화 효소는 143 mM의 km, 최적 pH는 4.5 를 보였으며 55°C에서 높은 효소 활성 및 열 안정성을 보였다. 젤의 효소 허용 부하량은 20 ml 당 150 mg이었고 6일동안 컬럼을 통한 설탕의 연속 가수분해에서는 효소 활성에 어떤 감소도 보이지 않았다.

Introduction

Various immobilization techniques have been developed to reuse expensive enzymes for various industrial purposes. Among them, physical entrapment of an enzyme within the interstitial spaces of gel matrix is often used by many workers because of the experimental simplicity and the relatively mild conditions of preparation.

Alginate, polymannuronic acid which is a linear polymer of β-(1-4)-D-mannosyluronic acid and α-(1-4)-L-gulosyluronic acid residues, is one of the most popular sources as a support material because beads of controllable and uniform size can be formed by a mild and simple

immobilization procedure. Also, it has often relatively high yields of enzyme activity and good operational stability compared to the other techniques.

Considering the diffusion of substrate into the gel and the stability of carrier material, we selected invertase as a model enzyme and entrapped it in calcium alginate beads. Invertase, an enzyme hydrolyzing β-1, 2-fructosidic linkage, converts sucrose into a 1 : 1 mixture of glucose and fructose, commonly known as invert sugar. Invert sugar is used in various confectionary products as a humectant to hold moisture and in the brewing industry. Although the production of invert sugar with the soluble enzyme is relatively inexpensive, attempts have been made to immobilize the enzyme for continuous production of invert syrups[1~7]

Therefore, we focused on those two points of view, which are to screen the optimum condition possessing high enzyme activity and to examine the reaction properties and kinetic behaviors of immobilized enzyme.

Materials and Methods

Materials

Invertase was obtained from Biocon Ltd. (Nagoya, Japan). The specific activity of invertase preparation was 26 units per mg (micromole/min/mg) at 50°C, pH 5.0, and an initial saccharose concentration of 3.4% (w/v). All other materials were of analytical grades.

Method of immobilization

Sodium alginate (2g, Kanto Chemical Co., Inc. Tokyo, Japan) was dissolved in 100ml demineralized water and deaerated under vacuum. Twenty ml of this solution was gently but throughly mixed with 20mg of invertase (particle size of 150~180μm) and deaerated again. The mixture was pumped dropwise through a needle into a 500ml 0.2M CaCl$_2$ solution. The size of the drops was controlled by needles

having different diameter sizes. Then the beads were gently stirred in the CaCl$_2$ solution at room temperature for 2hr. The supernatant was then decanted and the beads were washed with 100ml 0.1M sodium acetate buffer, pH 5. The resulting beads were immediately used for the pertinent experiments.

Physical characterization

From the 500 prepared beads the shape and mean wet particle size were determined by a microscope and a caliper rule. The swelling of beads was measured by suspending prepared beads (freed from adherent water on a sieve) in 3.4% sadcharose solution contained in a 500 ml graduated cylinder at 30°C and checking the increase in volume after equilibrium state.

Activity assays

The rato of hydrolysis of saccharose was measured by its ability to reduce 3,5-dinitrosalicylic acid. For that, the prepared beads were weighed and suspended in a 3.42% saccharose solution in 0.1M sodium acetate buffer, pH 5.0, at 30°C. The vessel diameter of the batch reactor was 7cm and the turbine type stirrer (size: 3cm×1cm) was placed at one third of the liquid level. The agitation speed was maintained at the rate of 98 to 100rpm. One unit of invertase activity is defined as the production of one micromole of free sugar per min at 30°C. Protein was determined according to the method of Lowry et al.[8] with bovine serum albumin as a standard.

Characterization of immobilized invertase

Since an immobilzed invertase activity was directly proportional to the incubation time during the swelling of beads, the activity was measured at regular time intervals. The effects of substrate concentration on the activity of immobilized invertase were determined under the standard assay conditions except for varing the sucrose solution from 0.34 to 17%. In the measurement of the activity as a function of

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pH, an 0.1M acetate solution (pH 2.9) and a 0.1M sodium acetate solution (pH 7.7) were used in addition to the sodium acetate buffer. Temperature effects on enzyme activity were determined at the various temperatures from 30 to 60°C. For the temperature stabilities, the immobilized invertase was suspended in 500ml of the substrate solution with stirring at different temperatures. During the reaction, 1ml aliquots were taken at time intervals of 10 min. The effects of enzyme load in the carrier were studied under the standard assay conditions except for varing the concentration of invertase from 10mg to 150mg. All other assay conditions further specified in the text and figures.

Operational stability

The operational stability of the immobilized beads (about 5000 beads; bead size: 1mm) was determined in a packed bed column reactor (3cm diameter) for 6 days. The fed substrate was 0.1M sucrose solution. The temperature was constant at 30°C and the pH was at 6.1. The feed flow rate was 2.9ml/min initially and was checked at regular time intervals. Sucrose solution was pumped via a preheating coil through the column with upward flow mode. To prevent microbial contamination dur-
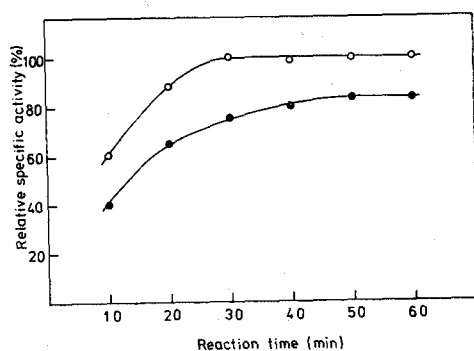

Fig. 1. Swelling effects of invertase entrapped in calcium alginate gel
○—○; swelled beads which were pre-incubated in 0.1M sodium acetate buffer, pH 5 at 30°C for 1hr before enzyme reaction, ●—●; beads which were not preincubated

ing operational precess whole column systems (including demineralized water for substrate solution) were autoclaved before operation.

Table 1. Shape and mean wet particle size distribution of immobilized invertase.

Diameter(mm) (mean±standard deviation)	Shape
2.2±0.05	Sphere
1.6±0.05	Sphere
1.0±0.05	Sphere

Results and Discussion

Physical characterization

For evaluation of an immobilized enzyme for preparative and/or industrial application, physical properties are an essential part of the description of the system because other important quantities-such as reaction rates, the compression behavior of packed beads, and the conditions prevailing within the catalyst particles in the course of their use depend on them. In this study, the shape and mean wet particle size of immobilized beads are given in Table 1. Although beads showed egg shapes due to the highly viscous gel at high enzyme load (over 100mg enzyme per 20ml gel), beads extruded by standard immobilization procedure had excellent uniform size and configuration. In the presence of substrate solution (3.42% sucrose in 0.1M sodium acetate buffer, pH 5, at 30°C) calcium alginate beads (1mm diameter) swelled 2.5 times in volume as big as initially prepared beads after 30 minute in a batch system. The degree of swelling was affected by time, temperature, substrate concentration and bead size. During the batch reactor operation, swelling effect of the prepared beads was investigated (Fig. 1). The activity increased with incubation time until the maximum swelling of the beads was reached. Hydration of gel network by water molecules can lead to
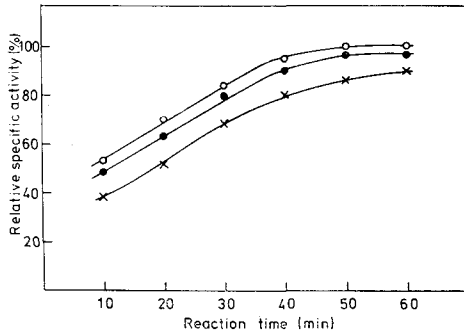
Fig. 2. Effects of sodium alginate concentrations on the immobilized enzyme activity
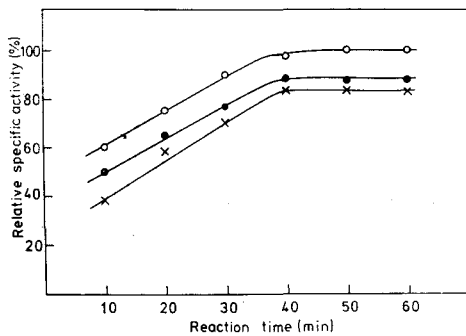
○—○; 2%, ●—●; 2.5% ×—×; 3%

Fig. 3. Effects of CaCl₂ concentrations on the immobilized enzyme activity
○—○; 0.2M, ●—●; 0.1M, ×—×; 0.05M

the enlargement of geometric pores inside beads. As a result, intradiffusional rate will be increased.

Optimal conditions for immobilization

To screen the optimal conditions possessing high enzyme activity, sodium alginate concentration, CaCl₂ concentration, and the incubation time of beads in CaCl₂ solution during the immobilization procedure should be considered. In this case, the major factors affecting enzyme activity can be diffusional limitation in gel and enzyme leakage throughout the gel network. Solutions of 2, 2.5, and 3%(w/v) sodium alginate were selected for experiments, while the lower concentrations were rejected for their weak gel strength. From Figure 2, the highest

activity was obtained at 2% sodium alginate. By examining data, the reaction rate is found to decrease with increasing sodium alginate concentration. During the 1 hr batch reaction any leakage of enzyme molecules from the beads was not detected. Also, the film diffusion region around the gel surface was minimized by controlling the agitation speed. Therefore, it assumed that high alginate concentration caused the intradiffusional limitation by the compactness of gel structure. Calcium chloride concentration was one of the major factors to optimize the immobilization because of the insolubilization of alginate by the substitution between two ions. From the data (Figure 3),
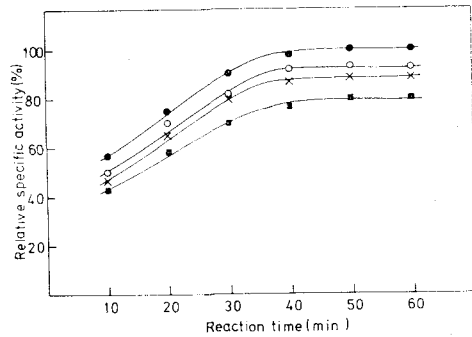

Fig. 4. Effects of incubation times of immobilized invertase in 0.02M CaCl solution
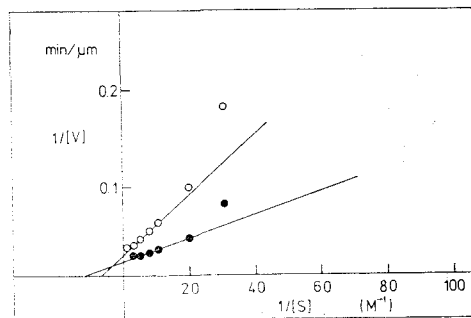●—●; 2hr, ○—○; 1hr, ×—×; 0.5hr, ■—■; 4hr

Fig. 5. Lineweaver-Burk plots for the hydrolysis of sucrose by the soluble and immobilized enzymes
○—○; immobilized enzyme, ●—●; soluble enzyme

0. ?M CaCl₂ was selected for enzyme immobilization. The interaction between Ca^{2+} and alginate is considered to be almost entirely electrostatic[9,10]. In low CaCl₂ concentration, the exchange between sodium and calcium ions in alginate would be lowered. As a result, the beads would form loose inter and intra molecular ionic bonds which result in leakage of enzyme molecules from the beads during the washing procedure. When beads were incubated in CaCl solution, the activity of immobilized beads varied with incubation time of beads in CaCl₂ solution (Figure 4). Initially, the activity increased up to 2 hr of incubation and further incubation lowered the enzyme activity. It seemed that further compactness of gel structure by long contact of beads with the gellant makes diffusional problem.

From the optimal conditions screened for immobilization, immobilized beads were prepared. The activity was 17.7 units per ml of gel, which is equivalent to 68% of the activity of soluble enzyme.

Immobilized enzyme kinetics

The initial rates vs. substrate concentration for both the free and the immobilized enzymes were determined under the standard assay conditions except for varing the sucrose solution from 0.34 to 17% and is shown in Figure 5. Km value of the immobilized invertase was 143
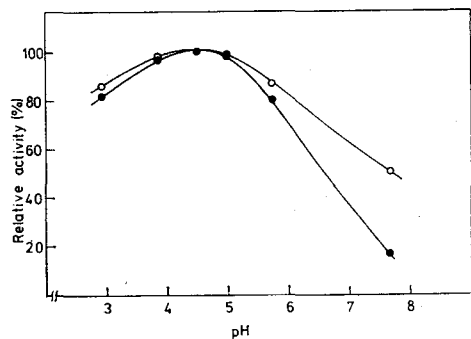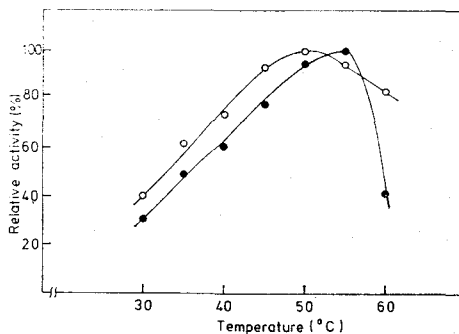

Fig. 7. Effect of temperature on the immobilized enzyme activity
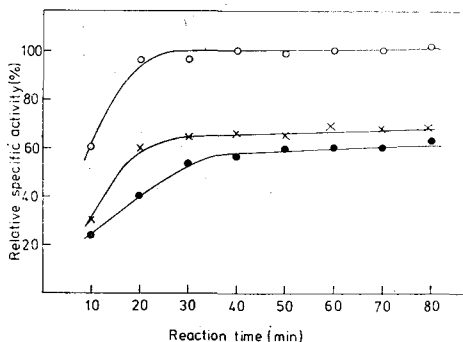○—○; soluble enzyme, ●—●; immobilized enzyme

Fig. 8. Thermal stablity at various temperatures for 80 min
○—○; 55°C, ×—×; 35°C, ●—●; 30°C

mM; the corresponding Km value of the soluble enzyme was 9I.0mM. Data obtained from Lineweaver-Burk plot indicate that there exists an intradiffusional problem in the immobilized enzyme. Generally, the apparent Km during the immobilization is altered by several environmental factors. These changes are caused by steric, microenvironmental, and diffusional effects. In this case, steric and microenvironmental effects were not significant to alter Km value. Also, external diffusion resistance was minimized by controlling agitation speed. Therefore, the alteration of Km value is mainly due to the intradiffusional resistance.

The activity of free and immobilized enzyme as function of pH is shown in Figure 6. The

Fig. 6. Effect of pH on the rate of hydrolysis of sucrose by immobilized invertase
●—●; soluble enzyme, ○—○; immobilized enzyme

optimum pH was 4.5 in both case. The profile for the immobilized enzyme was somewhat flattered than that for the free enzyme. This phenomenon suggested that at optimum pH region of the immobilized enzyme, diffusional resistance and product through the gel caused the activity ratio small.

The temperature-activity profile (Figure 7) showed that the optimum temperature for sucrose hydrosis by the immobilized enzyme is 55 °C, which shifts to the higher temperature than that of the free enzyme. However, the activity decreased dramatically at 60°C. Heat stability of the immobilized invertase was measured under standard assay conditions except for the various temperatures. As shown in Figure 8, the immobilized enzyme was stable at 55°C for

the incubation of 80min. But, the immobilized enzyme was very unstable at over 60°C for the same incubation time. Generally, organic carriers have higher thermal coefficients of expansion than the inorganics. The thermal stability of an enzyme may be influenced by a thermal expansion of a carrier. Therefore, the conformational change of an enzyme by the high thermal expansion of the carrier may be responsible for the thermal instability of the immobilized enzyme.

Enzyme loading capacity of immobilized beads was studied under standard assay conditions except for various concentration of invertase from 10 to 150 mg. Higher concentration than 50mg caused the problems in extruding the gel through a syringe due to the high viscosity. Higher invertase concentration increased the activity of immobilized enzyme. Althoug the total activity increased, there was a decrease in the percentage of activity retained. However, percentages of total activity retained showed almost same values at the concentrations of invertase from 50 to 150mg (Fig. 9).
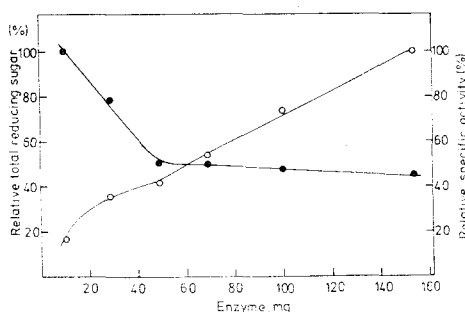

Fig. 9. Effect of enzyme concentration on the activity of immobilized enzyme ○—○; the relative total reducing sugar, ●—●; the specific activity of immobilized beads
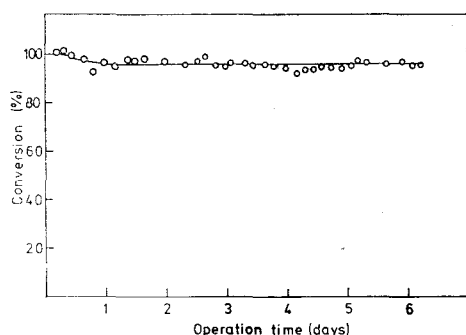
Operational stability

The operational stability of immobilized invertase was determined by continuously passing 3.42% sucrose solution at a flow rate 2.9ml/min through a column (diameter: 3cm) packed with about 5,000 beads (bead size:1mm) at 30°C for 6 days. No significant decrease in sugar conversion was observed during the whole experiment (Fig. 19).

From these results, invertase entrapped in calcium alginate has a high potential for the industrial applitions. Although main stream of the production of invert sugar is the use of glucose isomerase, invertase is a good source for the production of fructose so far. Further research including whole cell immobilization will be performed for the production of sweetners.

Fig. 10. Operational stability of immobilized enzyme in a packed bed column reactor

Abstract

Inverase was entrapped in calcium alginate gel. The immobilized beads had excellent uniform size and configuration. To screen the optimal conditions possessing high enzyme activity, effects of sodium alginate concentration, $CaCl_2$ concentration, and the incubation time of beads in $CaCl_2$ solution during the immobilization procedure were investigated. Immobilized beads prepared from the optimal conditions had 18.8 units per ml of gel, which is equivalent to 68% of the activity of soluble enzyme. Several kinetic parameters were determined: Km value, 143 mM; optimum pH, 4.5; optimum temperature, 55°C. Enzyme loading capacity was 150 mg per 20 ml gel. No significant decrease in sugar conversion was observed during the column operation for 6 days.

Acknowledgement

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