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Comparative Studies on Immobilized Invertase on Sepharose and Phenoxyacetyl Cellulose

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Sepharose와 Phenoxyacetyl Cellulose에 고정화 시킨 Invertase에 관한 비교 연구

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Abstract

Yeast invertase was immobilized on the 2 kinds of matrices : one is an indirectly coupled enzyme to the cyanogen bromide activated Sepharose by using ω-aminohexyl group as an extension arm, and the other is a tightly adsorbed enzyme on the modified hydrophobic cellulose derivative which has a phenoxyacetyl group as a linkage. The enzyme preparation coupled on Sepharose retained 26.0% of the original activity against sucrose as a substrate, while the preparation immobilized on phenoxyacetyl cellulose retained 72.9%. The immobilized invertase preparation on ω-aminohexyl Sepharose showed the optimal pH 4.5, optimal temperature 60°C, activation energy 5,941 *cal/mole · deg* and Km' 22.2 *mM* against sucrose, while the preparation adsorbed on phenoxyacetyl cellulose showed the optimal pH 4.0, optimal temperature 60°C, activation energy 7,769 *cal/mole · deg* and Km' 69.9 *mM*.

Introduction

Enzyme immobilizations no longer need to be a tedious task. Proteins can be immobilized by covalent attachment, by entrapment in hydrophilic gels, by adsorption on polymer matrices, by crosslinking, or by whole cell immobilization[1~3]. Immobilization by any of the first 3 methods usually calls for some rather difficult chemistry or the resulting enzyme activity is too low. Other adsorption techniques show that the adsorption generally has not been strong enough to immobilize a protein completely, some or all of it would be washed away in the course of whatever operation was being carried out, although the technique is much simple and less expensive.

Techniques for hydrophobic chromatography have recently been developed in order to exploit the affinity of proteins for hydrophobic materials

as means of purification[4,5]. Glycogen phosphorylase[5] was reversibly retained on hydrocarbon-coated Sepharose that varied in the length of their alkyl side chains, (Sepharose-NH-$(CH_2)_n$-H), passing from no retention (n=1), through retardation (n=3), up to very tight binding (n=6). Certain highly hydrophobic adsorbents that contain no ionizable group bind enzymes so strongly that they are effectively immobilized[6].

The strongly hydrophobic phenoxyacetyl groups on cellulose[6] interact with hydrophobic regions on the surface of the protein molecule : the attractive force arises from common repulsion of the aqueous medium by the hydrophobic pockets. The attachment is essentially irreversible in all normal operations.

In this study, two different immobilization techniques were applied and compared to the yeast invertase[7,8], whose characteristics are well known : preparation method, specificity, mechanism, pH and temperature dependence, and other kinetic constants. Moreover, the resulting invertase was compared with the enzyme immobilized by another methods[9~13].

Materials and Methods

Materials

Commercial yeast invertase (grade Ⅵ) was obtained from Sigma Chemical Co. (St. Louis, MO). This preparation contained 30~50 units of invertase activity against sucrose as a substrate per *mg* of dry powder. Sepharose 4B was a product of Pharmacia Fine Chemicals (Uppsala, Sweden). Cellulose powder, cyanogen bromide, phenoxyacetyl chloride, 3,5-dinitrosalicylate, pyridine and other chemicals were extra pure reagent grades of Aldrich products (Milwaukee, WI).

Enzyme assay

Invertase activity was determined by measuring the release of reducing sugar from sucrose[14]. To 5 *ml* of mixture of 0.05 *M* sodium acetate-0.3 *M* sucrose buffer, pH 4.7, 1.0 *ml* of the enzyme solution was added and mixed rapidly at 30°C. After exactly 10 min, 5 *ml* of 0.1 *N* NaOH was added to stop the reaction and mixed.

To this mixture, 5 *ml* of 3,5-dinitrosalicylate reagent was added, mixed and heated for 5 min in a boiling water bath. After cooling 5 min in cold running tap water, the absorbancy at 560 *nm* was measured. The amount of glucose produced by invertase was determined by using glucose as standard and converted to the amount of sucrose hydrolyzed. One unit of invertase activity was defined as 1.0 *μmole* of sucrose hydrolyzed per minute at pH 4.7 at 30°C.

For the immobilized enzymes, the above mentioned assay system was used except using the immobilized enzyme instead of free enzyme solution and the mixture was stirred well during the reaction with a submerged magnetic stirrer. Before adding dinitrosalicylate reagent, the mixture was filtered on a filter paper for the purpose of clarification.

Enzyme Immobilization

1. Pepraration of invertase immobilized on Sepharose 4B : Twenty *g* of the suctioned Sepharose 4B was activated with cyanogen bromide according to the procedure of March *et al* [15]. The activated Sepharose gel was coupled with 1,6-diaminohexane to form *ω*-aminohexyl Sepharose by the method of Byun *et al* [16]. The resulting *ω*-aminohexyl Sepharose was mixed with 0.2 *g* of invertase in 60 *ml* of 0.05 *M* sodium acetate buffer, pH 4.7 containing 120 *mg* of 1-cyclohexyl-3 (2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate. The mixture was swirled slowly at 4°C with a Rotor Toque rotaton. After overnight, the gel was filtered and washed with cold water, 0.5 *M* NaCl, and 2 *M* urea thoroughly. The activity yield of enzyme was determined by measuring activity of the final product to compare the original activity applied.

2. Preparation of invertase adsorbed on modified hydrophobic derivatives of cellulose (phenoxyacetyl cellulose) : Invertase was adsorbed on the hydrophobic derivatives of cellulose mainly according to the method of Butler[6,17]. Twenty-five *ml* of phenoxyacetyl chloride was added to 30 *g* of cellulose powder suspended in 300 *ml* of pyridine: dimethylformamide solution (1 : 1, v/v) with vigorous stirring. After 1.5 hr (occasional

stirring), the dark yellow suspension was heated at 70°C for 10 min. After standing overnight, the product was filtered by suction on a Büchner funnel and washed with 95% ethanol 6 times to remove pyridine completely. After drying at room temperature, 4 *g* of the resulting white phenoxyacetyl cellulose was suspended in 100 *ml* of 0.05 *M* Tris buffer, pH 7.4 containing 2 *M* $(NH_4)_2SO_4$ and 0.2 *g* of the invertase. The mixture was swirled overnight at 4°C.

Kinetic studies

To characterize the immobilized enzymes, invertase preparations immobilized on ω-aminohexyl Sepharose and phenoxyacetyl cellulose were examined with respect to pH dependence, temperature dependence, substrate concentration effect and stability according to the same procedures for immobilized pronase[16].

Since the invertase activities were directly proportional to the incubation time to 30 min for sucrose, relative activity at 10 min were used. The pH effect was measured under the same reaction condition as the assay condition except changing the pH of the buffer solution : 0.05 *M* citrate-phosphate buffers between pH 3.0 and 7.0, 0.05 *M* Tris-HCl buffers between pH 8.0 and 9.0 and 0.05 *M* carbonate-bicarbonate buffers between pH 10.0 and 11.0. Temperature effect was investigated at 8 different temperatures in the range from 30 to 75°C in a temperature controlled water bath (±0.1°C). Kinetic constants, Km and Vm were obtained from Lineweaver-Burk plots for the sucrose concentrations ranging from 7.8×10^{-3} to 2.5×10^{-1} *M*. Stabilities of the 2 immobilized enzymes were determined by incubation of the enzyme preparation at 0.05 *M* sodium acetate buffer, pH 4.7 and the remaining activity was measured at the assay condition.

Results and Discussion

Immobilized invertase preparation

Immobilized invertase on the two carrier matrices, ω-aminohexyl Sepharose and phenoxyacetyl cellulose, showed good results as shown in Table 1.

Invertase immobilized on ω-aminohexyl Sepharose retained 26.0% of the enzyme activity applied, while invertase adsorbed on phenoxyacetyl cellulose retained 72.9%. The specific activities of the immobilized invertase were determined and calculated on the basis of wet gel weight which was dehydrated by suction on Büchner funnel.

Table 1. The comparative results and activities of the immobilized invertases on ω-aminohexyl Sepharose and phenoxyacetyl cellulose

Immobilized enzyme	Yield (%)	Specific activity (Unit/*g* of gel)
Invertase immobilized on ω-aminohexyl Sepharose	26.0	9
Invertase adsorbed on phenoxyacetyl cellulose	72.9	15.3

Compared to those results of the previous works[16,17], enzyme preparation of ω-aminohexyl Sepharose revealed lower yield (26%). Invertase adsorbed on phenoxyacetyl cellulose, however, showed good yield of the enzyme activity (72.9%). In the process of hydrophobic adsorption on phenoxyacetyl cellulose, environmental factors appeared to be important.

Adsorption of invertase on the hydrophobic matrix was increased 2 folds in the presence of 2 *M* $(NH_4)_2SO_4$ (72.9%) in comparison to that of invertase adsorbed in the absence of $(NH_4)_2SO_4$ (34.2%). No considerably significant difference was observed between the concentrations of $(NH_4)_2SO_4$. But 2.0 *M* concentration was the most appropriate for the purpose. Similar effects of sulfate and phosphate ions have previously been observed[20].

Invertase was immobilized on Sepharose by covalent attachment and 26% of yield by this method appeared relatively good to compare with other enzymes which were immobilized covalently[16]. In contrast to covalent attachment, invertase was immonilized on phenoxyacetyl cellulose by adsorption. The strongly hydrophobic groups on the support interact with hydrophobic regions on the surface of protein molecule : the attractive force arises from common repulsion at the aqueous medium by the hydrophobic regions. The attach-

ment is essentially irreversible in all normal operations[5]. In this connection, stabilization with $(NH_4)_2SO_4$ would be due to salting-out effect and would cause more tight binding of protein to the hydrophobic support. The 72.9% yield of enzyme activity by this hydrophobic adsorption is considered as the most attractive compared to other adsorption methods, as on IRA 93 resin (50% yield) [18], ionic binding on DEAA cellulose (31~46 % yield) [10] and metal link on a hornblende (20%) [19]. Butler[17] also obtained good immobilized enzymes other than invertase using this hydrophobic adsorption method. Since preparation of the adsorbent is very versatile, inexpensive and cheap, it would give a good potential for utilization of this technology.

Stability and kinetic properties of the immobilized invertases

Stabilities of 2 immobilized invertase preparations during the storage in 0.1 *M* sodium acetate buffer, pH 4.7 at 4°C were determined by assaying the activity remained. Fig. 1 shows the results of this stability study. The soluble enzyme was also compared. Sepharose-bound invertase maintained 80% of its activity after 4 days and 76.7% after 10 days. This preparation was less stable than pronase which was immobilized by the same procedure[16]. Although the reason was not clearly understood, it may be due to the different kinds of enzyme studied.

Phenoxyacetyl cellulose-adsorbed invertase also showed almost same stability as Sepharose-bound enzyme. In the case of phenoxyacetyl cellulose-adsorbed enzyme, some adsorbed enzyme was washed out and this may cause the decrease of stability. Fig. 2 shows the washing effect of the phenoxyacetyl cellulose invertase. By every washing, about 5% of activity was washed away and plateaued after 5 washings. This tight hydrophobic adsorbed enzyme, however, was desorbed by washing with 0.1% detergent, Triton X-100 (dotted line in Fig. 2). This detergent in buffer very effectively desorbed the enzyme from phenoxyacetyl cellulose.

Fig. 3 shows the effects of pH on the activities of 2 immobilized invertases and free invertase. The effects were similar on both enzyme preparations and optimal pH was 4.5~5.0. The effects of temperature on the immobilized invertases were

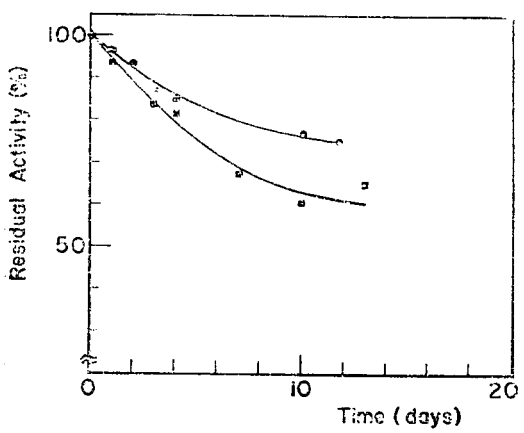

Fig. 1. Stability of immobilized invertase on ω-aminohexyl Sepharose or phenoxyacetyl cellulose during storage in 0.1 *M* sodium acetate buffer, pH 4.7 at 4°C

●—●—● : Invertase immobilized on ω-aminohexyl Sepharose

■—■—■ : Invertase adsorbed on phenoxyacetyl cellulose

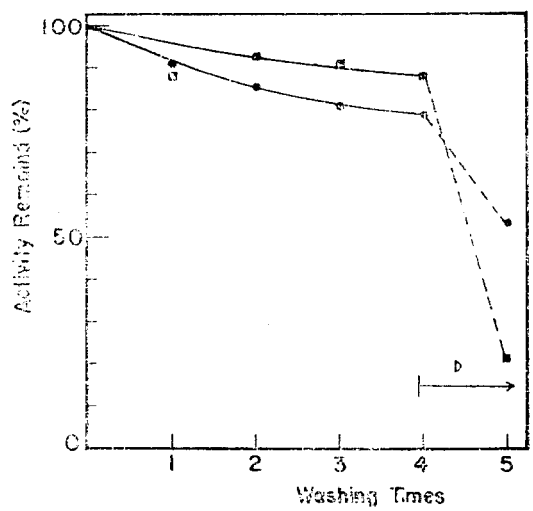

Fig. 2. Effects of washing on the enzyme activity of phenoxyacetyl cellulose-adsorbed invertase

●—●—● : Washing with water

■—■—■ : Washing with sodium acetate buffer, pH 4.7

• • • • • : Washing with detergent, Triton X-100 (D region)

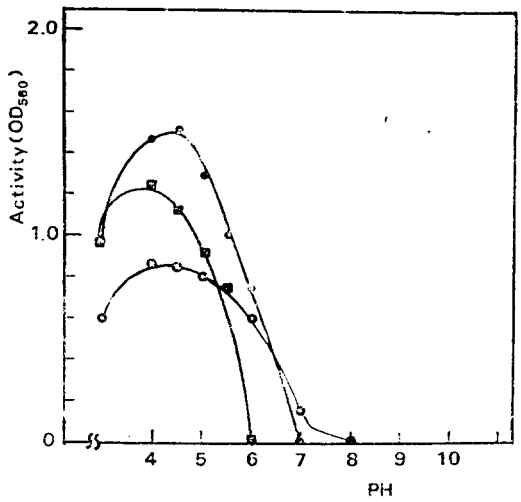

Fig. 3. **Effects of pH on the activities of soluble and immobilized invertases**
○—○—○ : Soluble invertase
●—●—● : Invertase immobilized on ω-aminohexyl Sepharose
■—■—■ : Invertase adsorbed on phenoxyacetyl cellulose
The enzyme activity was presented with absorbancy at 560 *nm* after the incubation mixture was treated with 3,5-dinitrosalicylate reagent

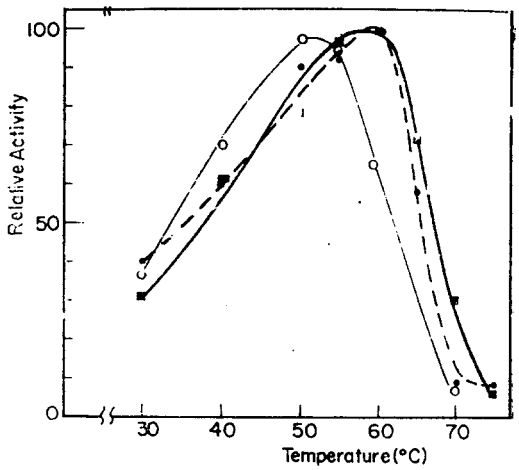

Fig. 4. **Effects of temperature on the activities of soluble and immobilized invertases**
○—○—○ : Soluble invertase
●—●—● : Invertase immobilized on ω-aminohexyl Sepharose
■—■—■ : Invertase adsorbed on phenoxyacetyl cellulose

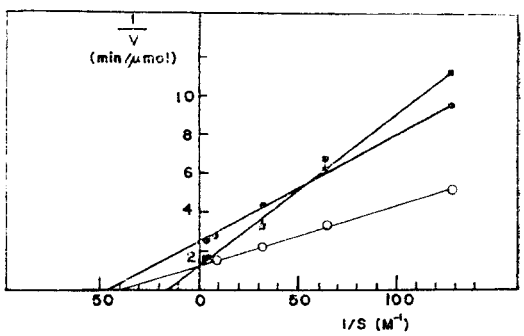

Fig. 5. **Lineweaver-Burke plots for hydroysis of sucrose by soluble and immobilized invertases**
○—○—○ : Soluble invertase
●—●—● : Invertase immobilized on ω-aminohexyl Sepharose
■—■—■ : Invertase adsorbed on phenoxyacetyl cellulose

also investigated. The results showed that reaction velocity increased with the temperature rising by the Arrhenius relationship up to 60°C and declined the velocity above 65°C for both two immobilized enzymes, while soluble invertase showed the optimal temperature at 50°C (Fig. 4). Activation energies obtained from the Arrhenius plot were calculated to be 5,941 *cal/mole·deg* and 7,769 *cal/mole·deg* for Sepharose-bound and phenoxyacetyl cellulose-adsorbed enzymes, respectively, while it was 5,995 *cal/mole·deg* for the soluble enzyme.

Fig. 5 shows Km values of various invertase preparations for sucrose as a substrate when Lineweaver-Burk plots were analyzed at the substrate concentrations from 7.8 to 275 *mM*. From this analysis Km's for sucrose appeared 22.2, 69.9 and 28.6 *mM* for Sepharose-bound, phenoxyacetyl cellulose-adsorbed, and free enzymes, respectively. Table 2 summarized these results. Generally Km value for an immobilized enzyme is higher than soluble form of enzyme, because affinity of the enzyme decreased due to volume increase by carrier matrix or other enviromental factors. It was of interest, therefore, that Sepharose bound invertase showed lower Km than that of soluble enzyme. Further study for the structure of the Sepharose-bound invertase would contribute information to understand this result.

Table 2. Summary of the characteristics of immobilized invertases on ω-aminohexyl Sepharose and phenoxyacetyl cellulose

Enzyme	Optimal pH	Optimal temp. (°C)	E_a (*cal/mole. deg*)	Km (*mM*)	Vm (*μ mole/min*)
Invertase immobilized on ω-aminohexyl Sepharose	4.5	60	5,941	22.2	0.4
Invertase adsorbed on phenoxyacetyl cellulose	4.0	60	7,769	69.9	0.95
Soluble invertase	4.0~5.0	50	5,995	28.6	1.25

요 약

Invertase의 고정화에 대해서 두가지 carrier matrix를 사용하여 연구하였다. Sepharose에 ω-aminohexyl arm을 붙인 후 효소를 결합시키는 indirect coupling method와 cellulose에 phenexyacetyl group의 linkage를 만들어 변형시킨 후 (modified cellulose), 여기에 효소를 흡착시키는 hydrophobic adsorption method로서 제조하였다. 각각의 고정화 수율(immobilized yield)은 ω-aminohexyl sepharose의 경우 첨가한 효소의 26.0%의 activity를 고정화 시킬수 있었으며, phenoxyacetyl cellulose의 경우는 72.9%였다. 제조한 고정화 효소의 안정성, pH 영향, 온도 영향 및 Km 값을 조사하였다. ω-Aminohexyl Sepharose에 고정화시킨 invertase는 최적 pH 4.5, 최적 온도 60°C, 활성화 에너지 5,941 *cal/mole·deg*, Km 값 22.2 *mM*이었으며 phenoxyacetyl celluloes에 고정화시킨 invertase는 최적 pH 4.0, 최적 온도 60°C, 활성화 에너지 7,769 *cal/mole·deg*, Km 값 69.9 *mM*이었다.

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