

Production of Fructose from Jerusalem Artichoke Tubers by Enzymatic Hydrolysis

I. Preparation and Properties of Immobilized Inulase

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호소 가수 분해에 의한 돼지 감자로 부터 과당 생산

제 1 보 : 고정화 이눌라아제의 제조와 성질

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Abstract

Partially purified β -fructosidase (inulase) from *Kluyveromyces fragilis* was immobilized on Tygon tube and aminoethyl-cellulose, respectively and both preparations were characterized. Silanization of Tygon tube in chloroform at 65°C and treatment with 10 % glutaraldehyde were critical for the immobilization of inulase on Tygon tube, while 2 % glutaraldehyde was effective for the immobilization on aminoethyl-cellulose.

The derivative of Tygon tube showed 11.5 units of inulase activity per g of dried matrix with retention of 22.5 % of original activity against inulin, whereas one of aminoethyl-cellulose showed 39.3 units per g of dried matrix with 53.4 % of retention. Studies of enzyme stability, pH and temperature dependences, and K_m values are presented for inulase and invertase activities of both immobilized enzymes.

Introduction

The application of enzymes as catalysts in large scale processes has been limited mainly because of the high cost of enzymes as chemical reagents. However, the development of enzyme immobilization technology has made it possible to recover enzymes from large operations for repeated reuse and the

number of applications of enzymes in industrial operations is definitely on the rise. The significance of enzyme immobilization has been reviewed in the areas of therapeutics, syntheses, analyses and food processings⁽¹⁻³⁾. The fact that many soluble enzymes are not on the GRAS list⁽⁴⁾ has in particular hampered the use of soluble enzyme in food processing. In this area, the immobilization techniques have again provided an easy solution to the

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problem of removing an enzyme from the consumable product. Thus, immobilized glucose isomerase⁽⁶⁻⁸⁾ for isomerization of *D*-glucose to fructose and immobilized invertase⁽⁹⁻¹¹⁾ for hydrolysis of sucrose have been extensively investigated, although immobilization of inulase has been scarcely studied so far. Fructose is an important sweetener as food stuff and for pharmaceutical purposes.

Tubers of Jerusalem artichoke (*Helianthus tuberosus* L.) contain a large quantity of inulin, whose structure is *G-F-Fn*, where *G* and *F* represent glucose and fructose respectively, and *G-F* a sucrosyl group. The subscript *n* indicates the number of extra-sucrosyl fructose residues in the polymer and varies from 1 (inulobiose) to about 35 (inulin)⁽¹²⁾. The total sugar content of the tuber, as inulin, varies from 10~20 % of fresh weight and fructose varies from 75~98 % of the total sugar⁽¹³⁾. Hence, the artichoke is an excellent potential source for production of fructose.

The extra-cellular inulase preparation of *Kluyveromyces fragilis* grown in a medium containing inulin shows both inulase and invertase activities^(14,15), whereas invertase of *Saccharomyces cerevisiae* cannot hydrolyze inulin. Using the inulase of *K. fragilis*, Byun *et al.*⁽¹⁶⁾ hydrolyzed extract of the artichoke tubers to yield 77 % fructose and 23 % glucose and separated fructose in the hydrolyzate by Dowex-1-X8 ion exchange chromatography with overall recovery 61 %.

To maximize the production rate of fructose from the artichoke tubers and for its continuous work, use of the immobilized inulase is required. In this communication, therefore, we studied immobilizations of the inulase on Tygon tube and on aminoethyl cellulose and the resulted enzyme preparations were characterized.

Materials and Methods

Materials

Commercial materials used in this experiment were as follows ; Sepharose 6B from Pharmacia Fine Chemicals (Upsala, Sweden), 3-aminopropyltriethoxysilane from Aldrich Chem. Co. (Milwaukee,

WI), aminoethyl-cellulose from Bio-Rad (Richmond, NJ), Tygon plastic tubing from Norton Co. (Akron, U.S.A.), and inulin from E. Merk (Darmstadt, W. Germany). Other chemicals were of analytical grades.

Jerusalem artichoke tubers, harvested in late fall, were stored at -4°C until used. Tubers were peeled and cut into slices. The slices were then vacuum dried at 30°C and packed in laminated polyethylene packs. Culture of *K. fragilis* and partial purification of inulase from the culture media with Sepharose 6B (column size, 5.0×95 cm) were carried out by the method of Nahm and Byun⁽¹⁶⁾.

Immobilization of inulase on Tygon tube

The method of Senyk *et al.*⁽¹⁷⁾ was used with a slight modification for the immobilization of inulase on Tygon tube. Tygon plastic tubing (inside diameter, 0.24 cm, wall thickness, 0.08 cm) was cut into small pieces (3 mm length) and then milled into coarse particle forms of 40 mesh using Thomas Mill (Arthur H. Thomas Co., U.S.A.) These small particles were refluxed either in chloroform (below 70°C), or in toluene (over 70°C) for 24 hr at various temperature conditions to remove plasticizers. After washing thoroughly with absolute methyl alcohol, they were silanized by refluxing in a 10 % solution of 3-aminopropyltriethoxysilane [$\text{NH}_2-(\text{CH}_2)_3-\text{Si}(\text{O}-\text{C}_2\text{H}_5)_3$] in chloroform for 48 hr. After washing again with methyl alcohol, 3 g of the silanized Tygon tube particles was treated with 50 ml of glutaraldehyde, pH 7.0, varying concentration at room temperature for 24 hr. Residual glutaraldehyde was removed by successive thorough washing with 0.1 M sodium phosphate buffer, pH 7.0. To the glutaraldehyde-treated Tygon tube product, 20 ml of the inulase solution (0.6 mg protein/ml) in the buffer was added and the mixture was stirred gently at 4°C for 24 hr. After the reaction was completed, it was washed with 0.1 M sodium acetate buffer, pH 5.0 and 1 M NaCl in the same buffer 5 times in turn to remove non-covalently bound enzyme. The final preparation was stored at 4°C as wet until used.

Immobilization of inulase on aminoethyl-cellulose

Coupling of inulase to aminoethyl-cellulose (AE-cellulose) was carried out according to the method of Lilly⁽¹⁸⁾. Twenty g of AE-cellulose was pretreated with 400 ml of 0.5 N NaOH and washed completely with distilled water until the washing became colorless for a phenolphthalein test. To the filtered AE-cellulose, 500 ml of 1 to 5 % glutaraldehyde was added, respectively. After stirring for 2 hr at room temperature, the AE-cellulose derivative was recovered by filtration and washed 5 times with 200 ml of the same buffer to remove residual glutaraldehyde. To it, 80 ml of the inulase solution (0.6 mg protein/ml) was added and stirred gently for 24 hr at 4°C. The immobilized enzyme was recovered by filtration on glass filter under vacuum and washed with 400 ml of 0.1 M sodium acetate buffer, pH 5.0 and same buffer containing 1 M NaCl 5 times in turn to remove non-covalently bound inulase. The final preparations was stored at 4°C as wet until used.

Enzyme assays

Since the enzyme activities were directly proportional to the incubation time up to 30 min for inulin and sucrose hydrolyses, the activities at 5 or 10 min were used instead of initial velocities. Invertase activity was measured by determining the reducing sugar released from sucrose. The reaction mixture, which contained 1.0 ml of 0.1 M sucrose in 0.1 M sodium acetate buffer, pH 5.0 and 0.1 ml of diluted enzyme solution, was incubated for 5 min at 30°C and the reaction was stopped by heating the reaction mixture in a boiling water bath for 5 min. One unit of invertase activity is defined as one μ mole of sucrose hydrolyzed per min at 30°C. Inulase activity was measured by determining the reducing sugar released from inulin. The incubation mixture contained 1.0 ml of 5 % inulin in 0.1 M sodium acetate buffer, pH 5.0 and 0.1 ml of diluted enzyme solution. After incubation for 10 min at 30°C, the reaction was stopped as above. One unit of inulase activity is defined as the production of one μ mole of free sugar per min at 30°C.

To measure the invertase activity of the immobilized enzyme, incubation mixture, which consisted of 3 ml of 0.1 M sucrose in 0.1 M sodium acetate buffer, pH 5.0, and various amounts of the immo-

bilized enzyme, was incubated at 30°C of water bath for 10 min with vigorous stirring with a submerged magnetic stirrer. After the incubation was completed, 1 ml of aliquot was taken and released reducing sugar was determined. Same procedure was used for the assay of inulase activity of the immobilized enzyme, using the 3 ml of 5 % inulin solution as substrate.

Assays of protein and sugar

Protein was determined according to the method of Lowry *et al.*⁽¹⁹⁾ with bovine serum albumin as standards. Reducing sugar was measured by the Somogyi-Nelson method⁽²⁰⁾. Glucose was assayed using Glucostat according to a Worthington manual⁽²¹⁾. Fructose was determined as difference between the amounts of total reducing sugar and glucose.

Kinetic studies

Two immobilized inulase preparations, Tygon tube and AE-cellulose derivatives, were characterized with respects to pH, temperature, and substrate concentration. Both sucrose and inulin were used as substrates. The pH and temperature dependences of activity were determined by the same procedures for the soluble inulase⁽¹⁵⁾. Substrate kinetics were also carried out by the same procedure⁽¹⁵⁾. For pH stability the enzyme product was treated at various pH's for 30 min and the remaining activities were measured by the standard assay method. For temperature stability, the immobilized inulase was preincubated at 4 different temperatures in the range from 40 to 55°C for 4 hr with substrate. Remaining activities were measured according to the standard assay procedure.

Results

Enzyme immobilization

Using Tygon tube as a carrier matrix, active immobilized inulase preparations were obtained only when pretreatment of the tube, silanization, glutaraldehyde treatment, and enzyme coupling steps were performed. Since commercial Tygon plastic tubing contained plasticizers, they should be removed before it was silanized. Fig. 1 shows the effect of solvent pretreatment of commercial Tygon plastic

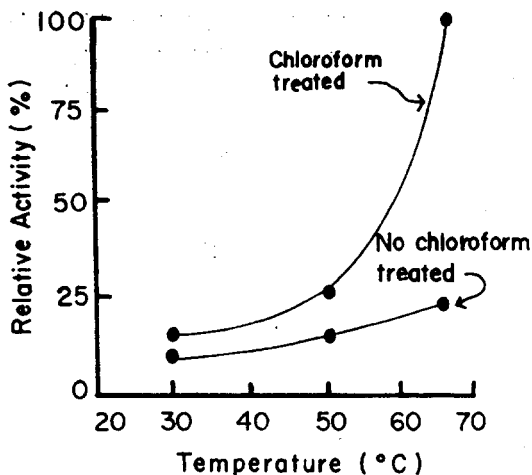


Fig. 1. Effects of solvent pretreatment on the degree of silanization of Tygon tube at various temperatures

Commercial Tygon tube was refluxed either in chloroform or in toluene for 24 hr at the given temperature. Degree of silanization was measured by the activity of inulase after the enzyme was immobilized on the silanized Tygon tube by the procedures described in the text

tubing on the immobilization of inulase. As result, the activity of chloroform-pretreated Tygon tube increased more rapidly than non-pretreated one with increase of temperature.

In chloroform or chloroform-silane treatment, Tygon tube lost weight and became more rigid because of the removal of plasticizers. It appeared that removal of plasticizers exposed reactive groups of the polymer to 3-aminopropyltriethoxysilane. As shown in Fig. 2, silanization at 65°C showed the most salient effect on immobilization of inulase. The experiment was performed with chloroform below 70°C and toluene was used above this temperature due to boiling point of chloroform. As shown in Fig. 2, maximum activity of inulase was immobilized by silanization at 65°C in chloroform. Therefore, this process was used thereafter.

Fig. 3 shows that the concentration of glutaraldehyde was also an important factor in inulase immobilization on Tygon tube. Ten % of glutaraldehyde was the most effective on immobilization. As results the final product, prepared by the proce-

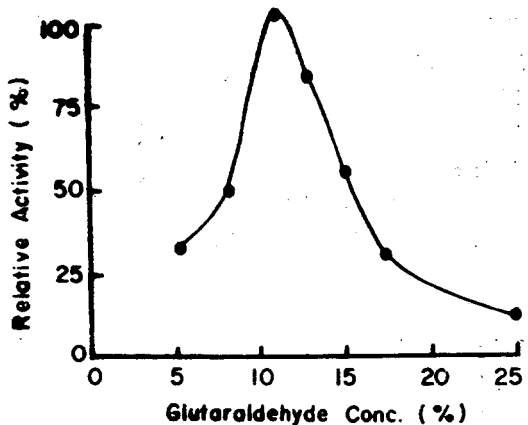


Fig. 2. Effects of temperature on the degree of silanization of Tygon tube

Tygon tube pretreated with solvent was silanized with 10 % solution of 3-aminopropyltriethoxysilane at the given temperature. Degree of silanization was measured by the activity of immobilized inulase by the procedure described in the text

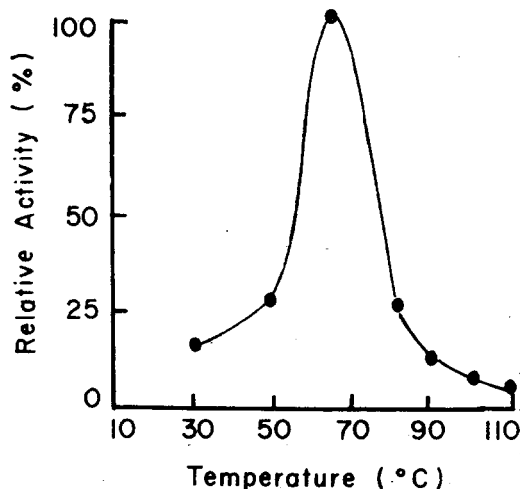


Fig. 3. Relative activity immobilized on Tygon tube as function of glutaraldehyde concentration

Inulase was immobilized with glutaraldehyde to Tygon tube which was pretreated and silanized by the procedures described in the text

cedure that Tygon tube was pretreated with chloroform at 65°C, silanized in chloroform at 65°C and followed by treatment with 10 % glutaraldehyde, pH 7.0 for 24 hr and enzyme coupling, showed 11.5 units of inulase activity per g of dried matrix with

22.5 % of activity recovery.

In the case of immobilization of inulase on AE-cellulose, 1 to 5 % glutaraldehyde in 0.02 M sodium phosphate buffer, pH 7.0, was added to AE-cellulose, respectively to examine effect of the glutaraldehyde concentration on immobilization of inulase. Fig. 4 shows that activity of inulase immobilized on AE-cellulose varied according to glutaraldehyde concentration. Differently from Tygon tube, 2 % glutaraldehyde was accepted as optimum. The final product showed 39.3 units of inulase activity per g of dried matrix with 53.4 % activity recovery.

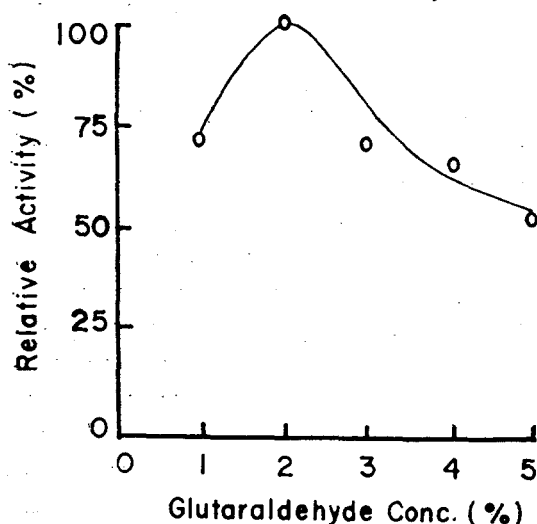


Fig. 4. Relative activity of inulase immobilized on aminoethyl-cellulose as function of glutaraldehyde concentration

Activities and yields of the two immobilized inulases are given in Table 1. It was shown that activity of AE-cellulose derivative was higher than that of Tygon tube derivative and so was coupling efficiency in activity. Using AE-cellulose as carrier matrix, coupling efficiency was 53.4 %, while yield on the basis of protein was 71 %. This indicated

Table 1. Results of Tygon tube and aminoethyl-cellulose immobilized inulase preparations

Carrier matrix	Inulase activity (units/g dry weight)	Activity yield (%)
Tygon tube	11.5	22.5
Aminoethyl-cellulose	39.3	53.4

that the bound enzyme was denatured to some extent during immobilization. Activity ratios (invertase/inulase) of soluble and two immobilized enzymes are given in Table 2. This showed that mass transfer of inulin was more limited than sucrose on immobilization.

Table 2. Comparative ratio of activity of soluble, Tygon tube- and aminoethyl-cellulose-immobilized inulases

Enzyme	Activity ratio (invertase/inulase)
Soluble enzyme	6.8
Immobilized enzyme on Tygon tube	10.0
Immobilized enzyme on aminoethyl-cellulose	7.5

Stability and kinetic properties of the immobilized inulase

The pH optima of Tygon tube derivative on sucrose and inulin hydrolyses were 3.5 and 4.5, respectively, while those of AE-cellulose immobilized one were 4.0 and 5.5, respectively. Compared with soluble inulase⁽¹⁵⁾, pH profile on sucrose hydrolysis by two immobilized enzymes shifted slightly toward the basic range, whereas those on inulin hydrolysis shifted slightly to acidic range for Tygon tube derivative.

An assessment of effect of temperature on the hydrolyzing activity of sucrose by the 2 kinds of immobilized inulase derivatives did not reveal any significant differences with soluble one. With both enzyme derivatives a temperature transition was observed at 60°C. For inulin hydrolysis, however, the temperature optima of Tygon tube derivative was 50°C, while one of AE-cellulose derivative was 45°C. The activation energies obtained from Arrhenius plots covering the temperature range from 30° to 65°C were as follows: for sucrose hydrolysis Tygon tube derivative, 5.9 kcal/mol/deg and AE-cellulose derivative, 7.0 kcal/mol/deg; for inulin hydrolysis Tygon tube derivative, 3.4 kcal/mol/deg and AE-cellulose derivative, 5.6 kcal/mol/deg.

In attempt to test the relative substrate accessibility of inulase derivatives, kinetic assays were conducted with sucrose and inulin as substrates.

Table 3. Summary of kinetic results of soluble, Tygon tube- and aminoethyl cellulose-immobilized inulases

Enzyme	Optimum pH		Optimum temperature(°C)		E_a (kcal/mol)		K_m (mM)	
	on sucrose	on inulin	on sucrose	on inulin	on sucrose	on inulin	on sucrose	on inulin
Soluble enzyme*	3.0	5.5	60	55	8.0	6.9	6.7	8.0 ^b
Immobilized enzyme on Tygon tube	3.5	4.5	60	50	5.9	3.4	10	11.0 ^b (5.5%)
Immobilized enzyme on aminoethyl-cellulose	4.0	5.5	60	45	7.0	5.6	7.7	10.0 ^b (5%)

a: Results from Ref. 15

b: Data were obtained by Michaelis-Menten plots and molecular weight of inulin was assumed 5,000

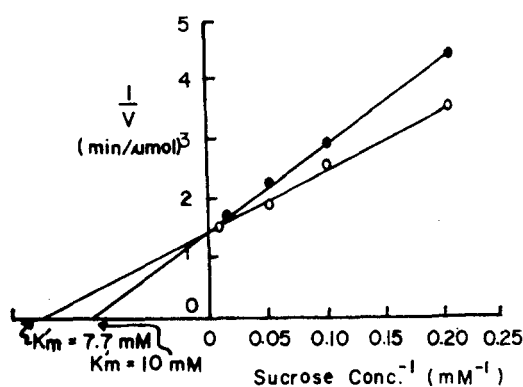


Fig. 5. Lineweaver-Burk plots for hydrolyses of sucrose by Tygon tube and aminoethyl cellulose immobilized inulase preparations
 ●—●: Tygon tube derivative, ○—○: aminoethyl-cellulose derivative

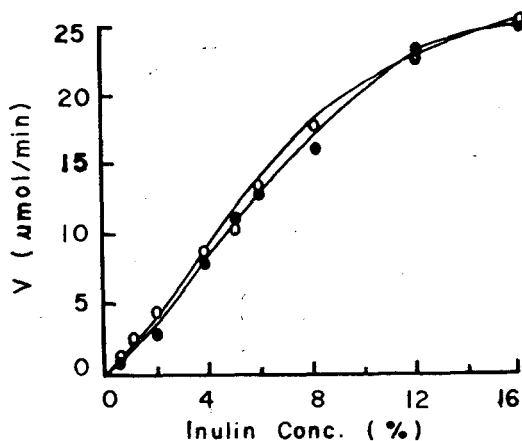


Fig. 6. Effects of inulin concentration on inulin hydrolyses by Tygon tube and aminoethyl cellulose immobilized inulase preparations
 ●—●: Tygon tube derivative, ○—○: aminoethyl cellulose derivative

The results are given in Fig. 5 and Fig. 6, respectively. For both sucrose and inulin hydrolyses

there was a significant increase of K_m for the derivatives in which the enzyme was coupled to the matrix. The inulin hydrolysis (Fig. 6) led to a non-hyperbolic behavior.

Kinetic parameters of two kinds of immobilized inulase are summarized and compared with soluble inulase in Table 3.

For the determination of stability of the immobilized enzyme, only AE-cellulose derivatives were examined, since AE-cellulose derivative showed higher activity than Tygon tube derivative per g of dried matrix. Fig. 7 shows retention of inulase activity of AE-cellulose derivatives of inulase which was kept for several time intervals at different pHs at 30°C. The immobilized inulase was stable at pHs 4, 5, and 6 for at least one week, while it was very unstable at pH 2 and most of activity was lost by 30 min treatment at that pH. The stability of the invertase activity was very similar to that of inulase activity.

Fig. 8 shows heat stability of AE-cellulose immobilized inulase which was treated for several time

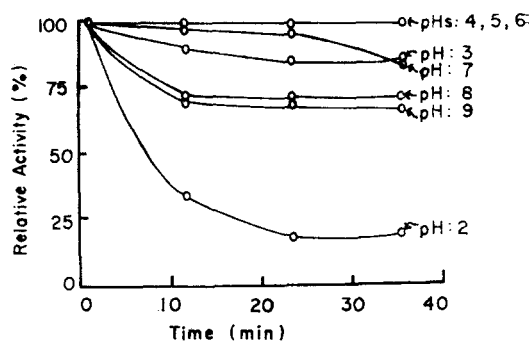


Fig. 7. pH stability of inulase activity of aminoethyl-cellulose immobilized inulase preparation

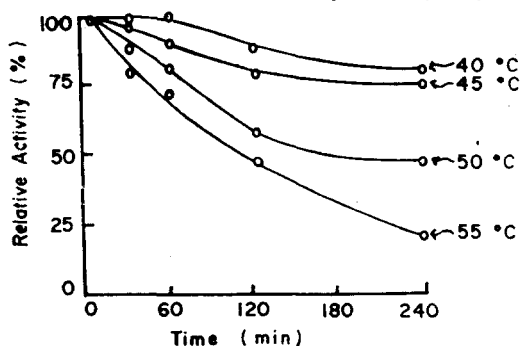


Fig. 8. Temperature stability of inulase activity of aminoethyl-cellulose immobilized inulase preparation

internals at pH 5.0 at different temperatures. Results showed that both invertase and inulase activities were recovered 80 % of original activity by 40°C treatment for 4hr. Although no inulase activity was lost by 30°C treatment for 240 min, the activity decreased rapidly over 50°C. Same results of pH and heat stabilities were obtained for the invertase activities of the AE-cellulose immobilized derivative of inulase preparation.

Discussion

Among several methods of β -fructosidase (invertase) immobilization, entrapment in cellulose triacetate⁽¹⁰⁾ is the most attractive method in many respects. This method, however, may cause difficult mass transfer for inulase immobilization, since β -fructosidase (inulase) hydrolyzes inulin and the molecular weight of inulin is relatively high. Indeed, we have tried the immobilization of inulase with cellulose acetate, but it showed only slight activity on inulin as substrate, while it showed relatively higher activity on sucrose.

For immobilization of inulase on Tygon tube, silanization in chloroform and glutaraldehyde treatment steps are requisite to immobilization. The exact mechanism for the enzyme immobilization of Tygon tube is unknown, while polymer backbone may be involved in immobilization. The storage of the immobilized enzyme as dry state caused the rapid decrease in enzyme activity.

The optimum concentration of glutaraldehyde in

AE-cellulose immobilization was lower than that in case of the Tygon tube. This phenomenon appeared by the fact that the increased glutaraldehyde concentration allowed more chemical inactivation of inulase activity on AE-cellulose due to multiplication of crosslinks⁽²²⁾ than one on Tygon tube, since AE-cellulose has more reactive groups for glutaraldehyde than the other.

In comparison of K_m for sucrose hydrolysis with soluble inulase (6.7 mM), K_m' values were 7.7 mM with the immobilized inulase on AE-cellulose and 10 mM with one on Tygon tube. These results represented the existence of diffusional limitation in both cases, especially in Tygon tube derivative. In this connection it was noticeable that activity ratios (invertase/inulase) were 6.8 for soluble enzyme, 7.5 for AE-cellulose derivative, and 10 for Tygon tube derivative as shown in Table 2.

Since sucrose has low molecular weight (M.W. 342) and inulin is a polysaccharide (M.W. 5,000), results represent difficulty of access of inulin to active site of the immobilized enzyme on Tygon tube in particular. In this experiment assay of the immobilized enzyme was carried out with vigorous stirring. This may eliminate the limitation of external diffusion. Therefore, we can infer that this effect mainly attributed to the limitation of internal mass transfer. In other words, there exists a significant internal diffusion limitation in case of the immobilized enzyme on Tygon tube using inulin as substrate. In fact, calculated diameter of the milled Tygon tube based on the sieve size was about 600 μm , while AE-cellulose particles has an average diameter of 18 μm and a length with an 80 μm in average. Generally the smaller the particle size is, less the internal diffusional limitation occurs. We can expect, therefore, that the immobilized enzyme on Tygon tube had more limitation of internal diffusion. This phenomenon was in good agreement with the activation energy decrease in the case of immobilization as summarized in Table 3. The presence of diffusional limitation may also be detected by measurement of the activation energy for the reaction. Wheeler⁽²³⁾ had shown that as effectiveness factor falls below unity, measured

activation energy also falls, tending toward arithmetic mean of activation energy for diffusion process and chemical reaction. Since the former is relatively small, measured activation energy in heterogeneous catalysis, E_{app} , is half of true activation energy, E_0 , where ϕ (Thiele modulus) is large, i.e., in case of existence of internal diffusion limitation. Experimentally activation energy of soluble enzyme with inulin is 6.9 kcal/mol, but that of immobilized inulase on Tygon tube is 3.4 kcal/mol, about half value of the former. Based on these three evidences, we can conclude that limitation of internal diffusion is existed in the immobilized inulase of Tygon tube using inulin as substrate, whereas slightly existed in the case of one on AE-cellulose.

요 약

*Kluyveromyces fragilis*로부터 정제한 β -fructosidase (inulase)를 Tygon 판과 aminoethyl-cellulose에 고정화 시킨 후 효소의 일반적 성질을 알아 보았다. Tygon 판에 효소를 고정화 시키는데 있어서는 크로로포름 용액에서 65°C의 조건으로 실란화(silanization) 시킨 다음 10% 글루타르알데히드로 처리하는 것이 매우 중요한 처리 조건인 반면 2% 글루타르알데히드 처리가 aminoethyl-cellulose에 inulase를 고정화 시키는데 매우 효과적이 었다.

Tygon판에 고정화 시킨 inulase는 g matrix당 11.5 units의 inulase 역가로 보여 주었고 효소 역가의 회수율은 22.5%인 반면 aminoethyl-cellulose에 고정화 시킨 제품은 g matrix당 39.3 units, 53.4% 효소 역가 회수율을 보여 주었다. 두 고정화 제품을 사용하여 invertase(기질이 sucrose) inulase(기질이 inulin) 역가에 대한 효소 안정성, pH 효과, 온도 효과 및 기질 효과를 살펴 보았다.

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