Kor. J. Appl. Microbiol. Bioeng.
Vol. 9, No. 4, 197—205 (1981)

Studies on Microbial Penicillin Amidase

(Part 6) Immobilization of Penicillin Amidase from *Bacillus megaterium* by Adsorption and Acrylamide Gel Entrappment

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(Received November 2, 1981)

미생물 페니실린 아미다제에 관한 연구

(제 6 보) 흡착효소의 아크릴아마이드겔 포괄방법에 의한 *Bacillus megaterium*의 변이주가 생산하는 페니실린 아미다제의 고정화에 관한 연구

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(1981년 11월 2일 수리)

Abstract

Penicillin amidase of *Bacillus megaterium* was recovered from the fermentation broth by adsorption on celite and immobilized by entrapping the adsorbed enzyme in acrylamide gel. The operational stability in column reactor was greatly increased by entrappment as compared with that of without entrappment. The optimum pH of the immobilized enzyme was 8.7 with broader activity profile than that of the free enzyme, while the most stable pH range appeared to be between pH 7.5 and 8.0. The optimum temperature was shifted to 50°C from 45°C for the soluble enzyme.

The values of K_m and the inhibition constants for 6-APA(K_{ia}) and phenylacetic acid (K_{ip}), were 4.55 mM, 36.5mM, and 10.5mM, respectively. No significant internal pore diffusion limitation was found since the value of effectiveness factor was 0.95. The operational half life in a column reactor at pH 8.0 was 6.8 days at 40°C and 47 days at 30°C, whereas that of without entrappment was only 1 day and 4 days, respectively. The performance of a batch and a column reactor was also discussed with respect to the productivity. The results demonstrated that the entrappment of an adsorbed enzyme for the enhancement of the operational stability of the immobilized enzyme was useful especially when an extracellular enzyme was used.

Introduction

To prepare the 6-aminopenicillanic acid (6-APA) by enzymatic hydrolysis of benzylpenicillin, various immobilization methods for penicillin amidase (E.C. 3,5,1,11) have been developed. These immobilization methods comprise, covalent coupling to cellulose derivatives,[1,2,3] Amberlite,[4] Sepharose 4B,[5] and nylon fiber,[6] entrappment in cellulose triacetate,[7] gelatin and cellulosic materials,[8] polyacrylamide gel.,[9] and copolymerization with acrylamide,[10] and adsorption on bentonite.[11] As enzyme sources, several microorganisms such as *Escherichia coli* and *Bacillus megaterium* are known to be useful for the practical application.

Penicillin amidase from *B. megaterium*, an extra-

cellular enzyme, was immobilized by adsorption to bentonite and its reactor performance in a stirred tank was reported.[11] One of the drawbacks of adsorption method in the application to the immobilization is its limited repetative use as an immobilized enzyme due to the rapid leakage of enzymes during the reactor operation, which may be ascribed to the weak binding force of enzymes to the carrier surface or/and the discrepancy in the optimum pH between the adsorption process and the enzymatic catalysis. Adsorbed enzyme on the fine particles such as bentonite or celite, moreover, cannot be used for plug flow reactor because of the pressure drop in column.

Entrappment or crosslinking after adsorption can strengthen the stability of the adsorbed enzyme. In the previous communication, an immobilization method of penicillin amidase by entrappment in the reinforced calciumalginate gel and the operation stability of the immobilized enzyme have been reported.[12]

In the present study, penicillin amidase of *B. megaterium* was immobilized by adsorption of the enzyme directly on celite from the fermentation broth and subsequently entrapping the celite particles in acrylamide gel to improve the enzyme stability. The characteristic properties of this new type of the immobilized enzyme were studied and compared with that of the free enzyme. The reactor performance of the immobilized enzyme in a column reactor was also reported in this communication.

Materials & Methods

Materials

Bacto-soytone and, yeast extract were purchased from Difco Lab. (U.S.A.). Acrylamide, N,N'-methylene-bis-acrylamide, and ammonium persulfate were obtained from Wako Chemical Co. (Japan). Penicillin G. TEMED, phenylacetic acid, p-dimethylaminobenzaldehyde, and 6-aminopenicillanic acid were purchased from Sigma Chemical Co. (U.S.A.). For enzyme adsorption, the purified diatomaceous minerals known under the trade name of celite 545 (Junsei Chem. Co., Tokyo, Japan) was used. Impurities or iron oxides were removed before use by extraction with 2N-HCl, followed by washing with water to neutrality and subsequently with methanol.

Cultivation of Microorganism

The culture of *B. megaterium* ATCC 14945 variant (*B. megaterium* KFCC 10029)[13] was maintained on soytone agar slant. For the mass production of penicillin amidase, the culture was grown in 28*l* fermentor (CMF—128 S, NBS, U.S.A.). The medium consisted of Bacto-soytone, 2.5%; glucose, 1.0%; yeast extract, 0.5%; and Neoline 202 as an antifoam, 0.01% (produced by Polyol Co., Korea). The fermentation process was carried out at temperature 30-33°C, agitation speed 500 rpm, and aeration rate 2 vvm.[13] The pH was adjusted to 7.0 before and after sterilization. 0.3% of phenylacetic acid was added after 8 and 12hr of incubation, respectively, to induce the enzyme production.[13]

Enzyme Preparation

After 24-36hr of cultivation, the fermentation broth was harvested and separated from cells by continuous centrifugation at 8,000 rpm (Kokusan Type H600, Japan). Since penicillin amidase from *B. megaterium* is an extracellular enzyme, the centrifuged supernatant can be used as a crude preparation of the soluble enzyme.

Determination of Enzyme Activity

The activity of penicillin amidase was determined by measuring the amount of 6-APA from a reaction mixture

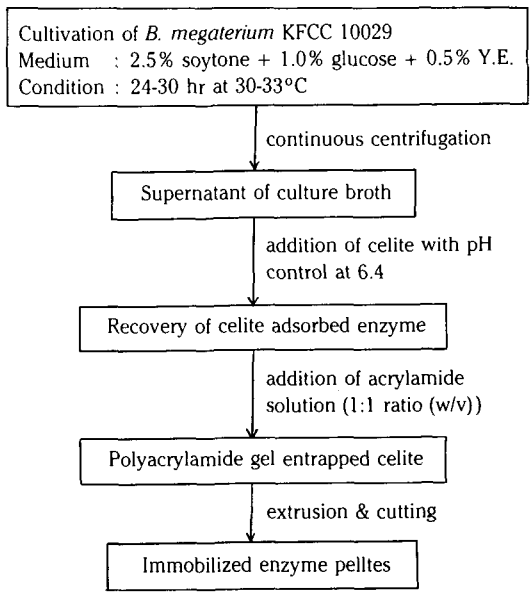

Fig. 1. Procedure of Immobilized Enzyme Pellet Preparation.

at 40°C with 20mM benzylpenicillin solution in 0.1M borate buffer (pH 8.7). The p-DAB (p-dimethylaminobenzaldehyde) method of Balasingham *et al.*[2] was used for the determination of 6-APA. One unit of the enzyme activity is defined as the amount of the enzyme required to produce 1 μmole of 6-APA per an hour under the given condition.

Immobilization Method

The immobilization of the enzyme was carried out by entrapping in the polyacrylamide gel matrix after the adsorption onto celite particles. For the enzyme adsorption, the supernatant was acidified with 20% acetic acid to pH 6.2-6.4 and was mixed with the acid washed celite at a ratio of 1,200 enzyme unit per g celite. The suspension was stirred for 2hr, during which sufficient acetic acid was added to maintain the pH 6.2-6.4. Celite particles were collected by filtration and washed with 1 volume of distilled water.

To 200ml of the acrylamide monomer solution (containing 10% acrylamide, 0.5% N,N-methylene-bis-acrylamide and 0.5% TEMED), 200g of celite slurry was added and made homogeneous suspension with stirring. After 30 seconds, 4ml of ammonium persulfate solution (0.6 g/ml) was poured into the suspension to initiate polymerization. Homogeneous gel of the polyacrylamide-celite mixture was obtained within 1-2 minutes.

The gel was cast into a pellet form (1.0-2.0mm in diameter) by extrusion in borate buffer (0.1M, pH 8.0). The pellet was washed thoroughly with the buffer and cut into a desired size (2-5mm). The immobilized enzyme was preserved in 0.1M borate buffer (pH 8.0) in the absence of other special preservatives. The overall procedure for the preparation of the immobilized enzyme is summarized in Fig. 1.

Reactor Operation

For batch reaction, the immobilized enzyme pellet was preincubated at 40°C for 20 min in 0.1M borate buffer (pH 8.0). After adding substrate solution, the reaction mixture was incubated at 40°C, except where otherwise stated, in a reactor agitated with a magnetic stirrer. The enzyme activity was determined at predetermined time intervals. A packed bed column reactor (1.3x12cm) was used for continuous operation. The column reactor was fritted with a sintered glass filter at the bottom of the column, and a thermostat unit to an inlet end of the water jacket of the column to maintain the isothermal condition. The substrate solution was continuously fed at a constant speed by MANOSTAT cassette pump (Junior model). In an attempt to attain the efficient performance in a plug flow reactor system, the pH drop effect along the column path was minimized by using 0.2M borate buffer solution (pH 8.0). The degree of conversion was measured after a steady state was attained.

Results

Preparation of Immobilized Enzyme

From 20*l* of culture broth (85 enzyme units/ml broth), 3.4kg (wet weight) of celite adsorbed enzyme was recovered. The total recovery of the enzyme activity was 94% by the adsorption process. The overall activity retention after the polyacrylamide gel entrappment was 46% of that of original broth, and the specific activity of the final preparation was 115 units/g (wet wt) or 740 units/g (dry wt). The activity recovery at each immobilization step is given in Table 1.

Table 1. Preparation of Immobilized Penicillin Amidase

Preparation step	Total volume or weight	Total activity (unit)	Activity recovery (%)
Culture broth	20,000 ml	1,700,000	100
Celite adsorption	3,400 g (wet wt.)	1,598,000	94
Polyacrylamide gel entrappment	6,800 g *	782,000	46

* Specific activity: 115 units/g wet wt. or 740 units/g dry wt.

Characteristics of Immobilized Enzyme

General properties of the immobilized enzyme were compared with those of the soluble enzyme. As shown in Fig. 2, the optimum pH for both soluble and immobilized enzyme was 8.5-8.7, but the activity profile of the immobilized enzyme was broadened in both acidic

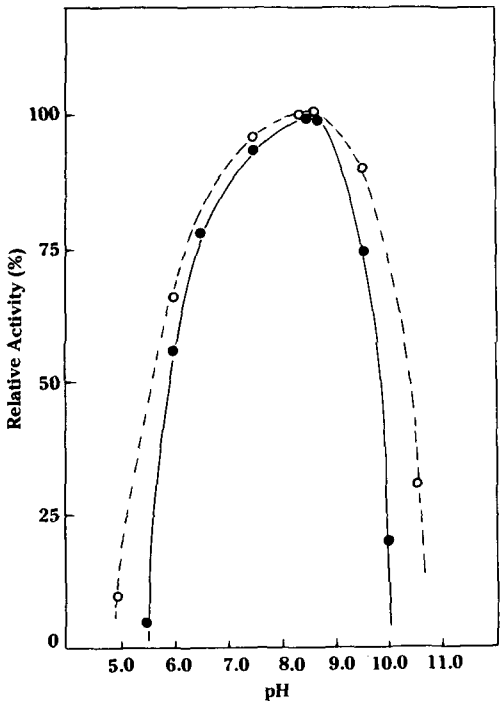

Fig. 2. pH-Activity Profile of Soluble and Polyacrylamide Gel Entrapped Enzyme.

—•— soluble enzyme, --o-- entrapped enzyme. Buffers used are 0.1M acetate (pH 5.0, 5.5), 0.1M phosphate (pH 6.0, 6.5), 0.1M Tris (pH 7.5, 8.5, 8.7), 0.1M bicarbonate (pH 9.5, 10.0, 10.5).

and alkaline regions. It was found that the enzyme was stable in the pH range from 6.0 to 8.5, while a rapid deactivation occurred below pH 5.5 and above 9.0 (Fig. 3). In the pH range of 7.0-8.0 the residual enzyme activity after 15hr incubation at 35°C was about 93% of the initial activity at the corresponding pH.

The optimum temperature of the immobilized enzyme was 50°C, whereas that of the soluble enzyme was 40°C (Fig. 4). The activity of immobilized enzyme was not changed during storage at pH 8.0 for 15 hrs in the temperature range of 20°C-35°C, while that of soluble

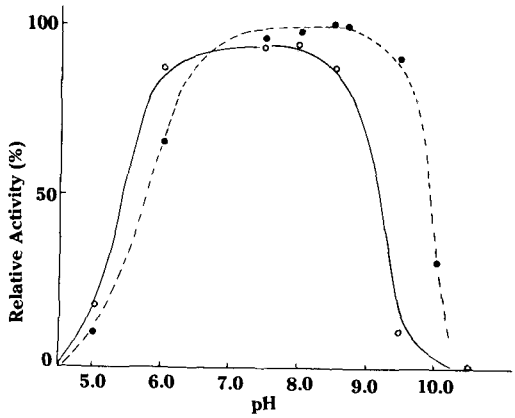

Fig. 3. pH stability of Polyacrylamide Gel Entrapped Enzyme.

Enzyme activities of respective pH values were measured after 15hrs of storage at each pH at 35°C. Buffers used are the same as those of Fig. 2. pH-activity profile is shown as a dotted line in comparison.

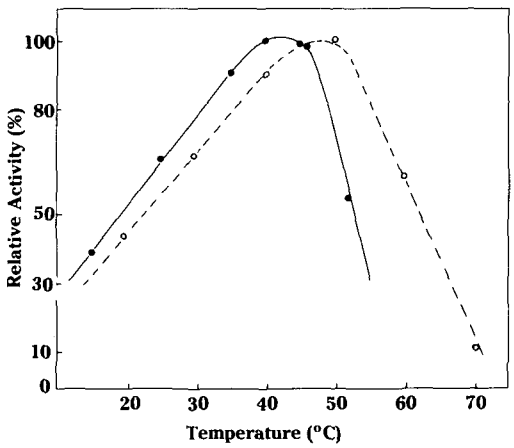

Fig. 4. Effect of Temperature on the Activity of Soluble and Polyacrylamide Gel Entrapped Enzyme.

—•— souble enzyme, ---o--- entrapped enzyme.

enzyme in the identical condition was 96% and 91% of initial activity at temperature 30°C and 35°C, respectively (Fig. 5).

The kinetic constants such as Michaelis constant (K_m) and the inhibition constants of 6-APA (K_{ia}) and phenylacetic acid (K_{ip}) were evaluated and compared with those of the soluble enzyme as shown in Table 2. There was no significant changes in the kinetic constants for the immobilized enzyme except for the K_{ip} value,

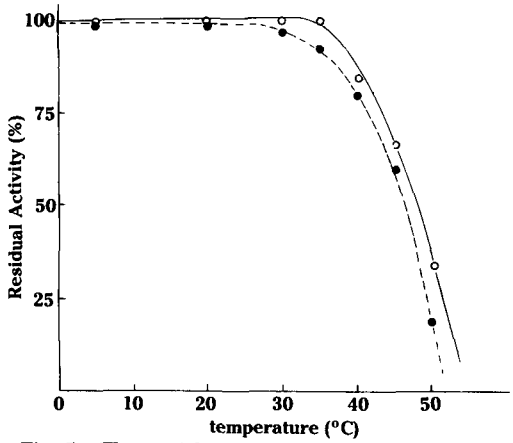

Fig. 5. Thermal Stability during Incubation.

—•— soluble enzyme, --o-- entrapped enzyme.

which was markedly decreased.

The pore diffusional limitation appeared to be negligible since the value of the effectiveness factor was 0.95 when 10mM substrate solution was used.

Table 2. Kinetic Constants of Penicillin Amidase

Enzyme kinetic const.	Soluble enzyme	Immobilized enzyme
K_m	4.5 mM	4.6 mM
K_{ia}*	45.0 mM	36.6 mM
K_{ip}**	26.0 mM	10.5 mM

* Inhibition constant for 6-APA
** Inhibition constant for PAA

Batch Operation

The time course of the conversion of benzylpenicillin solution (5mM-100mM) was tested in a batch reactor. Fig. 6 shows the result of batch reaction using 50ml of substrate solution containing 5g of the wet enzyme (565 units). 5-15mM benzylpenicillin solutions were converted completely within 2-3hr, while 30mM and 50mM of the substrate solution were converted to 60% and 40% within 4hr, respectively. Further conversion required much longer time especially in case of high concentration due to the strong inhibitory effect of the accumulated product. In a separate experiment on the repetitive use of the immobilized enzyme using 30mM of the substrate solution, the immobilized enzyme could be used 5 times without substantial loss of the activity maintaining the final conversion up to 95%.

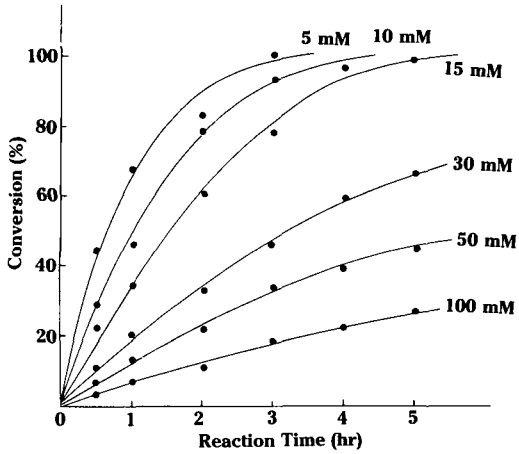

Fig. 6. Batch Progress of Conversion Using Polyacrylamide Gel Entrapped Enzyme.

Reactor volume, 50ml; enzyme loading, 565 units. Substrate solution of various concentration (0.2M borate buffer, pH 8.0) containing entrapped enzyme was continuously stirred at 40°C.

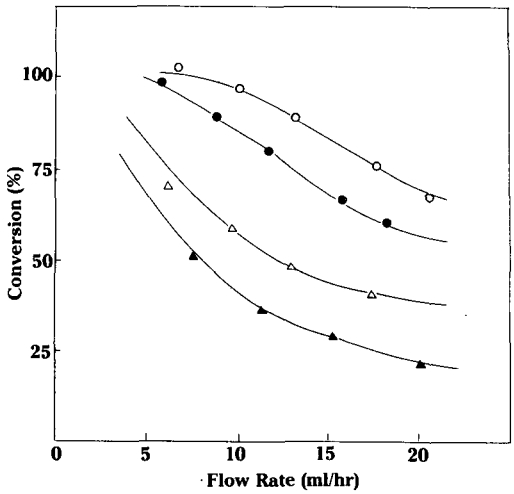

Fig. 7. Steady State Conversion in a Plug Flow Reactor as a Function of Flow Rate and Substrate Concentration.

Substrate solution of respective concentration (0.2M borate buffer, pH 8.0) was continuously fed at 40°C. 5g (wet wt) of immobilized enzyme was packed in column (1.3cm diameter, 10.8cm bed height). Substrate concentration; —o— 10mM, —•— 15mM, —△— 30mM, —▲— 50mM.

Plug Flow Reactor Operation

The bed height of a packed column was 10.8cm employing 5g (wet wt.) of enzyme loading. The steady state conversion at different flow rates for different concentration is shown in Fig. 7. The pH drop became increased as the substrate concentration increased and the flow rate decreased. The maximum pH drop was found which 50mM substrate concentration at 7.5ml/hr of flow rate. The effluent pH was as low as 6.8. It was demonstrated that the productivity decreased gradually

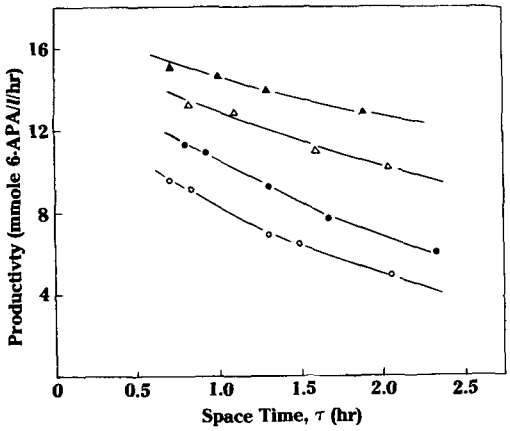

Fig. 8. Productivity of Immobilized Penicillin Amidase Plug Flow Reactor System.
The productivity was calculated as S_oX/τ where S_o, initial substrate concentration; X, conversion; , residence time. Substrate concentration; —o— 10mM, —•— /mM, —△— 30mM, —▲— 50mM.

with increased space time and decreased substrate concentrations (Fig. 8). The productivity, S_oX/τ, is defined as μmole of 6-APA produced per liter per hour. The residence time τ, was calculated from the specified column dimensions and the flow rate.

Storage and Operational Stability

Storage stability of the immobilized enzyme at 4°C in 0.1M borate buffer solution (pH 8.0) is shown in Fig. 9. The activity decay was not found during the storage for 80 days. During this period, the enzyme leakage from the immobilized enzyme was not detected since no enzyme activity appeared in the storage buffer. In a similar experiment on the enzyme adsorbed on celite, substantial amount of the enzyme activity was detected in the storage buffer after 20 days of storage.

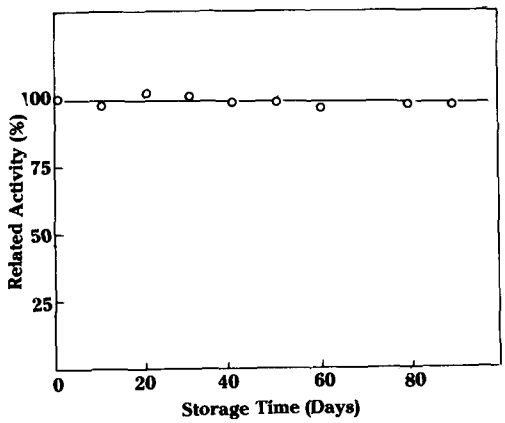

Fig. 9. Storage Stability of Entrapped Enzyme.
Storage condition; 0.1M (pH 8.0) borate buffer, 4°C.

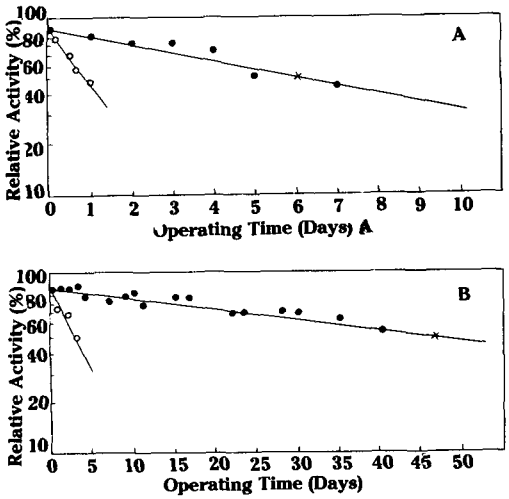

Fig. 10. Operational Stability of Entrapped Enzyme in a Plug Flow Reactor at 40°C (A) and 30°C (B).
10mM benzylpenicillin solution in 0.1M borate buffer (pH 8.0) was continuously fed with the flow rate of 6.1 ml/hr with 5g enzyme loading for the 40°C operation. The experiment was carried out at the flow rate of 6.9 ml/hr and 4g enzyme loading for the 30°C operation. (—•—) entrapped enzyme; (o—o) adsorbed enzyme without entrappment.

The enhancement of the enzyme stability by the en-

trappment was pronounced during the column reactor operation (Fig. 10). Under the operating condition with 10mM benzylpenicillin solution, 5g of enzyme loading, and 6.1ml/hr of flow rate, the half life of the immobilized enzyme at 40°C was 6.8 days. The enhancement in the operational stability was about 7 fold as compared to that without the entrappment. In a similar operating condition at 30°C, the half life was 47 days, whereas that without entrappment was about 4 days. The half life increased about 12 fold by entrappment.

During the reactor operation, substantial amount of the enzyme activity was detected in the effluent of column containing the celite-adsorbed enzyme. Difficulties of flow in a column also arised in case of the celite-adsorbed enzyme, when the amount of enzyme loading was above 2g. For substrate feed, a pump was required to overcome the pressure drop in a column which may be ascribed to the close packing of fine celite particles. Such difficulties of flow control was eliminated in the entrapped enzyme. Degradation or disintegration of the physical form of the immobilized enzyme was not found during the operation.

Discussion

Entrappment in acrylamide gel has been widely applied for immobilization of microbial cells or enzymes.[14-17] Copolymerization of soluble enzymes with acrylamide was also reported.[18,19] Any consideration of the techniques for preparing an immobilized enzyme system must be made in the light of understanding of the following attributes of the system: (1) kinetic behavior, (2) stability, (3) reactor type. In the present study, entrappment of an adsorbed enzyme in acrylamide gel was discussed with respect to the improvement of operational stability of immobilized penicillin amidase by preventing enzyme leakage and pressure drop in a plug flow reactor by increasing the particle size of the immobilized enzyme.

It was found that the enzyme leakage was successfully prevented by the entrappment as evidenced by the enhancement of the enzyme stability during storage and column reactor operation. The increased enzyme stability by immobilization is occasionally found. The changes in microenvironment of the immobilized enzyme or the enzyme carrier itself might play a stabilizing effect on the enzyme. Such stabilization can be accomplished by either the multi-point attachment of enzymes of by embedding the enzyme in a stabilizing and protecting environment.[20] It has also been shown that diffusion effect can cause an apparent increased in stability.[21,22] In the present enzyme preparation, however, the enhancement of stability may mainly be attributed to the prevention of the enzyme leakage although such effects as stated above may play a minor role.

The entrapped enzyme was found to have another advantage in choosing a suitable reactor type. A stirred tank reactor is considered to be suitable for the adsorbed enzyme without entrappment[11] due to the pressure drop in a packed column owing to the close packing on fine celite particles. This operational problem could be obviated for the entrapped enzyme since the flow property in a plug flow reactor was greatly enhanced. This advantage cannot be thought much pronounced especially for penicillin amidase. It is well known that the pH of penicillin amidase reactor gradually decreases due to the acidic product, phenylacetic acid. The design of a suitable plug flow reactor is rather difficult because the pH adjustment in a narrow range by alkali addition is essential. In this experiment, the use of substrate solution in a buffer with moderate strength (0.1M phosphate buffer, pH 8.0) was well compromised with the pH drop along the column path. The pH drop effect can also be solved by employing the multi-stage plug flow reactor.[23]

In a given condition of column reactor operation, the effluent pH dropped to as low as 6.8, showing pH drop of 1.2 pH unit. Since one cannot completely eliminate the pH drop even though a buffer solution is used in a plug flow reactor, the optimization of the reactor performance requires a careful consideration on various factors such as substrate concentration, residence time, desired coversion yield, and buffering capacity used. The use of moderate buffer solution even in case of a stirred tank with pH adjustment was recommended to increase the operational stability of immobilized penicillin amidase.[4] The use of a buffer solution in a plug flow reactor, in this regard, is not so great a sacrifice considering the simplicity of the reactor operation.

An example of industrial application of adsorbed enzyme is seen in the L-aminoacylase adsorbed on DEAE-Sephadex for the separation of racemic mixture of amino acids.[24,25] In this case, the adsorbed enzyme was suc-

cessfully used for a long period of time without substantial enzyme leakage, since the pH difference in adsorption process and the reactor operation was very narrow.

The optimal pH for the adsorption of penicillin amidase on celite or silica is below 7.0. The adsorption of the enzyme from *B. megaterium* on silicate materials such as celite or bentonite has been applied for the enzyme purification[26] and used as a technique for immobilization of the enzyme.[11] The optimal pH of adsorption is reported to be 6.4, while that of enzyme reaction was 8.5-8.7. In such a case where the optimum pH values of the adsorption and the enzyme reaction are different, subsequent entrappment after the adsorption of the enzyme improved remarkably the operational stability of the immobilized enzyme. It is thus concluded that the present immobilization method can be applied for other extracellular or cell free enzymes and will provide a wider choice of reactor systems in practice.

요 약

*Bacillus megaterium*의 발효액으로 부터 페니실린 아미다제를 셀라이트에 흡착시켜 분리한 후 이 흡착효소를 아크릴아마이드에 포괄시켜 고정화하였다. 관형식 반응조에서의 이 고정화효소의 안정도는 포괄시키지 않은 흡착효소에 비해 크게 증가하였으며 최적 반응 pH는 8.7, 그리고 최적안정도는 7.5~8.0이었고 최적온도는 50℃였다. Km과 6-APA, 페닐초산에 의한 저해상수는 각각 4.55mM, 36.5mM, 그리고 10.5mM이었다. Effectiveness factor 값은 0.95로 내부확산 효과는 무시할 수 있었다. pH8.0의 조건에서 관형식 반응조 내에서의 효소역가의 반감기는 40℃에서 6.8일 그리고 30℃에서는 47일로 포괄하지 않은 흡착효소에 비해 안정도가 각각 6.8배와 12배로 증가하였다. 이 고정화효소에 의한 회분식 및 연속식반응조에서의 6-APA의 생산성을 논의하였다. 실험결과로 미루어 보아 특히 흡착효소를 고정화효소로 사용하는 경우에 포괄방법을 이용함으로써 효소반응조의 안정도를 크게 증가시킬 수 있음을 시사하였다.

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