Korean J. Appl. Microbiol. Bioeng.
Vol. 9, No. 3, 159~164 (1981)

Studies on Microbial Penicillin Amidase

(Part 5) Application of Reinforced Calcium-Alginate Gel Entrappment Method for Immobilization of Penicillin Amidase from *Bacillus megaterium*

Hyeung Jin Son, Baik Lin Seong, Tae Ick Mheen and Moon Hi Han
Biotechnology Research Department, Korea Institute of Science and Technology,
P.O.Box 131, Dong Dae Mun, Seoul, Korea
(Received August 4, 1981)

미생물 페니실린 아미다제에 관한 연구

(제 5 보) *Bacillus megaterium* 페니실린 아미다제의 새로운 고정화 방법

손형진 · 성백린 · 민태익 · 한문희
한국과학기술원 생물공학 연구부
(81년 8월 4일 수리)

Abstract

Reinforced Calcium-alginate gel entrappment method for enzyme immobilization is described with an example of penicillin amidase from *Bacillus megaterium* KFCC 10029, a partially constitutive mutant of *B. megaterium* ATCC 14945. Penicillin amidase recoverd from the fermentation broth by adsorption on celite is mixed with alginate and gelatin solution, and cast into a pellet or noodle form by coagulation in calcium salt solution followed by crosslinking with glutaraldehyde.

Optimum pH and temperature of the immobilized enzyme preparation were 8.0 and 60°C, respectively. Kinetic constants such as Km value and the inhibition constant of 6-APA and phenylacetic acid were 2.6 mM, 7.4 mM and 21.2 mM, respectively. The enzyme leakage from the adsorbent during operation was successfully prevented owing to the increase of physical strength of gel coat. The half lives in a column reactor were 6 and 30 days at the respective temperature of 40°C and 30°C, which were the 6-8 fold increased values as compared with those of without entrappment.

The results highly recommended the use of reinforced Calcium-alginate gel entrappment method for the enhancement of physical strength and the operational stability of alginate gel entrapped enzyme.

Introduction

The immobilization of enzymes and microbial cells has been the subject of increased interest, and much work on immobilized enzymes and microbial cells have been carried out.[1,2] There are

several methods reported for the immobilization of cells by entrapping in polyacrylamide gels[3,4], agar gels[5,6], and polymers of cellulose triacetate [7,8]. An immobilization method of microbial cells entrapping in calcium alginate gels has been recently reported[9]. Although calcium alginate gel pellets appear to have excellent physical characteristics for practical use because of the carrier properties suitable for large-scale operation, there is some weaknesses that Ca^{++} chelating agents such as phosphate and certain cations such as Mg^{++} or K^{+} occasionally present in a reaction medium may disrupt the physical integrity of alginate gels either by removing or by attenuating the electrostatic interaction of Ca^{++} -alginate complex.

There are two different types of penicillin amidase, one intracellular enzyme generally produced by *Escherichia coli* and an extracellular enzyme produced by *B. megaterium*.

In the previous study, characterization and reactor performance of the immobilized whole cell penicillin amidase of *E. coli* have been carried out[10,11]. Biochemical properties and the reaction kinetics of purified enzyme of *E. coli* was also studied[12].

The reactor performance of the penicillin amidase from *B. megaterium* adsorbed on bentonite has been previousely reported.[13] However, immobilization of the soluble enzyme by a simple adsorption method is known to be inadequate for the practical purpose due to the low stability.

In the present communication, a method for the immobilization of penicillin amidase by entrapping the enzyme adsorbed on celite particles with the alginate and gelatin mixture followed by crosslinking with glutaraldehyde is described. The objectives of this work comprise, first, the reinformcement of physical and mechanical strength of the alginate gel preparation in the presence of some ions including substrates and products themselves; second, the enhancement of half life of the adsorbed enzyme by preventing the enzyme leakage during the operation owing to the reinforced alginate gel matrices around celite particles.

Materials and Methods

Materials

The culture media, Soytone and yeast extract were purchased from Difco Lab. (Detroit, USA); penicillin G, 6-APA, phenylacetic acid and *p*-dimethylaminobenzaldehyde from Sigma Chem. Co., (St. Louis, USA); glutaraldhyde from Aldrich Chem. Co. (Milwaukee, USA); sodium alginate from Kokusan (Tokyo, Japan). All other chemicals were obtained from Wako (Tokyo, Japan).

Enzyme preparation

The culture of a partially constitutive mutant of *B. megaterium* ATCC 14945 (deposited as *B. megaterium* KFCC 10029) was grown under the conditions of Son *et al*[14]. The supernatant of centrifuged culture broth was acidified with 20% acetic acid to pH 6.2-6.4 and was mixed with celite at a ratio of 1200 enzyme unit per g celite. The mixture was filtered after agitation for 2 hours maintaining pH 6.2-6.4 and the filter cake was used as the celite-adsorbed enzyme.

Enzyme activity assay

The enzyme activity of the penicillin amidase preparations was determined by measuring the amount of 6-APA from a reaction mixture containing penicillin G (10mg/ml) in 0.1M borate buffer (pH 8.7) and soluble or immobilized enzyme preparations with stirring at 40°C. The *p*-dimethylaminobenzaldehyde method was used for the determination of 6-APA[15]. A unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmole of 6-APA per an hour under the specified condition.

Enzyme immobilization

The celite adsorbed enzyme was used for the subsequent entrappment. 200ml of 4% sodium alginate solution pre-warmed at 40°C, was well mixed with 400g of celite slurry (water content, 60-67%) incubating at 40°C. 200ml of 60-80% gelatin solution cooled to 40°C was poured into the mixture with vigorous stirring. This mixture was extruded with a hypodermic needle (gauge

No. 17) into the coagulating bath containing 0.1M $CaCl_2$ solution.

After standing for 2 hours, the pellet or noodle type of gel was treated with 10% glutaraldehyde solution for 1 to 3 minutes followed by washing with 0.5% glycine solution and distilled water to remove the residual glutaraldehyde. The noodle type of gel was cut into small pieces (diameter, 1–2mm, and thickness, 0.2–0.5mm), and used as the immobilized penicillin amidase.

Measurement of hardness

The hardness of the immobilized enzyme treated with glutaraldehyde was measured with a Zenken Texturometer (Model GTX-2, Tokyo, Japan). The conditions of measurement were as follows; voltage 6V, chart speed 750mm/min, attenuator 1, plunger 18mm Lucite, clearance 0.28mm, and bite speed high. This measurement was carried out 20 times for every sample, whose size is 2mm (diameter) by 3mm (length).

Operation of plug flow reactor

The immobilized enzyme pellets of cylindrical form were stored at 4°C in 0.1M borate buffer (pH 8.0). For the performance of reactor, 5.5g (wet wt) of pellets (60U/g wet gel) were introduced into a column (1.2 cm in diameter and 5.5cm in bed height).

The column was thermostated at 30°C or 40°C throughout the operation. The various concentrations of Penicillin G in pH 8.0 borate buffer were fed into the column reactor at different flow rates with a cassette pump. The degree of conversion was measured after the steady state was attained.

Results and Discussion

Effect of glutaraldehyde treatment

In order to determine the optimal condition of glutaraldehyde treatment, the enzyme activity and the hardness (in textural unit) were measured with respect to the treating time and concentrations. The effect of treatment time with 10% glutaraldehyde solution is shown in Fig. 1. The hardness reached maximum after 5 minutes of treatment, while the recovery yield of the activity rapidly decreased. It was found that the proper treating time was within the range of 2 to 3 minutes with 10% glutaraldehyde solution.

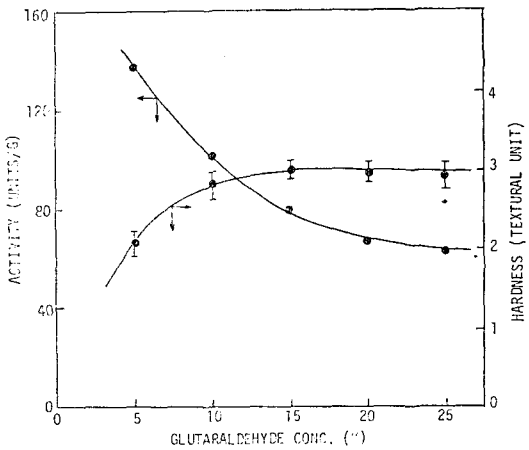

Fig. 1. Effect of Glutaraldehyde Treatment Time. 5g of wet pellets were treated with 10% glutaraldehyde solution in distilled water. The hardness of the pellets were measured with Zenken texturometer.

Fig. 2 shows the effect of glutaraldehyde concentration at a given treatment time, 3 minutes. Optimal concentration of glutaraldehyde was found to be between 5 to 10% at 3 minutes of treatment time. Practically, the desired hardness and the activity recovery can be determined ac-according to the type of a reactor desired to use.

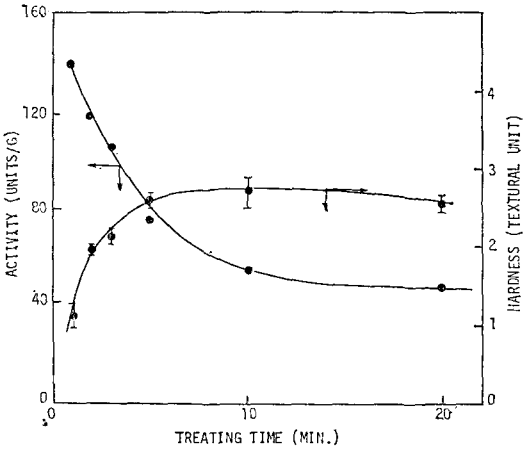

Fig. 2. Effect of Glutaraldehyde Concentration. 5g of wet pellets were treated with each glutaraldehyde solution for 3 min.

While the substantially high mechanical strength of the immobilized enzyme particles is often

required for a stirred tank reactor, the activity recovery can be increased with less hardness of the immobilized enzyme particle for the purpose of column reactor operation.

Characteristics of immobilized enzyme

The pH profile of the immobilized enzyme was broadened in wide range of pH while the optimum pH was shifted to 8.0 (Fig. 3). The result may be

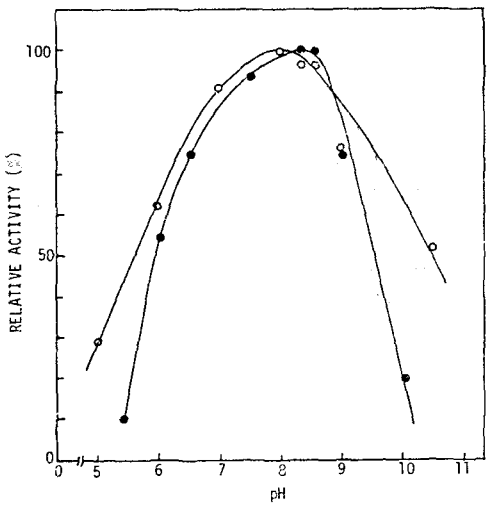

Fig. 3. pH Profile of Immobilized Enzyme Activity.
It was compared with that of soluble enzyme. Buffers used were; acetate buffer (0.1M) for pH 5.0, phosphate buffer (0.1M) for pH 6.0, and 7.0, Tris buffer (0.1M) for pH 8.0, 8.5, and 8.7, and bicarbonate buffer for pH 9.0 and 10.5. —●—, soluble enzyme; —○—, immobilized enzyme.

attributed to the resistance of mass transfer and the change of microenvironment in immobilized enzyme. As shown in Fig. 4, the optimum temperature was shifted to as high as 60°C and the marked increase in thermal stability was obvious after immobilization. The kinetic constants such as the Michaelis-Menten constant, Km, and the inhibition constants of both the products, Kia for phenylactic acid and Kip for 6-APA, were evaluated (Fig. 5). As compared with the values for the free enzyme[16], it was evident that there was substantial decrease in the apparent value of each kinetic constant after immobilization

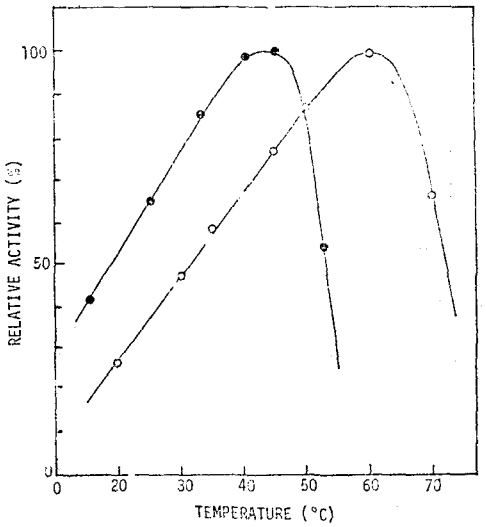

Fig. 4. Temperature Profile of Immobilized Enzyme Activity.
The immojilized enzymes were preincubated for 10 min at 20°C-50°C, and for 5min at 60-70°C. —●—, soluble enzyme;—○— immobilized enzyme.

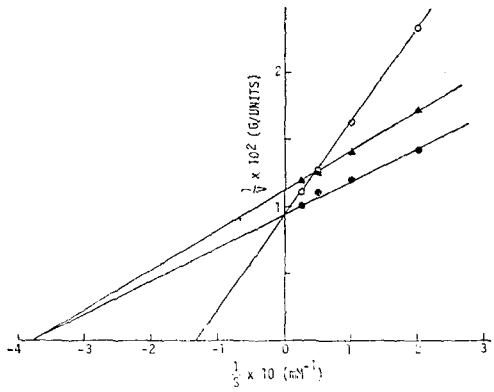

Fig. 5. Determination of Kinetic Constants of Immobilized Enzyme.
—●—, without any products; —▲—, with 2mM of 6-APA; —○—, with 40 mM of phenylacetic acid.

(Table I). This result is quite surprising, since classical diffusional limitation gives rise to an increase of the apparent kinetic constants after immobilization[17]. It suggests that another effects may also contribute to the apparent values of the kinetic parameters, which are composites of

Table I. Comparison of Kinetic Constants Between Free and Immobilized Enzyme

Kinetic constants	Immobilized enzyme(mM)	Free enzyme(mM)[14]
Km	2.6	4.5
Kia[a]	21.2	45
Kip[b]	7.4	26

a) Inhibitor constant for phenylacetic acid
b) Inhibitor constant for 6-APA

various rate constants involved in the reaction.

Stability and performance of plug flow reactor

While minimizing the pH drop in a column with a buffer solution (0.1M borate buffer, pH 8.0), the steady state conversion at different flow rates

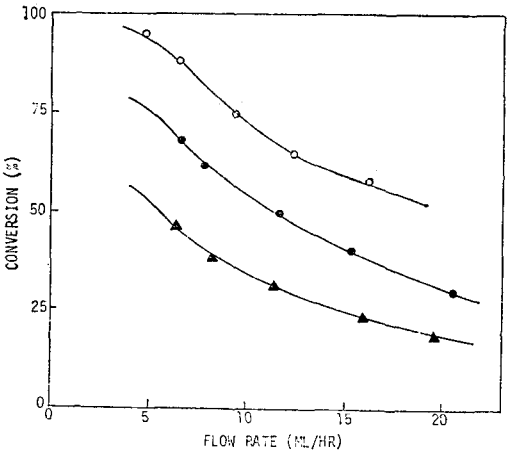

Fig. 6. Reactor Performance of Immobilized Penicillin Amidase Packed Column. 5.5g(wet wt) of enzyme pellets were introduced into a column (1.2 cm diameter ×5.5cm bed height). Substrate solutions in 0.1M borate buffer (pH 8.0) were continuousy fed to the column at 40°C Substrate concentration; —○—, 15mM; —●—, 30mM; —▲—, 50mM

was determined (Fig. 6). The enhancement of enzyme stability by entrappment and crosslinking was much pronounced during the plug flow reactor operation.

The physical integrity of the immobilized enzyme pellet without glutaraldehyde crosslinking was completely disrupted during the operation for a day. The loss of enzyme activity was also noted due to the enzyme leakage. During the operation for a day at 30°C and 5ml/hr of flow rate with 10mM substrate feed, the bed collapsed down to about half of the initial height and the enzyme leakage at the effluent amounted to 20% of the initial enzyme loading.

These operational difficulties were completely overcome by the entrappment and glutaraldehyde crosslinking. The physical integrity of the immobilized enzyme was retained during the operation over 30 days without supplying Ca^{++} salt. Furthermore, the enzyme leakage was successfully prevented since no enzyme activity was found in the effluent. The half life of the immobilized enzyme was estimated to be 30 days at 30°C and 6 days at 40°C as shown in Fig. 7.

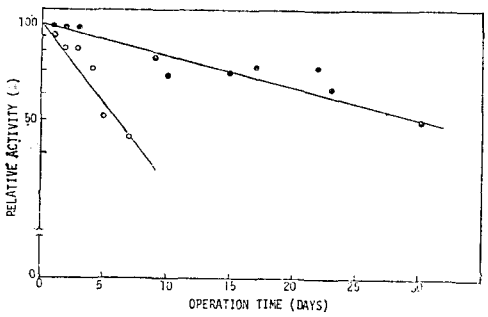

Fig. 7. Operational Stability of Immobilized Penicillin Amidase in a Plug Flow Reactor. Experimental conditions were same as described in Fig. 6. —●—, 30 C; —○— 40 C.

In a similar operating condition, the half life of the celite adsorbed enzyme without the entrappment was only 1 day at 40°C and 4 days at 30°C, respectively. By the reinforced alginate gel entrappment, the half life of the enzyme activity increased about 6 fold at 40°C and 7.5 fold at 30°C, respectively.

Conclusion

The physical and mechanical strength of Ca^{++} -alginate gel was reinforced by crosslinking with

glutaraldehyde in the presence of gelatin. The disruption of the physical form of alginate gel was successfully overcome by this treatment. In addition, various forms of immobilized enzyme such as pellets, noodle type, plate form and so on, was possible, which was difficult by using of Ca^{++} - alginate or gelatin only.

The half life of celite adsorbed penicillin amidase was greatly increased by the reinforced alginate gel entrapment, since the enzyme leakage during the operation was prevented. The results highly recommended the use of the reinforced Ca^{++} -alginate gel entrappment method for the enhancement of the operational stability of adsorbed enzymes.

요 약

부분적 항상성변이주인 *Bacillus megaterium* (KFCC 10029)가 생산하는 페니실린 아미다제를 예로하여 강화된 Ca^{++}-alginate gel에 의한 포괄 방법을 이용하는 효소 고정화 방법을 제시하였다. 발효액으로 부터 celite 흡착법에 의해 효소를 분리한 후 alginate의 gellatin 용액에 혼합하고 Ca^{++} 용액에서 응고시키고 glutaraldehyde로 처리하여 성형하였다. 이렇게 하여 얻은 고정화효소의 최적 pH 및 온도는 각각 8.0과 60°C였다. Km value와 6-APA 및 페닐초산에 의한 저해 상수는 각각 2.6mM, 7.4mM, 21.2mM이었다. Gel의 증가된 물리적 강도때문에 반응조 조작중 흡착효소의 유실을 성공적으로 없앨수 있었다. 관형식 반응조에서의 고정화 효소의 반감기는 40°C의 30°C에서 각각 6일 및 30일이었으며, 이것은 흡착효소의 비교해 볼때 6-8배의 증가치이다. 결론적으로 alginate gel 포괄방법에 의한 효소고정화 방법에 있어, 본 연구에서 개발된 개량된 방법을 사용함으로써 고정화효소의 물리적 강도 및 안정도를 크게 증가시킬 수 있었다.

References

1) K. Mosbach: Methods in Enzymology, Academic press, New York, Vol. 44 (Immobilized Enzymes) (1976)
2) O. R. Zaborasky: Immobilized Enzymes, CRC press, Cleveland, Ohio (1973)
3) K. Mosbach and P. O. Larsson: *Biotechnol. Bioeng.*, **12**, 19 (1970)
4) N. E. Franks: *Biotechnol. Bioeng. Symp.*, **3**, 327 (1972)
5) K. Toda and M. Shoda: *Biotechnol. Bioeng.*, **17**, 481 (1975)
6) K. Toda: *Biotechnol. Bioeng.*, **17**, 1729 (1975)
7) D. Dinelli: *Process Biochem.*, **7**, 9 (1969)
8) W. Marconi, F. Cecere, F. Morisi, G. Della Penna, and B. Rappuoli: *J. Antibiotics*, **26**, 4, 228 (1973)
9) Peter S. J. Cheetham, Kevin W. Blunt, and Christopher Burke: *Biotechnol. Bioeng.*, **21**, 2155 (1979)
10) B. H. Kim, B. L. Seong, T. I. Mheen, and M. H. Han: *Korean J. Appl. Microbiol. Bioeng.*, **9**, 29 (1981)
11) B. L. Seong, B. H. Kim, T. I. Mheen, and M. H. Han: *Korean J. Appl. Microbiol. Bioeng.*, **9**, 35 (1981)
12) M. H. Han, B. L. Seong, B. H. Kim, and T. I. Mheen: *Korean Biochem. J.* in press (1981)
13) D. Y. Ryu, C. F. Bruno, B. K. Lee, and K. Venkatasubramanian : Fermentation Technology Today (G. Terui, ed), Society of Fermentation Technology, Japan, p. 307 (1972)
14) H. J. Son, T. I. Mheen, B. L. Seong, and Moon H. Han: *J. Gen. Appl Microbiol.* Sumitted for publication.
15) K. Balasingham, D. Warburton, P. Dunnill, and M. D. Lilly: *Biochim. Biophys. Acta*, **276**, 250 (1972)
16) C. Chiang, and R. E. Bennett: *J. Bacteriol.*, **93**, 302 (1967)
17) D. Thomas, C. Bourdillon, G. Broun, and J. P. Kernevez: *Biochemistry*, **13**, 2995 (1974)