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Kinetic Study on the Immobilized Penicillin Amidase in a Differential Column Reactor

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Differential column reactor에 있어서 고정화페니실린 아미다제의 반응속도론에 관한 연구

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Abstract

The penicillin amidase from *Escherichia coli* (ATCC 9637) was immobilized by entrappment in gelatin and DEAE-cellulose mixture cross-linked with glutaraldehyde, and the kinetics in a differential column reactor was studied. The optimal operating condition of a differential reactor was reasonably met when the enzyme loading was 1g, and 30 mM substrate solution in 0.1M phosphate buffer (pH 8.0) was fed at flow rate 4ml/min and 40°C.

The optimal pH and temperature were found to be 8.0 and 55°C, respectively. The Michaelis-Menten constant was 4.8 mM while the maximum velocity was 308 units/g of the immobilized enzyme under the condition of the differential reactor. The effect of substrate inhibition disappeared in the immobilized enzyme preparation. The differential reactor was proved to be good for studying the true kinetics since the pH drop and the external diffusional resistance could be eliminated.

Introluction

Penicillin amidase (penicillin amidohydrolase, E. C. 3,5,1,11) hydrolyzes penicillin molecule to produce carboxylic acid and 6-aminopenicillanic acid (6-APA). There are different sources of this enzyme including some plant and animal tissues as well as several species of microorganisms such as *E. coli* and *B. megaterium*[1].

Much investigation has been carried out for

the immobilization of microbial penicillin amidase and the reactor performance of the respective immobilized enzyme.[2~13] Among these, penicillin amidase of *E. coli* is an intracellular enzyme, which has been used for the immobilization either after purification[2,3,6,7,10] or as a whole cell preparation.[9] During the course of studies on microbial penicillin amidase in our laboratory, the whole cell immobilized enzyme was also successfully prepared by entrapping *E. coli* cells in gelatin matrices crosslinked with glutaraldehyde.[13]

Although there are several studies on the reactor performance of the immobilized penicillin amidase,[6,7,9,13] it has been a difficult problem to conduct a basic kinetic study in a plug flow reactor system due to the changes in pH along the column path by the accumulation of the acidic reaction product, phenylacetic acid. The present paper deals with the evaluation of kinetic constants of the whole cell immobilized penicillin amidase from *E. coli* in a differential column reactor after the optimization of the operating condition of this reactor to minimize the pH effect and the external mass transfer effect.

Materials and Methods

Materials

The potassium salt of benzylpenicillin (1595 i.u./mg), 6-APA, and DEAE cellulose were purchased from Sigma Chemical Co. (U.S.A.). Glutaraldehyde was purchased from Aldrich Chemical Co. (U.S.A.). Other chemicals used in this research were of the Extra Pure Grade from Wako Chemical Co. (Japan).

Enzyme preparation and immobilization

The cuture of *E. coli* (ATCC 9637) was grown according to the method previously described by Kim *et al.* [12], and Seong *et al.* [13] using a 28*l* jar fermentor (New Brunswick, U.S.A.). The media consisted of tryptone 3.5%, yeast extract 0.5%, mono sodium-L-glutamate 1.5%, and phenylacetic acid 0.15% as an enzyme inducer. The pH was adjusted to 7.0 before sterilization. After about 24 hr of incubation at 30°C with agitation speed 400 rpm and aeration rate 0.5vvm, the culture broth was centrifuged at 3000 rpm for 30 min and the cells were harvested.

The immobilization of whole cell penicillin amidase was carried out according to the procedure described previously.[13] The whole cells were mixed with gelatin (40% solution in distilled water) and DEAE cellulose by the ratio of 10:1:3. The mixture was extruded by an extrusion apparatus and cut in an appropriate size as a cylindrical type, followed by crosslinking with glutaraldehyde (5% solution) for one minute. After washing, it was dried at room temperature overnight.

Determination of enzyme activity

The enzyme activity of the penicillin amidase preparation was determined by measuring the product (6-APA) formation, according to the method described by Balasingham *et al.*[3] The specific activity of the enzyme was defined as the equivalent of μmoles of the product formed per an hour under the assay condition.

Reactor operation

The hydrolysis of benzylpenicillin was carried out in a column reactor which consisted of a glass column (1.3cm×12cm) with a water-jacket that is connected to a thermostat for temperature control. Dried immobilized enzyme pellets were preincubated in distilled water at 40°C for swelling and degassed before packing in a column.

In order to eliminate any operational problems with flow pattern due to bubbles and the end effect at the inlet and outlet regions of a packed column, a supporter made of steel wire screen was installed inside the column. The substrate used was potassium benzylpenicillin in 0.1M phosphate buffer (pH 8.0) and was continuously fed to the reactor with a peristaltic pump (Model No. 7015, Cole Parmer, U.S.A.). The reaction was continuously monitored until the steady state was reached.

In order to obtain the true kinetics, it was intended to find the optimal condition for a differential reactor. The flow rate was adjusted as fast as possible in order to eliminate external mass transfer effect, and the conversion was maintained below 4%, where the condition for a

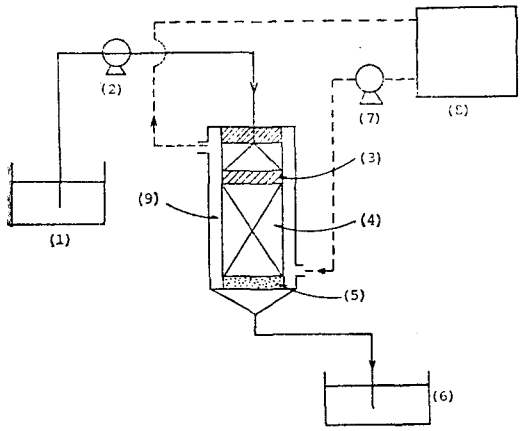

Fig. 1. The Diagramatic Scheme of Differential Column Reactor System.
1. substrate reservoir 2. peristalitic pump
3. steel wire screen 4. immobilized enzyme
5. sintered glass filter 6. product bottle
7. centrifugal pump 8. water bath
9. water jacket

differential reactor is assumed. A detailed design and arrangement of the reactor system is shown is Figure 1.

Determination of kinetic constants

In order to determine kinetic constants of the enzyme reaction, the following rate equation was considered. This rate equation is based on the ping-pong bi-bi mechanism with double inhibition by both reaction products and substrate as proposed by Lilly *et al.*[6]

$$v=\frac{V_m \cdot S}{S+K_m+\frac{S^2}{K_{ss}}+\frac{K_m}{K_{ip}}P+\frac{K_m}{K_{iq}}Q+\frac{K_m \cdot P \cdot Q}{K_{ip} \cdot K_{iq}}+\frac{S \cdot P}{K_{ip}}} \quad \cdots\cdots(1)$$

were S is the concentration of benzylpenicillin; P, phenylacetic acid; Q, 6-APA. Km is the Michaelis-Menten constant for S; Kaa, the substrate inhibition constant; Kip and Kiq, the inhibition constants for P and Q, respectively; and Vm, the maximum reaction velocity. Since we are dealing with a differential reactor system where the conversion is below 4%, the product formation can be considered negligible and one can neglect the terms involving products in the rate equation. The rate equation can thus be simplified as,

$$v=\frac{V_m \cdot S}{S+K_m+\frac{S^2}{K_{ss}}} \quad \cdots\cdots(2)$$

According to eq. 2, kinetic contants, Km and Vm were determined by either the double reciprocal plot of v and S or the direct linear plot proposed by Eisenthal *et al.*[14,15] The value of v was evaluated by determining the substrate conversion in a differential reactor conditioned as described aobve.

Results

Characteristics of immobilized penicillin amidase

The enzyme activity of whole cell was 0.59 units/mg dry cell weight. From 360g (wet weight) of whole cell, we obtained 166g (dry weight) of the immobilized enzyme and the activity of the immobilized enzyme preparation was 308 units/g dry weight of the immobilized enzyme. The recovery of the enzyme activity in the immobilization process was about 70%.

The pH-rate profile of the immobilized enzyme obtained by changing the pH of the substrate

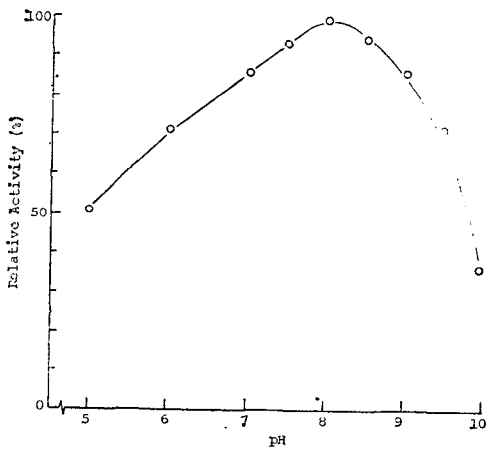

Fig. 2. pH-Rate Profile of the Immobilized Enzyme in a Differential Reactor.
Enzyme loading 1g, flow rate 2ml/min, temperature 40 C, substrate concentration 30mM. The progress of reaction is continuously monitored and the effect of pH was compared after the steady state is reached.

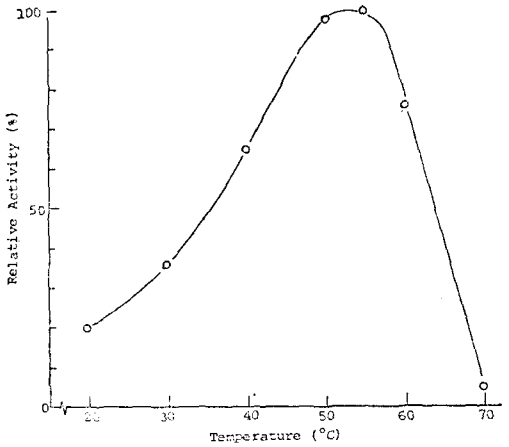

Fig. 3. Temperature-Rate Profile of the Immobilized Enzyme in a Differential Reactor. Enzyme loading 1g, flow rate 2ml/min, pH 8.0, substrate concentration 30mM. The steady state conversion is compared.

feed ranging from 5.0 to 10.0 is shown in Fig. 2. The optimal pH was demonstrated to be 8.0, and there was no significant change in activity in the pH range from 7.5 to 8.5. As shown in Fig. 3, the optimal temperature was found to be 55°C. Most of experiments, however, were carried out at 40°C to avoid the enzyme deactivation at high temperarature.

Optimization of differential reactor

One must choose a differential reactor in order to have the constant reaction rate throughout the reactor volume. Since the rates are dependent upon the substrate concentration and pH, the condition of a differential reactor is reasonably met for only small conversion through a shallow reactor bed volume. The portions of the substrate converted to 6-APA at different flow rates at a given substrate concentration are shown in Fig. 4. It shows that the conversion can be maintained below 4% when the flow rate is higher than 4ml/min with 1g of enzyme loading.

In order to find the optimal amount of enzyme loading, the conversion and the effluent pH at steady state were examined by varying the amount of enzyme loading from 0.4 to 2.0g at constant flow rate (4ml/min). As sholn in Fig. 5,

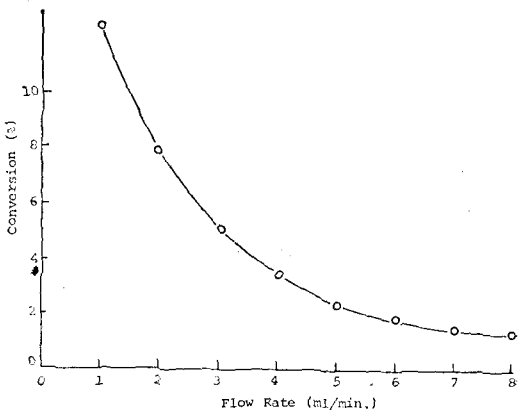

Fig. 4. The Effect of Flow Rate on the Conversion Efficiency. Enzyme loading 1g, pH 8.0, temperature 40 C, substrate concentration 30mM. The steady state was reached within 10 min. of operation at each pH, respectively.

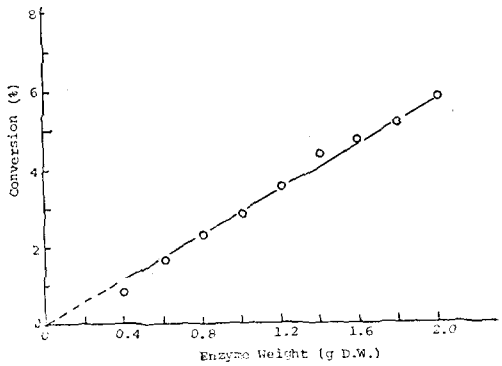

Fig. 5. The Effect of Enzyme Loading on the Conversion Efficiency. Flow rate 4ml/min, pH 8.0, temperature 40 C, substrate concentration 30mM

a good linear relationship between the enzyme loading and the conversion is demonstrated in the experimental range, although a slight decrease from the linearity is expected at higher enzyme loadings. It is readily seen that the conversion can be maintained below 4% in a given experimental condition within 1.4 g of enzyme loading. As shown in Fig. 6, the pH drop from the initial value 8.0, became larger as the flow rate decreases. However, the pH drop could be maintained within a very narrow range by employing a buffer with moderate strength. With 1g of enzyme loading,

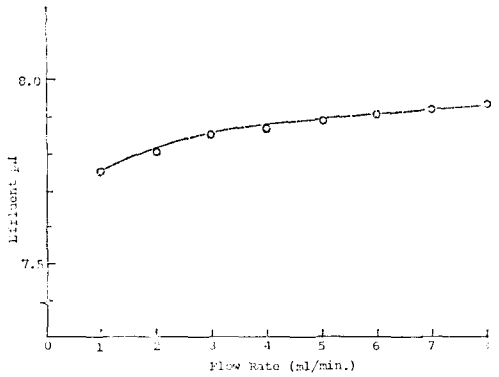

Fig. 6. The Effect of Flow Rate on the pH Drop. Initial pH of substrate 8.0 (0.1M phosphate buffer), enzyme loading 1g, temperature 40 C, substrate concentration 30mM.

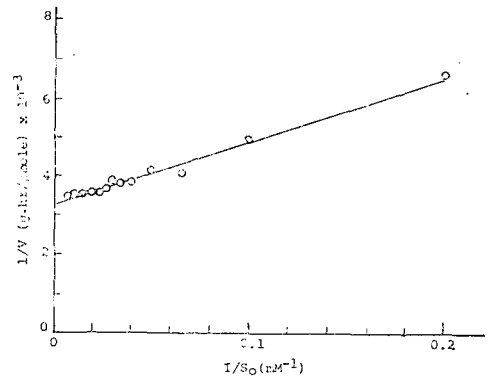

Fig. 7. Determination of Michaelis-Menten Constant by a Double Reciprocal Plot. The experimental conditions are the same as described in Table II except for the substrate concentration.

Table I. Optimal Condition for the Operation of Differential Reactor

Variables	Optimal condition
Initial pH	8.0 (0.1M phosphate buffer)
Enzyme loading	1g
Temperature	40 C
Substrate concentration	30mM
Flow rate	4ml /min

one could maintain the pH drop within the limit of 0.2 (final pH 7.8) and the conversion within the range of 4% at a flow rate above 2ml/min. Thus, the optimal condition for a differential reactor was found under a given set of experimental conditions as summarized in Table I.

Kinetic constants..

By a conventional double reciprocal plot (Fig.7), the values of K_m and V_m were calculated as 4.8mM and 308 units/g of the immobilized enzyme, respectively. These values were also confirmed by the direct linear plot (Fig. 8) proposed by Eisenthal *et al.*[14,15] From the latter plot, the values of K_m and V_m were calculated as 6.1mM and 313 units/g of the immobilized enzyme, respectively. No substrate inhibition at high substrate concentration was observed in the immobilized penicillin amidase system as shown in the double reciprocal plot.

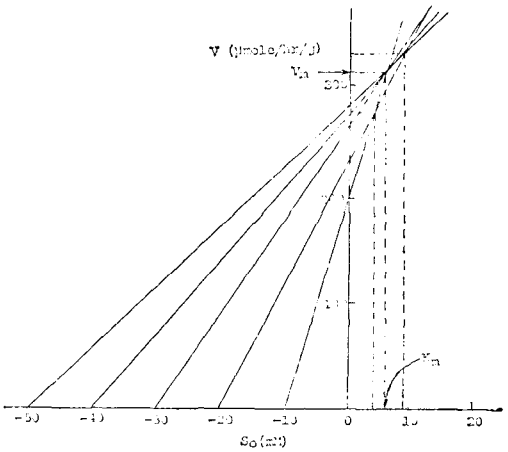

Fig. 8. Determination of Michaelis-Menten Constant by a Direct Linear Plot. Km and Vm values are evaluated with a mean value of the intercepts of each lines

Discussion

In a given set of experimental range, the optimal operating condition of a differential reactor for the immobilized penicillin amidase system was evaluated in terms of pH, temperature, substrate feed rate, and enzyme loading. The variation of kinetic constants involved in the rate equation with flow rate in a column reactor has been well noted, and explained as a consequence of the external diffusion effect. The attainment of optimal sets of the experimental condition fro

a differential reactor is necessary in order to obtain the true reaction kinetics for immobilized enzymes by eliminating the external diffusional resistance completely. The prerequisite for a differential reactor is to find the experimental range in which the environmental changes along the whole column path is reduced as negligible as possible. Variables that should be considered for the penicillin amidase system are pH, temperature, enzyme loading, feed rate, substrate concentration, and enzyme inactivation.

The use of 30mM substrate solution is well compromised since the concentration is 50 times as high as the numerical value of the Michaelis-Menten constant. This concentration is high enough to saturate the enzyme during the reaction. In a given temperature and pH (40°C and the initial pH 8.0), the conversion could be reduced below 4% when 1g of enzyme loading is employed and the flow rate is maintained at 4ml /min.

An important factor that should be considered for the penicillin amidase reaction is the pH drop in the reaction mixture due to the formation of acidic product, phenylacetic acid. Without pH adjustment and buffer solution, pH of the effluent decreases as low as 4.5. In order to prevent the decrease in pH along the column path, 0.1M phosphate buffer solution was used as described in the previous paper.[13]

It was noted that the effect of pH drop became more pronounced at low range of the flow rate. However, the pH drop could be reduced as low as 0.015 pH unit at 4ml/min of flow rate. As shown in the pH-rate profile curve, changes in the enzyme activity within this pH drop are 1 to 2% as compared to the maximum activity at pH 8.0. Therefore, the assumption of a differential flow reactor is well vindicated by the above experimental evidences. Another evidence may be drawn from the fact that the effect of pH and temperature was similar to the results obtained from the stirred tank.

Two types of graphical method, the direct linear plot and the conventional Lineweaver-Burk plot, were compared to evaluate the Michaelis-Menten constant Although the conventional Lineweaver-Burk plot has been conveniently used for routine determination of kinetic parameters, the statistical error involved in the plot cannot ve obviated. However, it was found that the K_m value obtained by the direct linear plot as the average mean value of the intercepts of each straight lines agreed well with that of a double reciprocal plot. This type of plot is considered as of potential use for its convenience and the validity on the statistical ground.

The disappearance of substrate inhibition by immobilization is an interesting feature. The reaction rate was not slowed down by the experimental range of high substrate concentration. This result may be ascribed to the characteristics of the immobilized enzyme. The amino acid residues responsible for the substrate binding site exerting substrate inhibition might be blocked or crosslinked with the gelatin matrix used in immobilization by the glutaraldehyde treatment. Another possibility is that the second substrate binding site may be blocked by the conformational change of the enzyme molecule due to the immobilization.

Quantitative studies on the kinetic properties of immobilized penicillin amidase in a plug flow reactor have not yet been made because of the formation of pH gradient along the column path. However, this operational difficulty was overcome in the present study by employing a substrate solution with moderate buffer strength, and by controlling the feed rate and the enzyme bed height. It is believed that the results presented here demonstrate its usefulness for the study of external diffusion effect on enzyme catalysis and the feasibility of a column reactor instead of a batch or a continuous flow reactor, where the immobilized enzyme pellet is liable to degradation by mechanical agitation. Operation under the recyclic condition with pH adjustment and the further improvement in designing such reactors are under current investigation.

Acknowledgment

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요 약

E. coil ATCC 9637의 균체를 젤라틴과 DEAE-cellulose의 혼합 성형 후 글루트알데히드 가교법으로 제조된 고정화 penicillin amidase의 differential column reactor에서의 반응속도를 논의하였다. 이러한 반응조의 최적 조작조건은 효소충진량 1g, 기질농도 30mM(0.1M 인산완충액, pH 8.0), 유출속도 4ml/min, 온도 40C이었다. 이 최적조건에서 고정화효소의 일반적인 성질을 조사하였다.

Km 상수는 4.8mM이었고 specific activity 308 units/g 고정화 효소이었다. 또한 고정화효소에서는 기질에 의한 효소반응 저해효과가 보이지 않았다. 이러한 differential column reactor에서는 column내에서의 pH 감소효과 및 외부 확산효과가 없어지기 때문에 이러한 외부적 영향을 받지않는 고정화효소의 반응속도론적 연구에 적합함을 알았다.

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