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AUTHOR(S):

HASHIMOTO, Kenji; ADACHI, Shuji; YOKOYAMA, Masaru; IMAKOMA, Hironobu; GELLF, Gerard; IKEDA, Takahiro

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Methods for Estimating Effective Diffusivity of Substrate and Kinetic Parameters of Immobilized Enzyme

By

Kenji HASHIMOTO*, Shuji ADACHI**, Masaru YOKOYAMA***,
Hironobu IMAKOMA*, Gerard GELLF****,
and Takahiro IKEDA*****

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Abstract

Two methods were presented for estimating simultaneously the kinetic parameters in the Michaelis-Menten equation, K_m and V_{max}, and the intraparticle effective diffusivity of substrate, D_{eA}, from the results of the transient changes in a batch reactor. The methods were applied to the estimation of the K_m and V_{max} values of α-chymotrypsin immobilized into firebrick particles or acrylamide gel, and the D_{eA} values of substrate through the supports. The experimental data of conversions both in the batch and tubular reactors were found to be calculated successfully by using the kinetic and transport parameters estimated by the proposed methods.

1. Introduction

Information on kinetic parameters of an immobilized enzyme and intraparticle effective diffusivity of substrate is required for a rational design of an immodilized-enzyme reactor. Kobayashi and Laidler[1] have proposed methods for estimating the kinetic parameters, K_m and V_{max}, of an immobilized enzyme. Their methods are limited to the case where the effective diffusivity of substrate, D_{eA}, is known. The D_{eA} value, however, is often unknown. Therefore, their methods require a separate determination of D_{eA}.

In this paper, we propose two new methods which can estimate simultaneously the K_m, V_{max}, and D_{eA} values from the transient changes of substrate concentrations observed in an isothermal batch reactor. One method uses particles with two different diameters. In the other method, the initial substrate concentration is varied. These

* Department of Chemical Engineering.
** Present adress: Niihama National College of Technology, Niihama.
*** Present adress: Matsushita Denko Co. Ltd., Kadoma.
**** Present adress: Université de Technologie de Compiègne, Compiègne, France.
***** Present adress: Sumitomo Naugatuck Co. Ltd., Niihama.

methods have been successfully applied for the estimation of the kinetic and transport parameters for hydrolysis of N-glutaryl-L-phenylalanine *p*-nitroanilide by α-chymotrypsin immobilized into crushed-firebrick particles or acrylamide gel. The kinetic parameters estimated were compared with those observed by using the immobilized-enzyme particles which were carefully crushed. The effective diffusivity estimated was also compared with those measured by usual physical methods.

The methods proposed in this article are available when an immobilized-enzyme reaction is in an intermediate range between reaction- and diffusion-controlling ranges. With use of an immobilized enzyme as an industrial catalyst, a large amount of enzyme may be, in many cases, immobilized to elongate the apparent half-life of the catalyst. The reaction catalyzed by the enzyme prepared under such conditions may be in the intermediate range. Since the reaction is in this range, it is important to estimate both the kinetic and transport parameters.

2. Methods for Estimating Kinetic and Transport Parameters

2.1. Rate expression at pseudo-steady state

Some investigators[2~5] have presented approximate expressions of the effectiveness factor for the Michaelis-Menten equation at a pseudo-steady state. These expressions are represented in terms of the generalized Thiele modulus, m, proposed by Bischoff[6], and the ratio of the Michaelis constant to substrate concentration ν. The generalized Thiele modulus is defined as

$$m=(\phi/\sqrt{2})\cdot\{1/(1+\nu)\}\cdot\{(1/\nu)-\ln(1+1/\nu)\}^{-1/2} \tag{1}$$

where

$$\nu=K_m/C_A \tag{2a}$$

$$\phi=(R/3)\{V_{max}/(K_m\cdot D_{eA})\}^{1/2} \tag{2b}$$

Kobayashi *et al*[2]. have presented the following approximate expression for the effectiveness factor.

$$E_{f\infty}=(E_0+aE_1)/(1+a)\quad(a=2.6\nu^{0.8}) \tag{3}$$

where

$$E_0=\begin{cases}1 & (m<1/\sqrt{3})\\ 1-[(1/2)+\cos\{(\psi+4\pi)/3\}]^3 & (m>1/\sqrt{3})\end{cases} \tag{4}$$

$$\psi=\cos^{-1}\{(2/3m^2)-1\} \tag{4a}$$

$$E_1=(1/m)\{1/\tanh(3m)-1/3m\} \tag{5}$$

Under the assumption of the pseudo-steady state, the appropriateness of which has been showed by us in a previous paper[7], a decrease in the substrate concentration in an isothermal batch reactor is expressed by the following equation.

$$-\frac{dC_A}{dt}=\frac{W}{V\rho_p}\cdot E_{f\infty}\cdot\frac{V_{max}\cdot C_A}{K_m+C_A} \tag{6}$$

An overall reaction rate r_{obs} is experimentally obtained by

$$r_{obs} = -(V\rho_P/W) \cdot (dC_A/dt) \tag{7}$$

and is also represented by

$$r_{obs} = E_{f\infty} \cdot V_{max} \cdot C_A/(K_m + C_A) \tag{8}$$

Rearangement of Eq. (8) gives the following equations:

$$E_{f\infty} \cdot C_A/r_{obs} = K_m/V_{max} + (1/V_{max})C_A \tag{9}$$

$$C_A/r_{obs} = (1/E_{f\infty}) \cdot \{K_m/V_{max} + (1/V_{max})C_A\} \tag{10}$$

2.2. Method I

The transient changes in substrate concentrations of bulk solutions are separately measured by using immobilized-enzymes with two different particle radii, R_1 and R_2, for the same substrate concentration. The curves are graphically differentiated at the same concentration for the two different particles to yield the values of dC_A/dt. The overall reaction rates, r_{obs1} and r_{obs2}, are calculated from the values of dC_A/dt by Eq. (7). Since $\nu_1 = \nu_2$, Eq. (1) gives the following equation.

$$m_1/m_2 = \phi_1/\phi_2 = R_1/R_2 \tag{11}$$

The ratio of $E_{f\infty 1}$ to $E_{f\infty 2}$ is given by the following equation from Eq. (8).

$$E_{f\infty 1}/E_{f\infty 2} = r_{obs1}/r_{obs2} \tag{12}$$

The kinetic parameters, K_m and V_{max}, and the effective diffusivity of the substrate, D_{eA}, are estimated by the following procedures: 1) Assume a plausible Michaelis constant K_m'. The K_m value of a free enzyme can be conveniently used as an initial guess. 2) Assume m_1 for each C_A. 3) Calculate m_2 by using Eq. (11), and evaluate $E_{f\infty 2}$ from Eq. (3). 4) Calculate $E_{f\infty 1}$ by substituting m_1 into Eq. (3) and obtain $E_{f\infty 2}'$ from Eq. (12). 5) Compare $E_{f\infty 2}$ with $E_{f\infty 2}'$. If the difference between them is not within a permissible error, assume m_1 again. 6) The calculations from 2) to 5) are repeated to evaluate the m_1, m_2, $E_{f\infty 1}$, and $E_{f\infty 2}$ values for the various C_A values. 7) Since the relationship between $E_{f\infty} \cdot C_A/r_{obs}$ and C_A has been given by Eq. (9), estimate the K_m and V_{max} values by using the least squares method. 8) Compare K_m with K_m'. If the difference between them is not within a permissible error, the K_m value is substituted into the K_m' value and the calculations from 2) to 7) are repeated until the difference becomes within the error. 9) Evaluate the D_{eA} values by using Eq. (1).

2.3. Method II

The transient changes in substrate concentrations of bulk solutions are measured for two different initial substrate concentrations by using immobilized-enzyme particles with the same diameter. The curves are graphically differentiated to yield the values of dC_A/dt at the same conversion, which gives the different substrate concentrations C_{A1} and C_{A2}. The differentiation is carried out at various conversions. The overall reaction rates r_{obs} are calculated from the values of dC_A/dt by using Eq. (7). The ratio of $E_{f\infty 1}$ to $E_{f\infty 2}$, where $E_{f\infty 1}$ and $E_{f\infty 2}$ are the effectiveness factor

at C_{A1} and C_{A2} respectively, is represented by the following equation from Eq. (8).

$$E_{f\infty 1}/E_{f\infty 2}=\{(1+\nu_1)/(1+\nu_2)\}\cdot(r_{obs1}/r_{obs2}) \tag{13}$$

Since the radius of the immobilized-enzyme particle is the same for both C_{A1} and C_{A2} and hence $\phi_1=\phi_2$, Eq. (1) gives the following equation.

$$\frac{m_1}{m_2}=\frac{1+\nu_2}{1+\nu_1}\cdot\frac{\{1/\nu_2-\ln(1+1/\nu_2)\}^{1/2}}{\{1/\nu_1-\ln(1+1/\nu_1)\}^{1/2}} \tag{14}$$

The kinetic and transport parameters are estimated by the following procedures: 1) Assume a plausible Michaelis constant K_m'. 2) Assume m_1 for any C_{A1}. 3) Calculate m_2 for C_{A2} corresponding to C_{A1} from Eq. (14), and evaluate $E_{f\infty 2}$ by using Eq. (3). 4) Calculate $E_{f\infty 1}$ by substituting m_1 into Eq. (3), and then obtain $E_{f\infty 2}'$ from Eq. (13). 5) Compare $E_{f\infty 2}$ with $E_{f\infty 2}'$. If the difference between them is over a permissible error, assume m_1 again. 6) The calculations from 2) to 5) are repeated to evaluate the m_1, m_2, $E_{f\infty 1}$, and $E_{f\infty 2}$ values for all pairs of C_{A1} and C_{A2}. 7) Estimate K_m and V_{max} by using Eq. (9). 8) Compare K_m and K_m'. If the difference between them is not within a permissible error, the K_m value is substituted into the K_m' value, and the calculations from 2) to 7) are repeated until the difference becomes smaller than the error. 9) Evaluate the D_{eA} value by using Eq. (1).

3. Experimentals

3.1. Materials

α-Chymotrypsin (EC 3. 4. 21. 1), bovine serum albumin, and N-glutaryl-L-phenylalanine *p*-nitroanilide (GPNA) were purchased from Sigma. Firebrick LBP-13 (Isoraito Kogyo) was crushed and sieved to the desired size. The particles were washed with 0.01 mol/*l* potassium phosphate buffer (pH 7.4) and with distilled water successively, and then dried. Acrylamide monomer and N, N'-methylenebisacrylamide were purchased from Nakarai Chemicals. Other chemicals were of an analytical grade.

3.2. Immobilization of α-chymotrypsin

Firebrick was crushed and sieved into particles with the various mean diameters (0.0505, 0.0775, 0.1090, 0.1545, and 0.2190 cm). α-Chymotrypsin was immobilized on the firebrick particles by a cross-linking method with an inactive bovine serum albumin according to Gellf and Boudrant[8].

The enzyme was also entrapped into acrylamide gel by the method presented previously[9]. The particle size was regulated by controlling the speed of the magnetic stirring in polymerization, and by sieving the resulting particles.

3.3. Batch reactor

Figure 1 shows a schematic flow sheet of the experimental apparatus and a batch reactor in detail. The firebrick particles (5g) into which α-chymotrypsin was immobilized were put into four stainless steel baskets placed near baffles. The

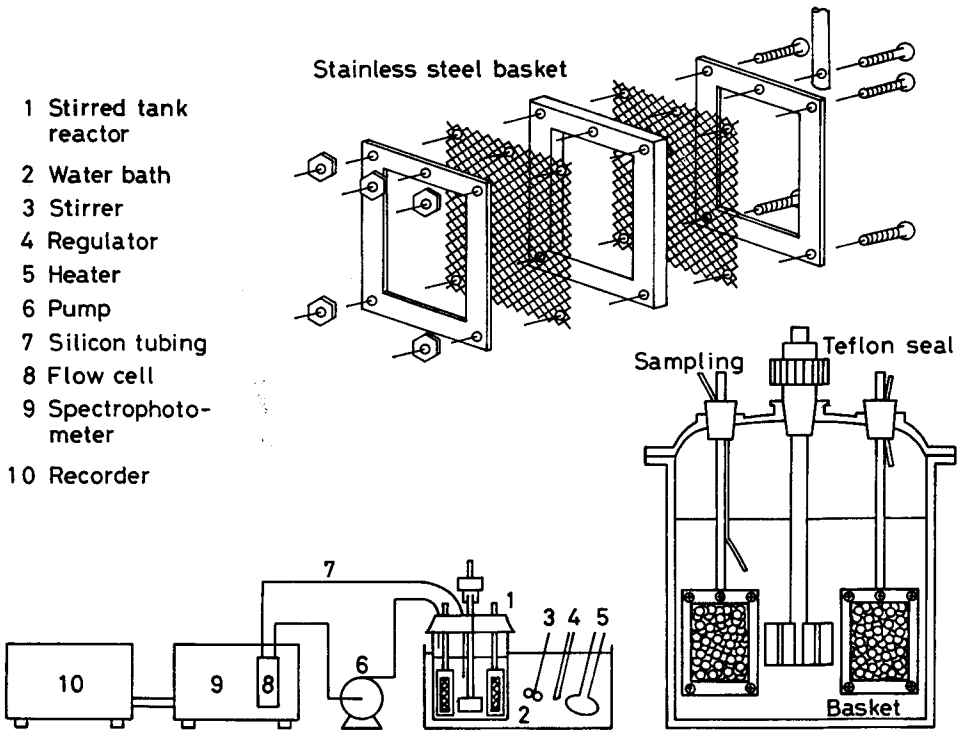

Fig. 1. Schematic diagram of apparatus and details of batch reactor and stainless steel basket.

particles were held between two stainless wire meshes. The reaction was allowed to run at pH 8.0 with 0.05 mol/*l* Tris-HCl buffer containing 2.0 mol/*l* NaCl and at 30°C. The volume of the substrate solution was 1000 cm³. The agitator with six paddles was revolved at 700~800 r.p.m. where the film mass transfer resistance was confirmed to be negligible. The initial concentration of GPNA, C_{A0}, was 0.88×10^{-4} or 3.52×10^{-4} mol/*l*. The concentration of the product, *p*-nitroaniline, was continuously monitored at 410 nm by passing the bulk solution through a spectrophotometer.

Some alterations of procedures were made in the experiments using α-chymotrypsin entrapped into acrylamide gel. The gels (0.7~3 g), held in one or two stainless steel baskets, were put into a GPNA solution of 275~700 cm³. The solution was agitated by a magnetic stirrer. The bulk solution was sampled at appropriate intervals and its absorbance at 410 nm was measured. The solution sampled was returned into the reaction vessel after a measurement of the absorbance.

3.4. Tubular reactor

The appropriateness of the kinetic and transport parameters estimated by the

proposed methods was verified by comparing the concentration profiles of GPNA observed experimentally in a tubular reactor with those calculated by using the parameters.

Figure 2 is a diagram of the experimental apparatus. The immobilized-enzyme firebrick particles (5 g) were packed in each column, which was kept at 30°C by circulating thermostatic water through the jacket. The inactive firebrick particles

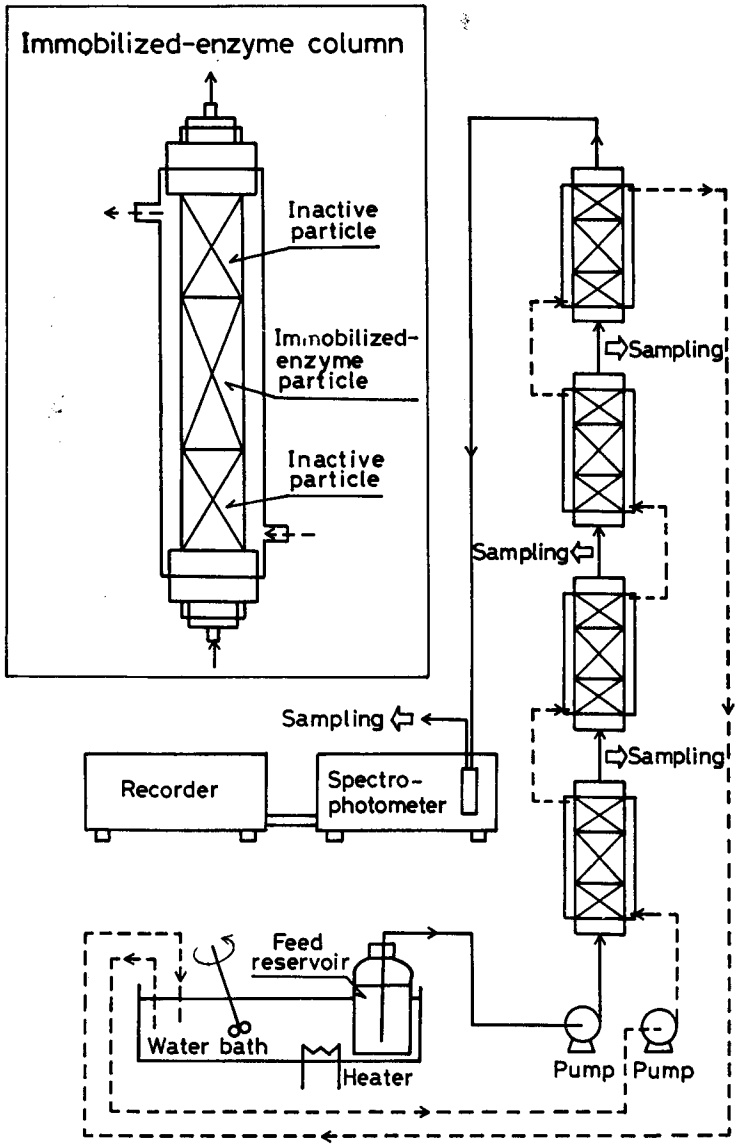

Fig. 2. Schematic diagram of tubular reactor.

with the same diameter as the active ones were packed above and below the active particles to preheat the substrate solution and to keep the flow pattern constant. The substrate solution was introduced upwards into the reactor by a constant-feeding-pump. The absorbance of effluent at 410 nm was continuously measured at the outlet of the last column. After the establishment of the steady state was confirmed, the concentration of the product sampled at the outlet of each column was measured.

The concentration profile of substrate in the tubular reactor was calculated by solving numerically Eq. (15) by the Runge-Kutta-Gill method.

$$\frac{dC_A}{dz} = -\frac{(1-\varepsilon_b)}{u_0} \cdot E_{f\infty}(C_A) \cdot \frac{V_{max} \cdot C_A}{K_m + C_A} \tag{15}$$

For the calculation of $E_{f\infty}$, the approximate expression proposed by Kobayashi *et al*[2]. was used.

3.5. Separate estimations of kinetic and transport parameters

The diameter of acrylamide gels can be reduced by grinding them carefully. Kinetic parameters free from intraparticle diffusional resistance were obtained by using granulated immobilized-enzyme.

The effective diffusivity of GPNA in acrylamide gel was observed by the following three methods. The first method is similar to that of Horowitz and Fenchel[10]. Beads soaked with GPNA were put into a buffer solution, and the increase of GPNA concentration in the buffer solution was measured. This method is called leakage one. In the second method, called penetration one, some fresh particles were poured into a GPNA solution, and the decrease of GPNA concentration in bulk solution was measured. The data were analyzed by the method of Carman and Haul[11]. For these two methods, inactive acrylamide gel beads with a large diameter (*ca.* 2 cm) were used. In the last method, the elution profile of the pulse input of a GPNA solution was analyzed by the moment method[9,12] to evaluate the effective diffusivity.

4. Results and Discussion

4.1. Parameters estimated by method I

Table 1 shows the kinetic and transport parameters estimated by method I for α-chymotrypsin immobilized onto firebrick particles. The values estimated may be reasonable because of the K_m value of the free enzyme (1.81×10^{-4}mol/l), of the molecular diffusivity of GPNA (in the order of 10^{-5}cm^2/s), of the tortuosity factor of the particle (in a range of 3-5), and of the porosity of the particle (about 0.5). Figure 3 illustrates a comparison of the experimental data with the curves calculated from Eq. (6) by using the parameters listed in the second row, which are near the average values. The calculated curves coincide well with the experimental results.

Table 1. Kinetic parameters of α-chymotrypsin immobilized onto firebrick particles and effective diffusivity of GPNA estimated by using method I.

$d_{p,ave}$ (cm)	$E_{f\infty,ave}$ (−)	$K_m \times 10^4$ (mol/l)	$V_{max} \times 10^6$ (mol/l·s)	$D_{eA} \times 10^{-6}$ (cm²/s)
0.0505 0.0775	0.945 0.873	1.12	3.33	2.18
0.0505 0.1095	0.915 0.679	1.74	3.90	2.13
0.0505 0.1545	0.932 0.601	2.93	4.93	3.43
0.0775 0.1545	0.899 0.601	0.50	2.70	2.18
0.1095 0.1545	0.870 0.765	2.49	3.55	6.43
Average		1.76	3.68	3.27

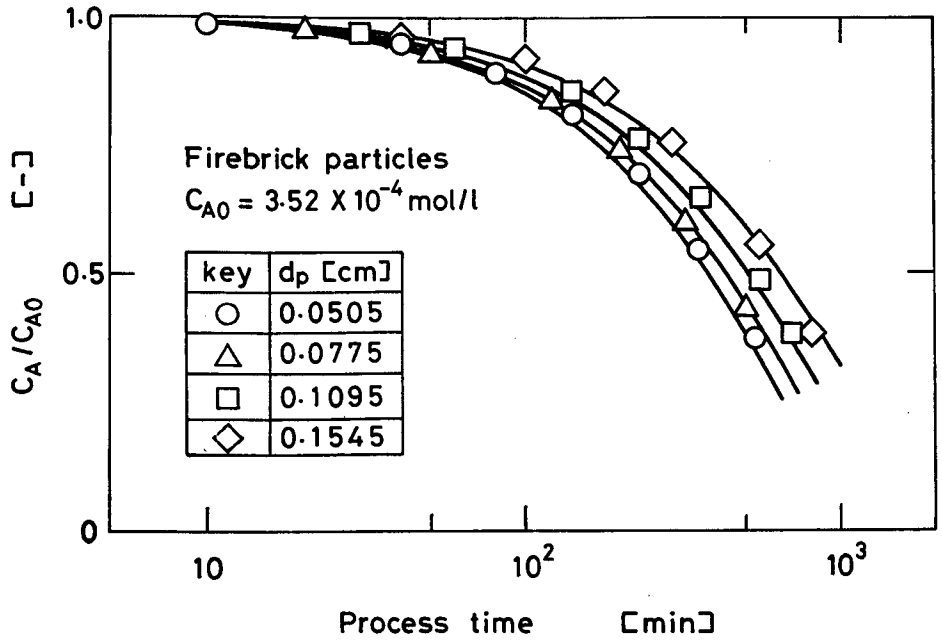

Fig. 3. Comparison of experimental and calculated transient changes of substrate concentrations for the parameters estimated by method I.

4.2. Parameters estimated by method II

The kinetic and transport parameters estimated by method II for α-chymotrypsin immobilized onto firebrick particles are listed in Table 2. Figure 4 illustrates the

Table 2. Kinetic parameters of α-chymotrypsin immobilized onto firebrick particles and effective diffusivity of GPNA estimated by using method II.

$d_{p,ave}$ (cm)	$K_m \times 10^4$ (mol/l)	$V_{max} \times 10^6$ (mol/l·s)	$D_{eA} \times 10^6$ (cm^2/s)
0.0505	2.91	4.87	7.07
0.0775	3.62	5.90	6.42
0.1095	0.98	3.28	4.44
Average	2.50	4.79	6.14

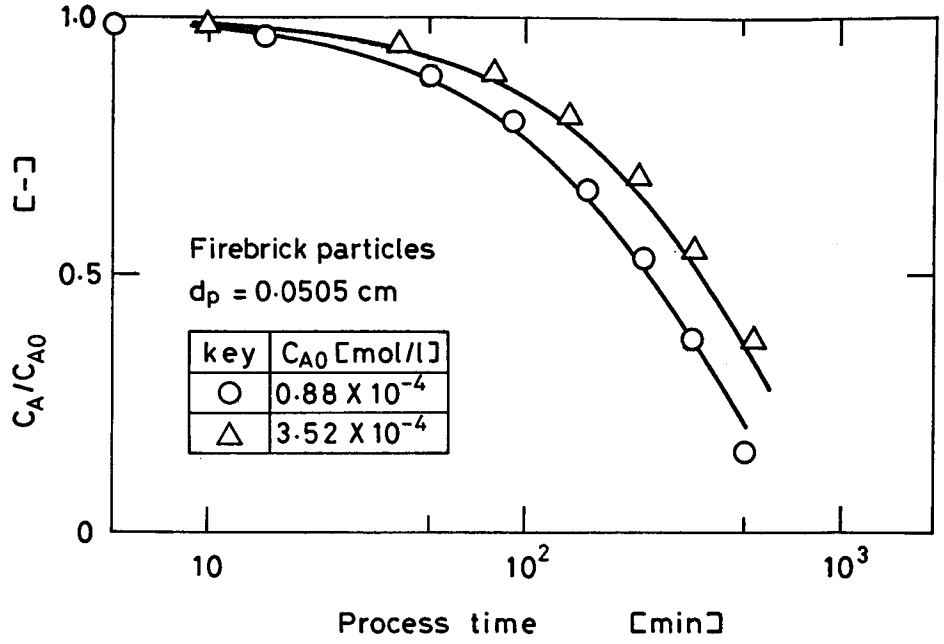

Fig. 4. Comparison of the experimental results with the curves calculated by using the parameters estimated by method II.

transient changes of the substrate concentrations. The curves were calculated by using the parameters estimated for particles with diameters of 0.0505 cm.

Table 3 shows the values of the parameters estimated by method II for the enzyme entrapped into acrylamide gel. In this table, the values in parentheses were obtained by using the immobilized enzyme granulated to reduce the diameter. The effective diffusivities estimated by the three different methods are also listed in the table. Figure 5 shows the transient change in conversion, which was observed in run 5 of Table 3. The initial concentration of the substrate was 7.57×10^{-5}mol/l,

Table 3. Kinetic parameters of acrylamide gel entrapped α-chymotrypsin and effective diffusivity of GPNA estimated by using method II and those by using granulated gel and physical methods.

Run no.	$K_m \times 10^4$ (mol/l)	$V_{max} \times 10^6$ (mol/l·s)	$D_{eA} \times 10^6$ (cm²/s)
1*	3.86	2.89	3.54
2	4.23	6.45	5.82
3	3.42 (3.29)	2.27 (4.20)	7.15 —
4	1.76 (2.20)	2.34 (6.40)	14.4 —
5	3.23 (3.70)	3.77 (5.49)	4.38 —
Leakage method			0.82
Penetration method			1.90
Moment method			2.48
Average	3.30	4.12	7.06 (2.60)

* The amount of enzyme entrapped was a half of that in other runs.

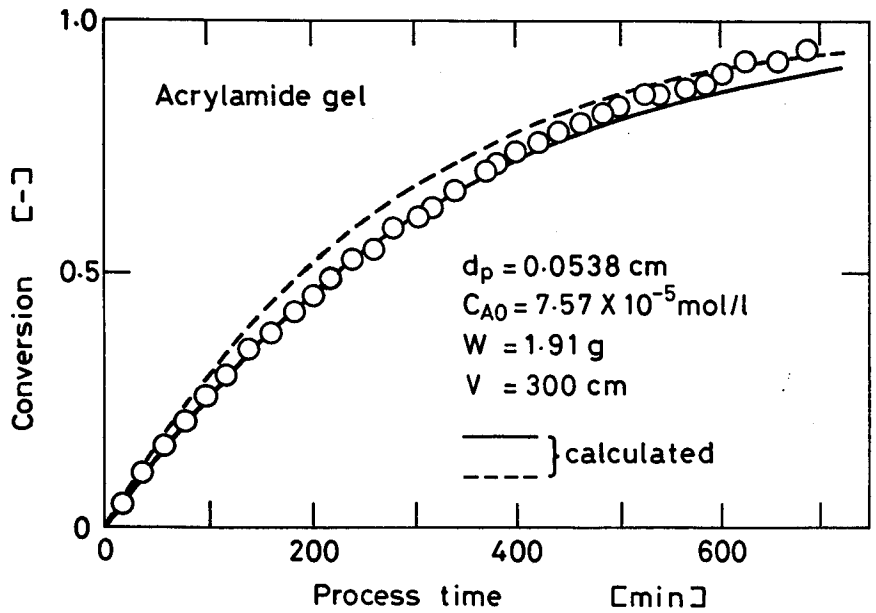

Fig. 5. Comparison of the experimental data with the calculated curves. The solid curve was calculated by using the parameters estimated by the proposed method II, and the broken one by the parameters estimated separately.

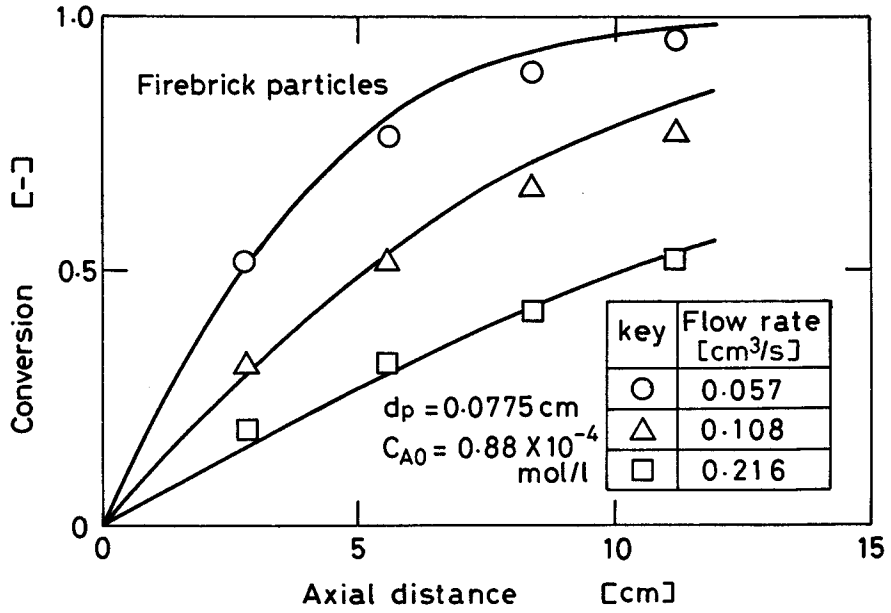

Fig. 6. Comparison of concentration profiles in tubular reactor with those calculated by using the parameters estimated by method I.

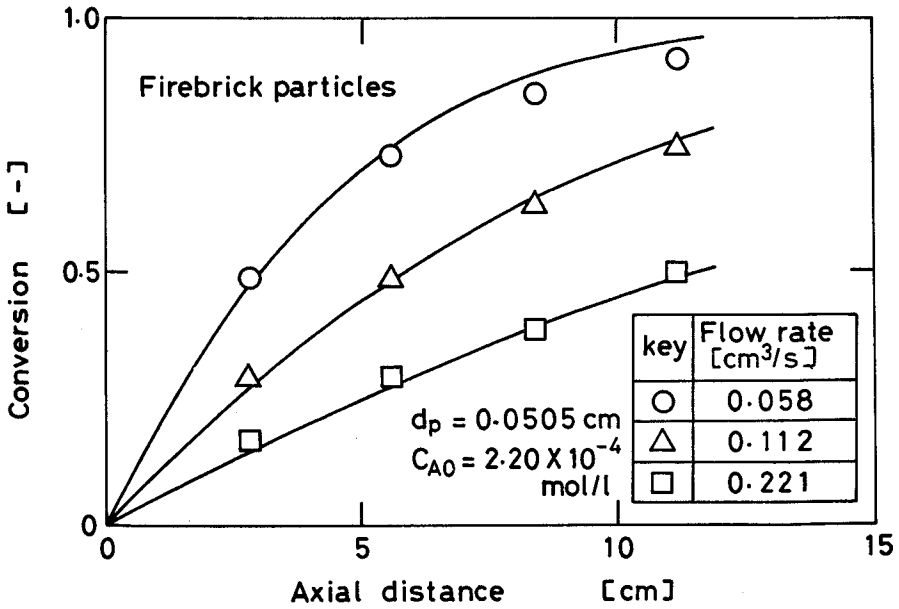

Fig. 7. Comparison of experimentally obtained profiles in tubular reactor with those calculated by using the parameters estimated by method II.

and the mean diameter of the particles was 0.0538 cm. The solid curve is calculated by using the parameters estimated by method II. On the other hand, the broken curve is obtained by using the kinetic parameters shown in parentheses and the effective diffusivity evaluated by the moment method. The solid curve coincides better with the experimental results than the broken curve. This may mean that the proposed method can evaluate reasonably the parameters.

4.3. Concentration profiles in a tubular reactor

Figures 6 and 7 show the concentration profiles for various flow rates in a tubular reactor packed with α-chymotrypsin immobilized onto firebrick particles. The curves in Figs. 6 and 7 were calculated by using the kinetic and transport parameters estimated by methods I and II, respectively. The calculated curves coincide well with the experimental results. This also indicates that the parameters, estimated by the proposed methods using an isothermal batch reactor, can be successfully utilized for designing a tubular immobilized-enzyme reactor.

4.4. Applicable range of the proposed methods

The two methods proposed here can estimate conveniently the kinetic parameters and the effective diffusivity of substrates by using the experimental data observed in a batch reactor. The methods, however, possess some limitations. Neither methods I nor II can be utilized when the reaction catalyzed by an immobilized enzyme is within the reaction- and diffusion-control regions. The methods are

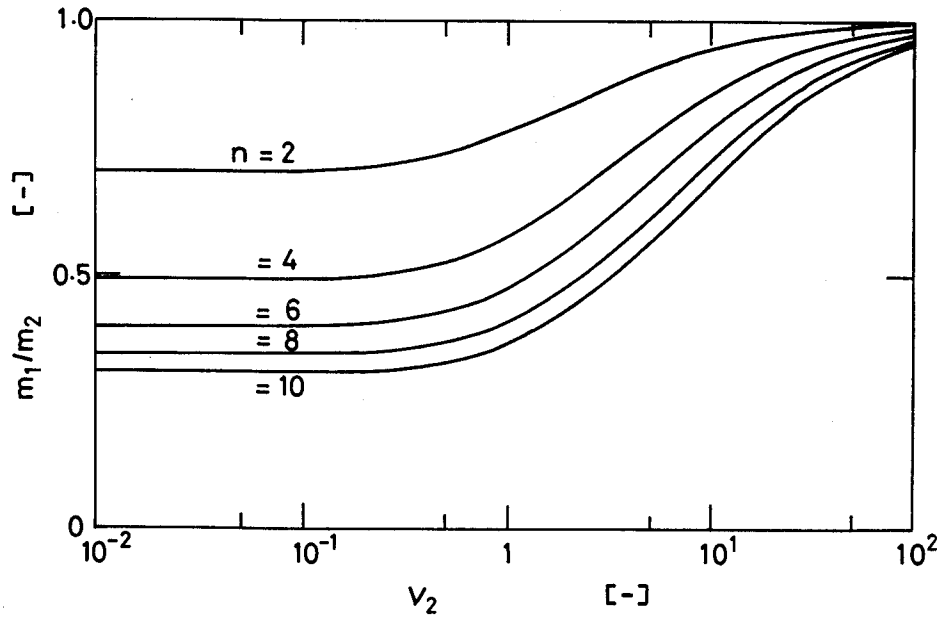

Fig. 8. Relationship between ν_2 and m_1/m_2 when $C_{A1}=n \cdot C_{A2}$ $(n>1)$.

effectively used in an intermediate range between reaction- and diffusion-controlling ranges. Method I becomes inapplicable when the radii of two particles are close to each other, and the ratio of the dC_A/dt value for R_1 to that for R_2 falls within the margin of error of a graphical differentiation. In method II, the initial substrate concentrations must be changed within a certain range. When C_{A1} and C_{A2} are chosen as two different substrate concentrations, and $C_{A1}=n \cdot C_{A2}$ ($n>1$), the relation between $\nu_2=(K_m/C_{A2})$ and m_1/m_2 is obtained from Eq. (14) as shown in Fig. 8. When the reaction is approximated to be of the first-order, that is $C_{A2} \ll K_m$, m_1/m_2 approaches 1.0. When $C_{A2} \gg K_m$, $E_{f\infty 1}/E_{f\infty 2}$ approaches 1.0. Neither method is available in such a region. Therefore, appropriate substrate concentrations must be selected to use the methods. The K_m value for the free enzyme, which is easily obtained, may offer a suggestion in the selection.

5. Conclusion

Two methods were proposed for estimating simultaneously the kinetic parameters, K_m and V_{max}, of an immobilized enzyme and the effective diffusivity of the substrate, D_{eA}, from the transient changes of substrate concentrations in an isothermal batch reactor. Both methods were applied for the estimation of the kinetic and transport parameters for the reaction catalyzed by α-chymotrypsin immobilized into crushed firebrick particles or acrylamide gel.

The transient changes of substrate concentrations calculated by using the parameters estimated showed good agreement with the experimental results. The concentration profiles in a tubular reactor were also correctly predicted by using the estimated parameters. These results indicate that the methods proposed here are very useful for estimating simultaneously the kinetic and transport parameters.

Nomenclature

C_A : concentration of substrate in bulk solution	(mol/cm^3)
D_{eA} : effective diffusivity of substrate	(cm^2/s)
d_P : diameter of the particle	(cm)
$E_{f\infty}$: steady state effectiveness factor	(—)
K_m : Michaelis constant	(mol/cm^3)
m : generalized Thiele modulus	(—)
R : radius of the particle	(cm)
r_{obs} : overall reaction rate	(mol/cm^3·s)
t : time	(s)
u_0 : superficial velocity	(cm/s)
V : volume of the solution	(cm^3)

V_{max} : maximum reaction rate (mol/cm³/s)
W : weight of the particles (g)
z : axial distance (cm)
ε_b : void volume of the bed (—)
$\nu = K_m/C_A$ (—)
ρ_P : apparent density (g/cm³)
$\phi = (R/3)\{V_{max}/(K_m \cdot D_{eA})\}^{1/2}$ (—)

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