PURIFICATION OF TYROSYL-tRNA SYNTHETASE BY AFFINITY CHROMATOGRAPHY

EIZO SADA, SHIGEO KATOH AND TSUNEO INOUE
Department of Chemical Engineering, Kyoto University, Kyoto 606
KAZUO MATSUKURA, MASAMI SHIOZAWA AND AKIRA TAKEDA
Research and Development Center, Unitika Ltd., Uji 611

Key Words: Adsorption, Affinity Chromatography, Biochemical Engineering, Chromatography, Elution Curve, Enzyme, Ionic Strength, Mass Transfer, Purification, Tyrosyl-tRNA Synthetase

Stable tyrosyl-tRNA synthetase (EC 6.1.1.1) was isolated from thermophilic bacterium *Bacillus stearothermophilus* and purified by use of two consecutive affinity chromatographic procedures on a large scale. The specific activity increased 25,900-fold after the purification. With a Mātrex Blue A column, used in the final-step affinity chromatography, the effects of factors influencing the performance of chromatography were studied. The column diameter was scaled up from 1.65 cm to 18.4 cm without appreciable change in the elution profiles. The degree of purification decreased gradually and the elution profile became sharper with the increase in concentration of potassium chloride from 0.1 to 0.6 mol·l^{-1}. Variation of the elution profile with liquid velocity was also studied. The experimental elution curves showed general agreement with the calculated values based on the mass transfer theory.

Introduction

Affinity chromatography, which depends on the specific interactions between pairs of biological materials, has become a widespread method for purification of a number of biological materials. However, affinity media are expensive and have relatively short lives, because biological ligands are usually used.

Recently, affinity media utilizing various kinds of dye as cheap and stable synthetic ligands have widely been used, especially in industrial purification, due mainly to economic reasons. The dye ligand has affinity for a group of materials that have common properties. Such ligands are thus called group-specific ligands. A suitable adsorption and elution condition has to be chosen for the isolation and purification of a targeted material by use of an affinity medium with dye ligand.

In the present work, stable tyrosyl-tRNA synthetase (EC 6.1.1.1) from thermophilic bacterium *Bacillus stearothermophilus* was isolated and purified with two consecutive group-specific affinity chromatographies after proper pretreatment. The effects of operating conditions such as the column scale, the ionic strength of the eluent and the liquid velocity on the performance of affinity chromatography were studied by use of the final-step affinity medium, Mātrex Blue A.

Tyrosyl-tRNA synthetase possesses a high degree of specificity for tyrosine and can discriminate among tRNA molecules of very similar structures. Tyrosyl-tRNA synthetase catalyzes the following reaction *in vivo*:

$$\text{tyrosine} + \text{ATP} + \text{tRNA} \longrightarrow \text{tyrosyl}-\text{tRNA} + \text{AMP} + \text{PPi}$$

This enzyme has a specificity for its substrate and can be used for synthesis of oligopeptides *in vitro*.[5] Therefore, a purification technique of it on an industrial scale is significant, though the purification on a laboratory scale has already been presented.[5] Since aminoacyl-tRNA synthetases generally are unstable and liable to be inactivated, it is preferable to obtain the enzymes from thermophilic bacteria. These are expected to have good storage stabilities for practical uses.

1. Experimental

1.1 Purification procedure

Bacillus stearothermophilus UK 788 strain, isolated at Unitika Ltd., was cultivated continuously in a low-cost medium with ammonium sulfate as a nitrogen source. Cells of 230 kg wet wt. (ca. 46 kg dry wt.) were harvested from the culture medium by centrifugation. As tyrosyl-tRNA synthetase is an intracellular enzyme, cakes of cells were first frozen below 273 K, broken into pieces, and then thrown into an appropriately prepared potassium phosphate buffer solution at room temperature. Thus the cell walls were broken

Received March 31, 1984. Correspondence concerning this article should be addressed to E. Sada.

by thermal shock. After removing cell debris and nucleic acids in the homogenate (800 l), salts and low molecular materials, which may have a bad effect in the following column chromatographies, were removed by gel chromatography with Cellulofine GH-25-m (Chisso Corporation). The bed height was ca. 100 cm. Volume of the sample applied was ca. 25% of the bed volume, and superficial liquid velocity was ca. 100 cm·h^{-1}. The eluate was loaded onto a DEAE-Cellulose 32E (Whatman) column of ca. 60 cm i.d. Volume of the sample applied was ca. 3 times the bed volume. The column was eluted with a linearly increasing gradient of KCl (from 0 to 0.3 mol·l^{-1}) in the potassium phosphate buffer solution (pH 7.5) and the eluate containing tyrosyl-tRNA synthetase was collected. Other valuable enzymes were obtained separately in this step. The phosphate buffer of the tyrosyl-tRNA synthetase fraction was exchanged to a Tris-HCl buffer (50 mmol·l^{-1} Tris-HCl, 2 mmol·l^{-1} EDTA, 10 mmol·l^{-1} 2-mercaptoethanol, pH 7.5) with the Cellulofine GH-25-m column. Then the protein fraction was applied to a column of cellulose phosphate P11 (Whatman). Cellulose phosphate is used not only as an ion exchange chromatographic medium but also often as an affinity medium for enzymes that have specific affinity for the phosphate group. In the final purification step the tyrosyl-tRNA synthetase fraction was loaded onto a Mātrex Blue A (Amicon Corporation) column and eluted with a step of KCl in the Tris-HCl buffer. Hollow-fiber sets (H1P10, Amicon Corporation) were used for concentration or dialysis needed after each purification step. For example, the eluate from DEAE-Cellulose 32 E was concentrated to an optical density of ca. 40 at 280 nm. The volume of the solution decreased to ca. one-ninth of the eluate. As the protein concentration grew higher, performance of the hollow-fiber sets decreased because of the high viscosity of the solution. A column of GAJ16×15 (1.65 cm i.d., Amicon Corporation) was used to investigate the effects of operating conditions on the performance of affinity chromatography. Other, larger columns were designed to obtain uniform distribution of liquid flow within them. The chemicals used for the purification were of reagent grade. Temperature was kept at 278–280 K during the purification.

1.2 Enzyme assay

The activity of tyrosyl-tRNA synthetase was assayed by measuring the rate of tyrosyl-tRNA formation at 303 K by use of ^{14}C-labeled L-tyrosine (1.82×10^{13} Bq·mol^{-1}, New England Nuclear). A tyrosine solution (mixture of the 203 μmol·l^{-1} ^{14}C-tyrosine (0.25 cm^3) and 50 μmol·l^{-1} L-tyrosine (10 cm^3)); a buffer solution, (200 mmol·l^{-1} HEPES (pH 7.9), 80 mmol·l^{-1} KCl, 50 mmol·$^{-1}$ $MgCl_2$, 20 mmol·l^{-1} 2-mercaptoethanol, 25 mmol·l^{-1} ATP); and a tRNA solution (1100 OD_{260} units, *B. stearothermophilus* UK 788 strain origin) were prepared. A mixture of the tyrosine solution (0.01 cm^3), the buffer solution (0.015 cm^3), the tRNA solution (0.01 cm^3) and distilled water (0.04 cm^3) was preincubated for 10 min at 303 K and 0.002 cm^3 of appropriately diluted tyrosyl-tRNA synthetase sample was added. The mixture was incubated for 4 min at the same temperature. The mixture of 0.05 cm^3 was sampled and applied to a GF/C filter paper (Whatman). The paper was washed in a 0.2 mol·l^{-1} of HCl three times to remove unreacted tyrosine, dried, immersed in a liquid scintillator (Econofluor, New England Nuclear) and counted in a scintillation counter (LSC-700, Aloka). One unit of tyrosyl-tRNA synthetase is defined as the amount of the enzyme that catalyzes the formation of 1 nmol of tyrosyl-tRNA in 10 min at 303 K. The concentration of protein was calculated from the absorbance of the solution at 280 nm on the assumption that the optical factor for protein is 1.0.

2. Results and Discussion

2.1 Large-scale purification of tyrosyl-tRNA synthetase

Table 1 shows the results of large-scale purification of tyrosyl-tRNA synthetase. Although similar purification on a laboratory scale has already been reported,[5] in the present work the operating conditions, specifications and related instruments were changed for large-scale operation to obtain a final purity as high as that on the laboratory scale. The increase in specific activity during the desalting by gel chromatography might be due to the elimination of some low-molecular weight inhibitor(s), because after this step the total activity of tyrosyl-tRNA synthetase also increased 81-fold.

2.2 Effects of operating conditions on affinity chromatography with Mātrex Blue A

1) Adsorption capacity The adsorption capacity of Mātrex Blue A was approximated by an isotherm of the Freundlich type,

$$\rho_b\bar{q}=KC^{\beta} \tag{1}$$

Here, the coefficient K and the exponent β were affected by the ionic strength of the solution and were approximately expressed by the following equations:

$$K=10^{2.54-7.62I},\quad \beta=1.38I+0.48 \quad \text{for } I\leqq0.38 \tag{2}$$

$$K=0.46,\quad \beta=1.0 \quad \text{for } I>0.38 \tag{3}$$

2) Volumetric coefficients of mass transfer In the present work, the height of adsorbent bed and the superficial liquid velocity sufficiently satisfied the conditions required for the constant pattern approximation.[2] In this case, the following equation is

Table 1. Large-scale purification

Step	Specific activity [units·mg^{-1}]	Purification [—]
Crude extract	0.4	1
Desalting	35	88
DEAE-Cellulose	128	320
Cellulose phosphate	1640	4100
Mātrex Blue A	10,350	25,900

derived:

$$\bar{q}=(\bar{q}_0/C_0)C \tag{4}$$

Using the linear driving-force relation, the mass transfer rate can be expressed as follows[3,8]:

$$\rho_b\frac{\partial\bar{q}}{\partial t}=K_f a(C-C^*) \tag{5}$$

Equation (5) can be integrated by use of Eq. (4), and, if the isotherm is of the Freundlich type, the integration can be conducted analytically[1]:

$$t_E-t_B=\frac{\rho_b\bar{q}_0}{K_f a C_0}\left(\ln\frac{X_E}{X_B}+\frac{\beta}{1-\beta}\ln\frac{1-X_B^{(1-\beta)/\beta}}{1-X_E^{(1-\beta)/\beta}}\right) \tag{6}$$

Figure 1 shows a breakthrough curve at a superficial liquid velocity of 11.1 cm·h^{-1}. From this curve, $\overline{K_f a}$, the averaged volumetric coefficient of overall mass transfer, was calculated between $X_B=0.1$ and $X_E=0.9$ by use of Eq. (6) and the value of 0.005 s^{-1} was obtained. From the material balance on a volume element of differential height within an adsorbent bed, the following equation is derived[3,8]:

$$u\frac{\partial C}{\partial z}+\varepsilon_b\frac{\partial C}{\partial t}+\rho_b\frac{\partial\bar{q}}{\partial t}-D_z\frac{\partial^2 C}{\partial z^2}=0 \tag{7}$$

The solid line in Fig. 1 shows the profile of the breakthrough curve calculated numerically from Eqs. (1), (5) and (7) by use of $\overline{K_f a}=0.005\,\mathrm{s}^{-1}$ and $\rho_b=0.75\,\mathrm{g\cdot cm^{-3}}$, and $\varepsilon_b=0.253$ and $D_z=3\times10^{-5}$ cm^2·s^{-1} determined by the impulse-response method[6,9] utilizing KCl. The calculation was performed by dividing the column height into 40 and with a time interval of 0.5 s. The profile shows good agreement with the experimental points.

3) Effect of column size **Figure 2** shows the elution profiles for the columns of 1.65 and 18.4 cm i.d. The abscissa indicates the time from the start of supply of the enzyme solution on the column. The height of the adsorbent bed was 8 cm in both columns. Though there were slight differences in operating conditions between these two columns, the elution curves show similar profiles. It is possible to scale up the column diameter to 18.4 cm i.d. in affinity chromatography provided the column is carefully designed and manufactured to obtain a uniform flow distribution over the cross-sectional area of the column. If elution of

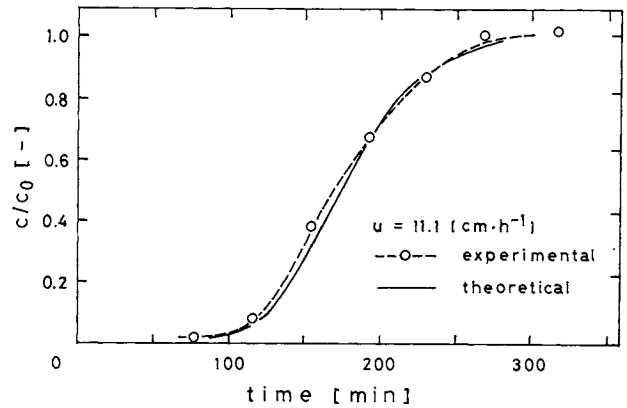

Fig. 1. Breakthrough curve. 50 mmol·l^{-1} KCl in Tris-HCl buffer.

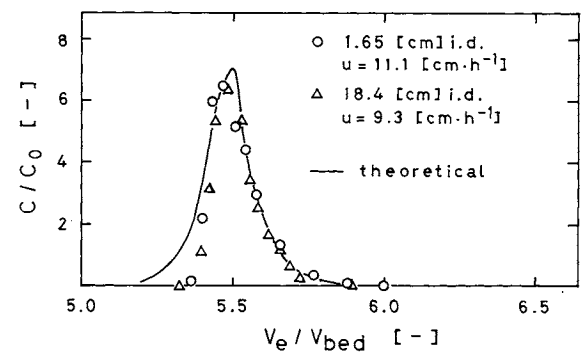

Fig. 2. Effect of column size. Charged samples: 1082 units·cm$^{-3}\times25\,\mathrm{cm^3}$ for 1.65 cm i.d. column, 1006 units·cm$^{-3}\times3140\,\mathrm{cm^3}$ for 18.4 cm i.d. column. Eluent: 0.3 mol·l^{-1} KCl in Tris-HCl buffer. Solid line: calculated for 1.65 cm i.d. column.

tyrosyl-tRNA synthetase from Mātrex Blue A is carried out with an eluent of high ionic strength, elution of impurities that have higher affinities for the medium results in a lower purity of the eluate. So in the present work, tyrosyl-tRNA synthetase was eluted with an eluent of a comparatively low ionic strength of 0.35 (0.3 mol·l^{-1} KCl in the Tris-HCl buffer). To calculate the elution curve, the following equations concerning KCl were used to obtain the average concentration of KCl in the adsorbent beads.

$$u\frac{\partial C_{Cl}}{\partial z}+\varepsilon_b\frac{\partial C_{Cl}}{\partial t}+\rho_b\frac{\partial\bar{q}_{Cl}}{\partial t}-D_{zCl}\frac{\partial^2 C_{Cl}}{\partial z^2}=0 \tag{8}$$

$$\rho_b\frac{\partial\bar{q}_{Cl}}{\partial t}=K_{fCl}a(C_{Cl}-C^*_{Cl}) \tag{9}$$

$$\rho_b\bar{q}_{Cl}=0.747C_{Cl} \tag{10}$$

By use of the average concentration and Eqs. (1)–(3) the adsorption equilibrium was determined, and the elution curve was calculated with Eqs. (5) and (7). D_{zCl} was regarded as equal to D_z. $K_{fCl}a$ was determined to be 0.05 s^{-1} by the step-response method.[1,7] The solid curve in Fig. 2 shows the elution curve calculated in this way, which agrees well with

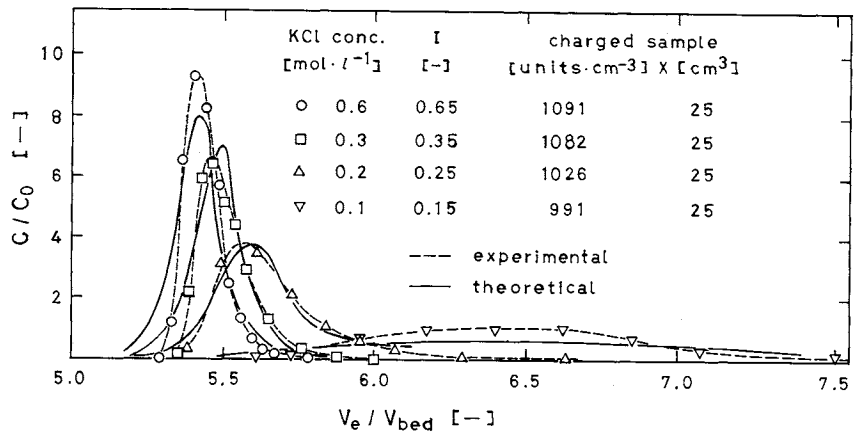

Fig. 3. Effect of ionic strength of eluent on elution profile. Column: 1.65 cm i.d. ×8 cm; $u = 11.1\ \mathrm{cm \cdot h^{-1}}$.

Table 2. Effect of ionic strength of eluent on purification

Conc. of KCl in eluent [mol·l⁻¹]	Specific activity of charged sample [units·mg⁻¹]	Specific activity of eluate [units·mg⁻¹]	Purification [—]	Yield [percent]
0.10	569	—	—	—
0.13	734	2760	3.76	68
0.16	635	2469	3.89	70
0.20	671	2329	3.47	75
0.30	707	2439	3.45	69
0.60	735	2237	3.04	74

the experimental points.

4) Effect of ionic strength of eluent As stated above, tyrosyl-tRNA synthetase can be eluted with an eluent of the comparatively low ionic strength. Variation of KCl concentration in the eluent varies the adsorption equilibrium of tyrosyl-tRNA synthetase as expressed by Eqs. (1)–(3) and results in a change in the elution profile. The elution profiles were measured by changing the ionic strength of the eluent from 0.15 to 0.65 and were compared with those calculated numerically by use of Eqs. (1)–(3), (5) and (7)–(10). As shown in **Fig. 3**, they agreed well with the experimental profiles. Values of the specific activity and the purification measured are shown in **Table 2**. With increasing KCl concentration the elution profile became sharper. On the other hand, elution of some impurities bound to the adsorbent more tightly than tyrosyl-tRNA synthetase resulted in a gradual decrease in purification, as shown in Table 2. The ionic strength of the eluent must be determined so as to be optimal for the purification by considering the specific activity, the yield and the operating time needed. In the present work, a KCl concentration of 0.3 $\mathrm{mol \cdot l^{-1}}$ in the Tris-HCl buffer was used except for the runs determining the effect of the ionic strength, because the lowering of the purification was not so prominent below this value and the elution profile was fairly sharp.

5) Effect of liquid velocity **Figure 4** shows the

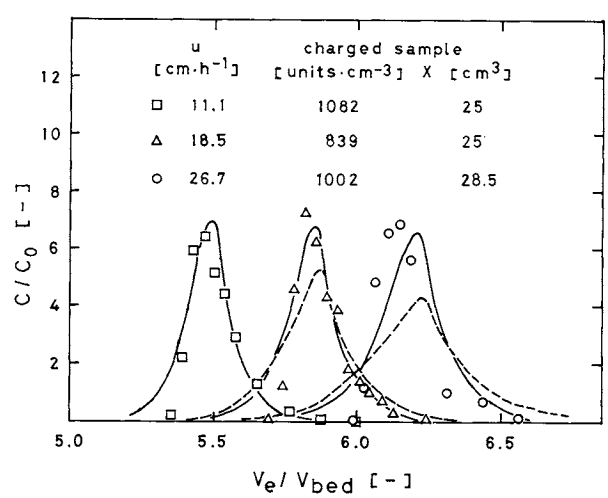

Fig. 4. Effect of liquid velocity on elution profile. Column, 1.65 cm i.d. ×8 cm; eluent, 0.3 $\mathrm{mol \cdot l^{-1}}$ KCl in Tris-HCl buffer; solid line, calculated using $\overline{K_f a}$ value dependent on liquid velocity; broken line, calculated using constant $\overline{K_f a}$ value of 0.005 $\mathrm{s^{-1}}$ independent of liquid velocity.

effect of the superficial liquid velocity on the elution profile. The solid lines are determined by use of $\overline{K_f a}$ values of 0.005 $\mathrm{s^{-1}}$ obtained from the breakthrough curve for the superficial liquid velocity of 11.1 $\mathrm{cm \cdot h^{-1}}$, 0.007 $\mathrm{s^{-1}}$ for 18.5 $\mathrm{cm \cdot h^{-1}}$ and 0.009 $\mathrm{s^{-1}}$ for 26.7 $\mathrm{cm \cdot h^{-1}}$ by considering the dependence of $\overline{K_f a}$ on the liquid velocity reported in the literature.[4] Broken lines are calculated by using the value of

$0.005\,s^{-1}$ for $\overline{K_f a}$ for 18.5 and 26.7 $cm \cdot h^{-1}$. The values of D_z used for the calculation were 6×10^{-5} $cm^2 \cdot s^{-1}$ for the superficial liquid velocity of 18.5 $cm \cdot h^{-1}$ and 1.4×10^{-4} $cm^2 \cdot s^{-1}$ for 26.7 $cm \cdot h^{-1}$. In all cases $K_{fCl}a$ of $0.05\,s^{-1}$ was used because of the slight effect of the K_{fCl} a value on the elution profile in this condition. These results show that the variation of the coefficient of mass transfer with liquid velocity affects the elution profile.

6) Storage stability The ammonium sulfate precipitate of the eluate from the cellulose phosphate column was dissolved in the Tris-HCl buffer and stored at 277–278 K in a liquid state (ammonium sulfate concentration of ca. 0.19 $mol \cdot l^{-1}$). The activity of the stored sample was assayed for 39 days. **Figure 5** shows that inactivation was negligible in this period. Enzymes from thermophilic bacteria are generally very stable, even in solutions. In the present case, tyrosyl-tRNA synthetase, which is said to be comparatively unstable, proved to be fairly stable in the case of *B. stearothermophilus* origin.

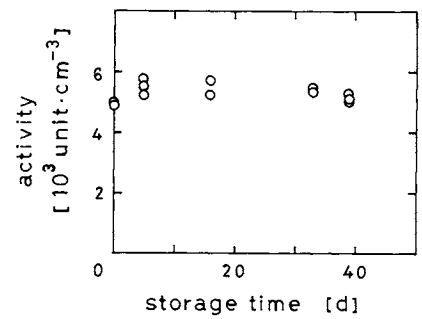

Fig. 5. Storage stability.

Conclusion

1) Stable tyrosyl-tRNA synthetase was isolated and purified from 230 kg (wet wt. cells) of *Bacillus stearothermophilus* by use of two consecutive affinity chromatographic procedures and the final purity was as high as 25,900-fold that of crude extract.

2) In affinity chromatography with Mātrex Blue A, the effects of operating conditions on performance were studied. Column diameter can be scaled up to 18.4 cm i.d. without appreciable change in the elution profile. The effect of ionic strength in the eluent due to KCl on purification and elution profile was studied within the range of KCl concentration 0.1–0.6 $mol \cdot l^{-1}$. The elution profile may be estimated numerically, taking the effect of KCl concentration on the adsorption equilibrium of tyrosyl-tRNA synthetase into account. The purification decreases gradually and the elution profile becomes sharper with increasing KCl concentration.

3) During storage of tyrosyl-tRNA synthetase in Tris-HCl buffer containing ammonium sulfate at 277–278 K the decrease of enzymatic activity was negligible after 39 days.

Nomenclature

a	=	specific particle surface area in bed	$[cm^2 \cdot cm^{-3}]$
C	=	fluid-phase concentration	$[units \cdot cm^{-3}]$
C^*	=	C in equilibrium with $\bar{q}$	$[units \cdot cm^{-3}]$
D_z	=	axial eddy diffusivity	$[cm^2 \cdot s^{-1}]$
I	=	ionic strength	[—]
K	=	constant in Eq. (1)	
K_f	=	overall mass transfer coefficient	$[cm \cdot s^{-1}]$
$\overline{K_f a}$	=	averaged volumetric coefficient of mass transfer	$[s^{-1}]$
$\bar{q}$	=	averaged solid-phase concentration	$[units \cdot g^{-1}]$
t	=	time	[s]
u	=	superficial liquid velocity	$[cm \cdot s^{-1}]$
X	=	dimensionless concentration, C/C_0	[—]
z	=	coordinate along bed height	[cm]
β	=	exponent of Freundlich-type isotherm	[—]
ε_b	=	void fraction of adsorbent bed	[—]
ρ_b	=	bed density	$[g \cdot cm^{-3}]$

〈Subscripts〉

B	=	break point
Cl	=	potassium chloride
E	=	exhaustion point
0	=	column inlet

Literature Cited

1) Hashimoto, K. and K. Miura: *J. Chem. Eng. Japan*, **9**, 388 (1976).
2) Hashimoto, K., K. Miura and M. Tsukano: *J. Chem. Eng. Japan*, **10**, 27 (1977).
3) Katoh, S., T. Kambayashi, R. Deguchi, and F. Yoshida: *Biotechnol. Bioeng.*, **20**, 267 (1978).
4) Katoh, S. and E. Sada: *J. Chem. Eng. Japan*, **13**, 151 (1980).
5) Nakajima, H., K. Yamamoto, I. Tomioka, Y. Echigo, T. Iwasaki and K. Imahori: 45th National Meeting of the Chemical Society of Japan, Abstract No. 2L15–16, p. 746–747, Tokyo, September (1982).
6) Nakanishi, K., S. Yamamoto and R. Matsuno: *Agric. Biol. Chem.*, **41**, 1465 (1977).
7) Rosen, J. B.: *Ind. Eng. Chem.*, **46**, 1590 (1954).
8) Sada, E., S. Katoh and H. Hieda: *Biotechnol. Bioeng.*, **21**, 341 (1979).
9) Suzuki, M.: *J. Chem. Eng. Japan*, **7**, 262 (1974).