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IMMOBILIZED AMINOACYLASE ON POROUS GLASS BEADS

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Aminoacylase was immobilized on porous glass by two different coupling methods. One aminoacylase preparation was covalently bound to an alkylaminosilane derivative of porous glass with glutaraldehyde [alkylamino–porous glass–CVB-aminoacylase]; the other aminoacylase derivative was prepared by covalently binding the enzyme to arylaminosilane glass by diazotization [arylamino–porous glass–CVB-aminoacylase]. The enzyme activities of the immobilized aminoacylases were 3.2–13.0 U/ml glass for the former and 1.9–6.8 U/ml glass for the latter. The alkylamino–porous glass–CVB-aminoacylase showed excellent stability at pH 6–9 and at temperatures below 50°C. The derivative could be stored for more than 6 mo without appreciable loss of the activity. Continuous hydrolysis using the alkylamino–porous glass–CVB-aminoacylase packed in column was carried out for 54 days at 37°C, with a calculated half-life of 78 days. It was determined that alkylamino–porous glass–CVB-aminoacylase would be applicable in an industrial preparation of various L-amino acids from their DL forms.

INTRODUCTION

Aminoacylases from various origins have been studied in relation to the optical resolution of DL-amino acids (1–3). A number of immobilization methods for aminoacylase have therefore also been studied (4–7).

In order to apply an immobilized enzyme in an industrial process, selection of a suitable carrier is of practical importance. The carrier must have mechanical durability, appropriate pore size, relatively large surface area, and a reasonable cost. Because of the advantages in the physical properties of porous glass particles described by Weetall (8), it seemed to be a promising carrier for immobilized enzymes.

The authors prepared two types of derivatives of aminoacylase covalently bound to porous glass particles. Porous glass–CVB-aminoacylase was prepared according to the method of Weetall et al. (8,9) and examined for its properties. Long-term operations of the immobilized enzymes were also studied. These results are reported in the present paper.

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MATERIALS AND METHODS

Enzyme Preparation. A commercial grade aminoacylase of *Aspergillus sp.* was purchased from the Amano Seiyaku Co., Ltd., Japan. It contained 56% protein, and its specific activity was 0.55 U/mg protein (1 unit of activity represents the production of 1 μmol L-methionine per minute at 37°C from acetyl-DL-methionine).

This crude enzyme was further purified by the following method: Commercial grade aminoacylase, 100 g, was dissolved in 1 liter 0.05 M phosphate buffer (pH 7.8) containing 1 mM $CoCl_2$. Precipitates from the solution by 45–75% saturation with ammonium sulfate showed a specific activity of 1.0 U/mg protein.

The precipitate was again dissolved in the buffer described above and passed through a column of Sephadex G-25, and the active fraction was collected. The active fraction was then passed through a column of DEAE-cellulose. The enzyme adsorbed on DEAE-cellulose was eluted with a linearly increasing gradient of sodium chloride from 0 to 1.0 M in the same buffer described above. The active fraction from the DEAE-cellulose column was treated by ammonium sulfate precipitation, and the enzyme precipitated was further purified by gel-filtration using a Sephadex G-100 column. The purified aminoacylase thus obtained showed a specific activity of 4.5 U/mg protein.

Porous Glass. Alkylamino and arylamino derivatives of 96% silica-porous glass particles coated with ZrO_2 (8) having an average pore diameter of 550 Å and particle size from 40 to 80 mesh were donated by the Corning Glass Works, Corning, New York.

Immobilization of Aminoacylase on Porous Glass. The aminoacylase was bound to the arylamino derivative of porous glass by azo-linkage, and also to the alkylamino derivative of porous glass by glutaraldehyde according to the method by Weetall et al. (8,9).

Methods of Analysis. (*a*) *Protein*: Protein content was determined by the colorimetric method of Lowry et al. (10) and calculated from a standard curve prepared with bovine serum albumin.

(*b*) *Activity of native aminoacylase*: A reaction mixture comprising 0.4 ml 0.1 M veronal buffer (pH 8.5), 0.2 ml 0.5 mM $CoCl_2$, 0.2 ml of 0.1 *N*-acetyl-DL-methionine (pH 8.0), and 0.2 ml of the enzyme solution was incubated at 37°C for 30 min. The reaction was immediately stopped by heating the mixture for 3 min at 100°C. The liberated L-mathionine was measured by a ninhydrin colorimetric method (11).

(*c*) *Activity of immobilized aminoacylase*: Determination of the enzyme activity of the immobilized aminoacylase was conducted by a column operation. Immobilized aminoacylase, 3 ml, was packed into a glass column

(10×80 mm, D/H) with an outer jacket in which temperature-controlled water was circulated to maintain the temperature at 37°C. Unless otherwise noted, a substrate solution containing 0.1 M *N*-acetyl-DL-methionine (pH 8.0) and 1 mM $CoCl_2$ was charged into the column at a flow rate of 30 ml/h (S.V. = 10). After several hours of continuous operation, the concentration level of the liberated L-methionine in the effluent became constant. Then, the concentration of liberated L-methionine in the effluent was determined by the ninhydrin colorimetric method (11).

The activity of the immobilized aminoacylase was expressed in terms of micromoles of the liberated L-methionine per minute by 1 ml of the immobilized enzyme (1 g immobilized enzyme corresponds to 2 ml packed volume.)

RESULTS

Preparation of Immobilized Aminoacylase on Porous Glass Particles and Factors Affecting the Preparation

Arylamino–porous glass-CVB-aminoacylase and alkylamino–porous glass–CVB-aminoacylase were prepared by using enzyme solutions of different concentrations and purities. The results are summarized in Table 1. In these experiments, native aminoacylase of three different purities—0.55, 1.0, and 4.5 U/mg protein—were reacted with the porous glass derivatives at three different concentrations of enzyme protein—25, 50, and 100 mg/ml glass.

As shown in Table 1, alkylamino–porous glass–CVB aminoacylase always gave higher enzyme activity than the arylamino–porous glass–CVB-aminoacylase under the same preparative conditions. Also, it can be seen that the activity of the immobilized aminoacylase depends on the purity of the native enzyme used, but does not depend on the concentration of enzyme protein used under the experimental conditions employed.

When more enzyme was used, more was bound, but less retention of enzyme activity was observed. A higher purity but lower amount of native enzyme gave better activity, as expressed by the immobilized enzyme.

A small amount of aminoacylase was adsorbed by the porous glass even in the absence of a coupling agent, and could not be removed by washing with 5 M NaCl. Such adsorbed enzyme was readily eliminated, however, when 0.1 M *N*-acetyl-DL-methionine solution was passed through the enzyme column (see Fig. 1). In the case of alkylamino–porous glass–CVB-aminoacylase, the activity was stabilized within several hours.

Stabilities of the two types of immobilized aminoacylases were compared using continuous column operations. The results are shown in Fig. 1.

TABLE 1. **Preparation of Immobilized Aminoacylase on Porous Glass Particles**

Carriers used (coupling agent used)	Aminoacylase added			Aminoacylase bound			Retention of aminoacylase activity		
	Specific activity (U/mg protein)	Protein (mg/ml glass)	(A) Total activity (U/ml glass)	Protein[a] (mg/ml glass)	(B) Activity[b] bound (U/ml glass)	Ratio of activity bound (B/A, %)	(C) Activity[c] of immobilized enzyme (U/ml glass)	Retention of bound activity (C/B, %)	Ratio to added activity (C/A, %)
Arylamino–glass (diazonium salt)	0.55	50	27.5	22.3	12.27	44.6	2.0	16.2	7.23
	0.55	100	55.0	37.0	20.35	37.0	1.9	9.3	2.54
	4.5	50	225.0	19.4	87.30	38.8	7.2	8.2	3.18
	4.5	100	450.0	28.7	129.15	28.7	6.8	5.3	1.52
Alkylamino–glass (glutaraldehyde)	0.55	50	27.5	14.0	7.70	28.0	3.4	44.2	12.41
	0.55	100	55.0	32.7	17.99	32.7	3.2	17.8	5.82
	1.0	50	50.0	16.8	16.80	33.6	5.7	34.0	11.42
	1.0	100	100.0	28.3	28.30	28.3	5.5	19.5	5.52
	4.5	25	112.5	7.6	34.20	30.4	12.8	37.4	19.11
	4.5	50	225.0	14.0	63.00	28.0	12.0	19.0	4.56
	4.5	100	450.0	30.7	138.15	30.7	13.0	9.4	1.22
Alkylamino–glass (no coupling agent)	1.0	50	50.0	3.9	3.90	7.8	1.47	32.0	2.94

[a] Calculated from the amount of protein in filtrate and washing water.
[b] Calculated from the amount of protein bound and specific activity.
[c] Activity was assayed by passing 0.1 M or 0.2 M *N*-acetyl-DL-methionine (pH 8.0, containing 1 mM $CoCl_2$) through the enzyme column at flow rate S.V. 10.

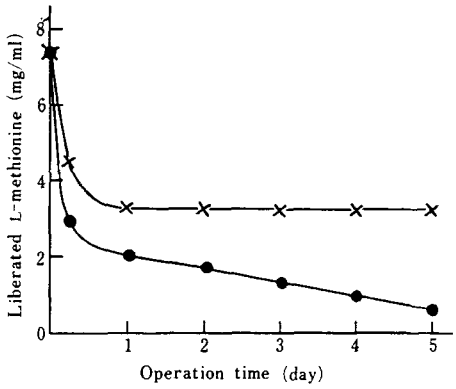

FIG. 1. Stability profiles for aminoacylase immobilized on alkylamino–porous glass (×) and arylamino–porous glass (●) in continuous column operation. Temperature was maintained at 37°C and flow rate was kept at 10 ml/h/ml glass. Substrate solution comprised 0.1 M acetyl-DL-methionine (pH 8.0) and 10 mM $CoCl_2$. The immobilized enzymes were prepared from crude enzyme (sp act 0.55) of Table 1.

The initial declines of activity within the first several hours are explained by leakage of adsorbed enzyme in the presence of the substrate solution. Even though the immobilized enzymes used in this experiment originally showed low activity, the alkylamino–porous glass–CVB-aminoacylase was apparently more stable than the arylamino–porous glass–CVB-aminoacylase.

From the data described above, it became evident that the alkylamino–porous glass–CVB-aminoacylase was superior to the arylamino–porous glass–CVB-aminoacylase. In the subsequent experiments, therefore, only alkylamino–porous glass–CVB-aminoacylase was employed.

Properties of Alkylamino–Porous Glass–CVB-Aminoacylase

Optimum pH. The pH–activity relationship of the immobilized enzyme and the native enzyme was examined in the pH region from 6.5 to 9.0. The results are shown in Fig. 2. The immobilized enzyme had a pH optimum at 8.0 and showed rather constant activity in the pH region from 6.5 to 9.0.

Optimum Temperature. The effect of temperature on the immobilized enzyme activity was examined in the column operation in which temperature was varied in the circulating water bath used with the jacketed column. With the increase in temperature, the reaction velocity was accelerated, but was concomitantly retarded due to the thermal inactivation of the

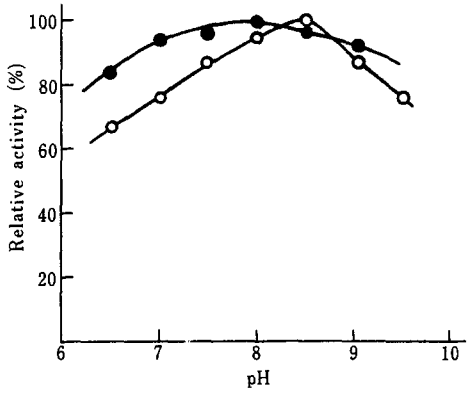

FIG. 2. Effect of pH on the activity of immobilized amino-acylase (●) and free aminoacylase (○). Immobilized aminoacylase on alkylamino–porous glass was used in a column. Substrate solution comprised 0.1 M acetyl-DL-methionine and 10 mM $CoCl_2$. The native soluble enzyme was incubated in the same substrate solution for 30 min at 37°C. The pH of substrate was varied from 6.5 to 9.0 with NH_4OH.

immobilized enzyme, particularly in long-term operations. As described below, the immobilized aminoacylase was not appreciably inactivated at temperatures below 50°C, but was significantly inactivated above 50°C. As a superposing effect of activation and inactivation, the maximum activity of the immobilized enzyme was observed at 55°C, as shown in Fig. 3. It is noteworthy that the effect of temperature on the activity of the immobilized aminoacylase was less remarkable than the effect on the native enzyme, as demonstrated by only a 27% increase in activity with a rise in temperature from 37°C to 55°C.

Heat Stability. The heat stability of the immobilized aminoacylase was also determined in the presence of the substrate. Both immobilized and native enzymes were exposed to different temperatures for 16 h, and the residual activities were then assayed at 37°C. The results are shown in Fig. 4. It is evident that the immobilized enzyme is more stable at higher temperature than the native enzyme. From the results shown in Figs. 3 and 4, the favorable temperature for long-term operation of the immobilized enzyme must be less than 45°C.

The stability of the immobilized enzyme stored at 5°C was also examined. It was found that the enzyme activity was completely kept intact even after 6 months' storage.

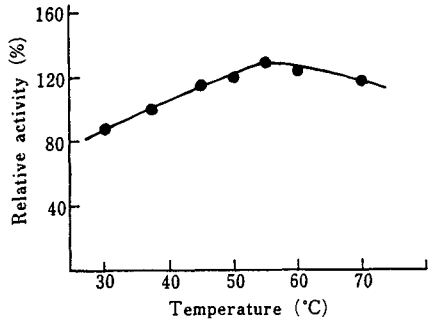

FIG. 3. Effect of temperature on continuous operation of immobilized aminoacylase columns. The columns of aminoacylase coupled to alkylamino–porous glass were charged with 0.2 M acetyl-DL-methionine (pH 8.0) containing 10 mM $CoCl_2$ at various temperatures.

Effect of Buffers and Salts on Enzyme Activity. *N*-Acetyl-DL-methionine was dissolved in deionized water and various buffer and salt solutions at a concentration of 0.2 M. All these solutions contained 1 mM $CoCl_2$ and were adjusted to pH 8.0 with sodium hydroxide. These substrate solutions were charged in the immobilized columns; the relative concentrations of L-methionine liberated in effluents from these columns are shown in Table 2.

As the results show, the substrate solution using deionized water gave the best activity. All the buffer and salt solutions gave lower activities.

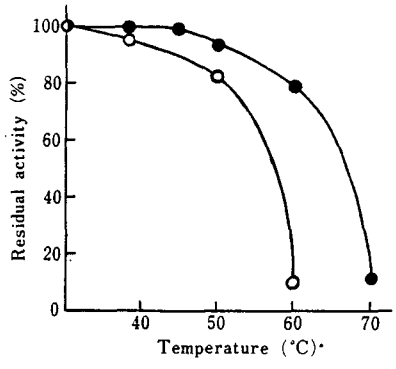

FIG. 4. Effect of temperature on stability of immobilized aminoacylase. Aminoacylase coupled to alkylamino–porous glass (●) and native soluble enzyme (○) were incubated for 16 h in 0.1 M acetyl-DL-methionine (pH 8.0) containing 0.5 mM $CoCl_2$. The temperature was varied from 5 to 70°C. The residual enzyme activity of the immobilized enzyme in a column was assayed at 37°C.

TABLE 2. **Effect of Buffer and Salt Solutions on Activity of Immobilized Aminoacylase**[a]

pH 8.0 Buffer or salt solution	Activity (%)[b]
Deionized water	100
0.02 M Na_2HPO_4–NaH_2PO_4	87
0.02 M H_3BO_4–Na_2CO_3	87
0.02 M $NaHCO_3$	87
0.02 M $Na_2B_4O_7$	68
0.02 M H_3BO_4–$Na_2B_4O_7$	82
0.02 M K_2HPO_4–KH_2PO_4	82
0.1 M K_2HPO_4–KH_2PO_4	64
0.1 M NaCl	86
0.5 M NaCl	73
0.1 M CH_3COONa	79
0.5 M CH_3COONa	42

[a] The activity was assayed in column operation at a flow rate of S.V. 10.
[b] Activity: Relative concentration of L-methionine liberated in effluent from each column.

Substrate Specificity. The relative activity of the immobilized aminoacylase on the alkylamino porous glass toward several *N*-acetyl-DL-amino acids was determined using column operation. The results are shown in Table 3.

Of the various substrates tested, *N*-acetyl-DL-methionine was hydrolyzed most rapidly. The specificity pattern of the immobilized enzyme was similar to that of the native enzyme.

TABLE 3. **Substrate Specificity of Immobilized Aminoacylase**[a]

Substrate	Relative activity (%)
N-Acetyl-DL-methionine	100
N-Acetyl-DL-valine	53
N-Acetyl-DL-tryptophan	65
N-Acetyl-DL-phenylalanine	98
N-Acetyl-DL-alanine	35
N-Acetyl-DL-phenylglycine	10

[a] A substrate solution of 0.1 M *N*-acetyl-DL-amino acids (pH 8.0) was charged on the immobilized enzyme column at a flow rate of S.V. 10 under standard conditions. The liberated amino acids were determined by the ninhydrin colorimetric method. The activity toward *N*-acetyl-DL-methionine was taken as the control.

Effect of Concentration and Flow Rate of Substrate Solution in Column Operation. The effect of substrate (*N*-acetyl-DL-methionine) concentration on the activity of the immobilized aminoacylase was examined using column operation. Substrate concentrations were varied from 0.025 to 0.6 M, and the flow rates of the substrate solution were varied up to S.V. 40. The results are shown in Fig. 5.

At the lowest concentration of substrate—0.025 M *N*-acetyle-DL-methionine—the reaction rate was constant in all the region of flow rate from S.V. 1 to 40. On the other hand, at substrate concentrations higher than 0.2 M, no distinct difference of reaction rate was observed at each flow rate used.

On the basis of the data shown in Fig. 5, the relationship between the concentration of the substrate and conversion rates at various flow rates was expressed by plotting according to the method by Bar-Eli and Katchalski (12) (Fig. 6). From the decline of each slope, apparent Michaelis constants were calculated. With increasing flow rates, the apparent Michaelis constant decreased. At a flow rate as high as S.V. 40, the apparent Michaelis constant became zero, indicating that the conversion of the substrate into the product was controlled by diffusion rather than reaction. At a flow rate as low as S.V. 3, the apparent K_m could not be obtained by this method, presumably due, again, to the effect of diffusion on the conversion.

In order to observe the leakage of enzyme from the immobilized enzyme used in the column operation, the effluent obtained in the case of 0.6 M substrate solution was incubated for 24 h at 37°C. No increase of L-methionine was observed, indicating no leakage of enzyme during the course of continuous operation.

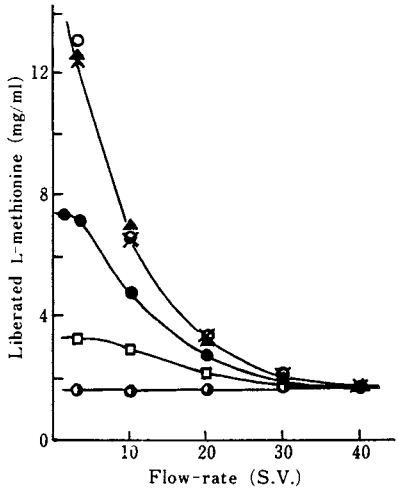

FIG. 5. Effect of substrate concentration and flow rate on the activity of immobilized aminoacylase. Two ml of aminoacylase coupled to alkylamino-porous glass was packed in a column and charged with acetyl-DL-methionine solution (pH 8.0) containing 0.5 mM $CoCl_2$ at a flow rate between S.V. 1 and 40. The concentration of substrate was varied from 0.02 to 0.6 M. When continuous reaction at 37°C was stabilized, L-methionine liberated in effluent was measured.

▲, 0.6 M; ○, 0.4 M; ×, 0.2 M;
●, 0.1 M; □, 0.05 M; ◑, 0.025 M.

$PS_o = K_m \log_e(1 - P) + K(E_o)/\text{S.V.}$

S.V.	K_m
3	?
10	7.70×10^{-2} M
20	3.45
30	1.64
40	0

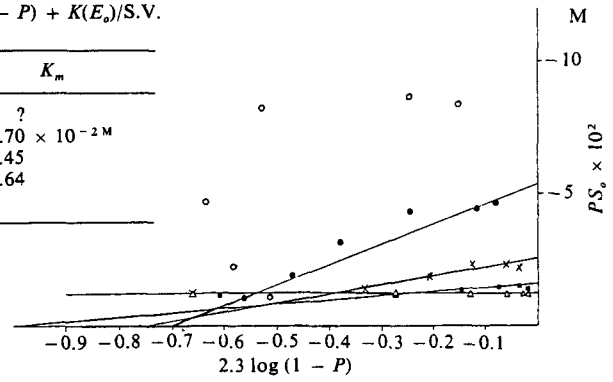

FIG. 6. Effect of flow rate on the Michaelis constant of the immobilized aminoacylase. ○, S.V. 3; ●, S.V. 10; ×, S.V. 20; ■, S.V. 30; △, S.V. 40.

Continuous Column Operation of Immobilized Aminoacylase

For the purpose of examining the applicability of the immobilized aminoacylase on alkylamino porous glass for the industrial resolution of D,L-amino acids, the long-term operation of the immobilized enzyme in a column was carried out.

A substrate solution (pH 8.0) comprising 0.1 M *N*-acetyl-DL-methionine and 1 mM $CoCl_2$ was passed through a column containing 3 ml alkylamino–porous glass–CVB-aminoacylase (6.85 U/ml glass) at flow rates of S.V. 5 and 10. The temperature in the columns was maintained at 37°C. The changes in activity during 54 days' operation are shown in Fig. 7.

At a flow rate of S.V. 5, the concentration of L-methionine liberated in the effluent was maintained around 7.1 mg/ml during the entire experimental period; this meant 48% hydrolysis of the racemic substrate, or 96% hydrolysis of the L form used. A slow decline was observed at a flow rate of S.V. 10 after continuous operation for 40 days. The observed decrease in the apparent activity of the immobilized aminoacylase in an operation with shorter residence time can be explained as follows: The enzyme molecules immobilized deeply inside the pores of the carrier did not contribute appreciably to the apparent activity, particularly in an operation with a short residence time of the substrate solution. It was mainly the enzyme molecules immobilized on the surface of the carrier that participated in the enzyme reaction, which proceeded in a zero-order fashion. From the declining line observed in this zero-order reaction, shown in Fig. 7, the half-life of the immobilized aminoacylase was calculated to be 78 days. When a substrate

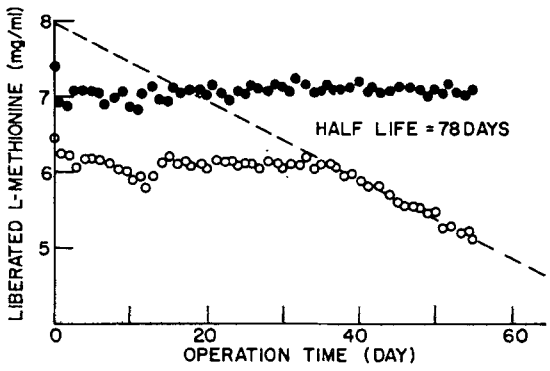

FIG. 7. Continuous operation of aminoacylase covalently bound to alkylamino–porous glass. Columns containing 3 ml immobilized enzyme were continuously operated at 37°C using a substrate solution (pH 8.0) comprised of 0.1 M acetyl-DL-methionine and 1 mM $CoCl_2$. The flow rates were S.V. 5 (●) and S.V. 10 (○).

solution containing 0.2 M *N*-acetyl-DL-methionine was charged into the column, complete hydrolysis of the L-form substrate was attained at a flow rate of S.V. 2.

L-Methionine was isolated by concentration of the effluent and crystallized by the addition of ethanol. The yield of L-methionine was 94% of the theoretical value, and $[\alpha]_D^{25}$ of it was +23.4° ($c = 1.0$, in 6 N HCl). On the other hand, *N*-acetyl-D-methionine was recovered from the crystallization mother liquor and racemized by heating in acidic condition. The racemized substrate could be hydrolyzed again by the immobilized enzyme without difficulty.

DISCUSSION

Immobilized enzyme technology has developed rapidly in recent years, but only a few immobilized enzymes have been applied to industrial processes. This limited application may be accounted for by the poor durability of the immobilized enzymes hitherto prepared and the extremely high cost of the support materials employed.

Aminoacylase is a well-known enzyme that has already been immobilized on DEAE-cellulose and DEAE-Sephadex and applied for the practical resolution of DL-amino acids (1). It is assumed, however, that support materials such as cellulose and dextran derivatives are not ideal for

large-scale column operation. In this study, the authors were interested not only in aminoacylase as a practical enzyme, but also in the physicochemical properties of porous glass. Porous glass has physical strength in a column operation and stability against microbial attacks. Also, its large surface area and desirable pore size resulted in the effective retention of enzyme activity (9).

The commercial aminoacylase used in this study contained some quantity of cellulase activity; therefore, cellulose and dextran derivatives used in the purification of the enzyme were often decomposed by the crude aminoacylase. For the same reason, dialysis of the enzyme using cellophane membranes also could not be employed. Cellulose and dextran derivatives therefore did not seem to be suitable support materials for immobilized aminoacylase. In the case of covalent binding of the enzyme of the porous glass, however, the contaminant cellulase did not show any inhibitory effect on the preparation of immobilized enzyme.

From the results shown in Table 1, it can be suggested that the more potent immobilized enzyme can be prepared if a more purified enzyme with higher specific activity is employed for the preparation. These data also suggest that the use of lesser amounts of enzyme per unit volume of porous glass may give better retention of enzyme activity and higher yields of enzyme activity expressed.

In the long-term continuous operation of the alkylamino–porous glass–CVB-aminoacylase, the half-life of the immobilized enzyme was calculated to be 78 days when a substrate solution—0.1 M *N*-acetyl-DL-methionine—was passed through the column at a flow rate of S.V. 10, but no loss of activity was observed at a lower flow rate—S.V. 5.0. The stability of the immobilized aminoacylase looks much better than that of the immobilized aminoacylase hitherto reported.

Calculating from the data of Fig. 7, if 0.1 M *N*-acetyl -DL-methionine is passed through the column at a flow rate of S.V. 5 at 37°C, about 25 kg L-methionine can be produced by continuous operation of 1 liter of the immobilized enzyme for 1 mo. This production capacity may be improved by preparing a more potent immobilized aminoacylase.

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