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PREPARATION, CHARACTERISTICS, AND APPLICATION OF AN ASPARAGINASE TUBE

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Conditions for the preparation of an asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) tube using polyacrylamide gel were investigated. The apparent yield of activity of the asparaginase tube was about 20%; the activity of the tube was dependent on inner surface area. The optimum pH of the asparaginase tube shifted by 1 pH unit to the acid side in comparison with that of native enzyme. The apparent Michaelis constant of the tube was about 200 times higher than that of the native enzyme. The half-life of the asparaginase tube in continuous operation was estimated to be 16 days at 37°C. The tube was found to be resistant to attack by proteolytic enzyme. The L-asparagine level in blood plasma of a dog was lowered to 1/4 by the performance of an extracorporeal shunt using the asparaginase tube.

INTRODUCTION

Recently, the use of immobilized enzymes has been noted as one of the potential methods of solving some of the problems in enzyme therapy, such as production of antibody and loss of effectiveness of enzyme administered due to the attack of proteolytic enzymes or neutralization of enzymes by its antibody in blood (1–14). Immobilized enzymes prepared by entrapping into microcapsules of semipermeable polymer membranes or polymer gel lattices do not leak out, but still act efficiently on permeable substrates. It is therefore possible that the above-stated problems in enzyme therapy might be overcome by the use of immobilized enzymes in direct administration or as an extracorporeal shunt system. From these points of view, immobilization of asparaginase employed for the suppression of lymphosarcoma has been investigated by the entrapment method. In the case of immobilized asparaginase, two forms for both types of applications described above could be used therapeutically.

For the purpose of direct administration, we prepared the nylon or polyurea microcapsules containing asparaginase. The preparation and

properties of these microcapsules has been reported on in detail (7,8). Further, for the purpose of therapeutic use of immobilized asparaginase in an extracorporeal shunt system, the entrapment of the enzyme into polyacrylamide gel lattice was carried out, since the latter is physically more stable than microcapsule membranes. These results were also presented previously (10).

In this paper, we describe the preparation of an asparaginase tube, which is considered to be superior to previously reported immobilized asparaginase in resistance to the bloodstream. The characteristics and application of the asparaginase tube are also presented.

MATERIALS AND METHODS

Materials

Asparaginase was prepared from *Proteus vulgaris* according to the method of Tosa et al. (15). Its activity was 5,100 or 8,600 μmol ammonia liberated/h/mg of the preparation by standard enzyme assay.

Acrylamide monomer and potassium persulfate were obtained from Katayama Chemical Industries Co., Ltd., Osaka, Japan. *N,N'*-Methylenebisacrylamide and β-dimethylaminopropionitrile were obtained from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan.

Methods

Standard Preparation of the Asparaginase Tube. To 4 ml 0.1 M phosphate buffer (pH 7.0) containing asparaginase, acrylamide monomer, *N,N'*-methylenebisacrylamide, and 300 mg L-serine were added 0.5 ml 5% β-dimethylaminopropionitrile and 0.5 ml 1% potassium persulfate solution. A quantity of 1 ml of this reaction mixture was poured into a glass tube in which a glass rod coated with Tween 20 was vertically set in the central part. The mixture was allowed to stand at 23°C for 5 min. After completion of polymerization, the glass rod was removed. A hollow asparaginase tube thus obtained was cooled by steeping in ice water, and washed by passing through it 0.1 M borate buffer (pH 8.0) and water.

Standard Enzyme Assay of Native Asparaginase. Unless otherwise noted, standard enzyme assay of native asparaginase was carried out by the method previously described (10). The enzyme activity was expressed in micromoles of ammonia liberated per hour.

Standard Enzyme Assay of Asparaginase Tube. Unless otherwise noted, 50 mM L-asparagine solution (pH 8.0) was passed through the asparaginase tube at 37°C for 2 h at a flow rate of 40 ml/h. The liberated ammonia in the effluent was measured by the colorimetric method, using Nessler's reagent.

Determination of Michaelis Constant. In the case of native asparaginase, determination of the Michaelis constant was carried out by the same method as described in the previous paper (10). In the case of the asparaginase tube, L-asparagine solution (pH 8.0, 1×10^{-3} M to 1×10^{-2} M) was passed through the asparaginase tube at a flow rate of 120 ml/h for 30 min. The liberated ammonia in the effluent was measured by the colorimetric method, using Nessler's reagent.

Determination of L-Asparagine and L-Aspartic Acid in Blood Plasma. Blood freshly drawn from an artery in the thigh of a dog was centrifuged at 1,000*g* for 15 min. The plasma obtained was boiled for 20 min and centrifuged at 100,000*g* for 1 h. L-Asparagine and L-aspartic acid in supernatant were assayed by the NAD-coupled method of Cooney et al. (16).

Model Extracorporeal Shunt System Using Asparaginase Tube. A quantity of 500 ml 32 μM L-aspargine solution was circulated through the asparaginase tube which had an activity of 600 μmol/h at 37°C at a flow rate of 2.4 1/h. The diagram for this circulation system is shown in Fig. 1. After appropriate circulation, L-asparagine and L-aspartic acid in the circulating solution were determined by Cooney's method.

Animal Experiment for Extracorporeal Shunt Using Asparaginase Tube. A dog (beagle strain, 6 kg body weight) was anesthetized with Nembutal, after which heparin and Ringer's solution were administered. The blood was circulated through an asparaginase tube, which had an activity of 800 μmol/h, from the femoral artery to the vein. The circulation was carried out at 37°C at a flow rate determined by the natural blood pressure of the animal. After an appropriate circulation time, blood was drawn at the inlet of the asparaginase tube.

RESULTS

Conditions for Preparation of Asparaginase Tube

Effect of Concentration of Acrylamide and N,N'-Methylenebisacrylamide. In order to prepare an asparaginase tube suitable for use in an extracorporeal shunt system, the effects of the concentration of acrylamide monomers on enzyme activity and the physical properties of the asparaginase tube were investigated. The results are shown in Table 1. When 150 mg acrylamide and 12 mg *N,N'*-methylenebisacrylamide are used in 1 ml of the reaction mixture, the activity of the asparaginase tube was found to be highest. The physical properties of the gel were also superior.

Protective Effect of Amino Acids on Immobilization of Asparaginase. As described in the previous paper (10), to prevent the inactivation of asparaginase during immobilization, sodium L-aspartate was added to the

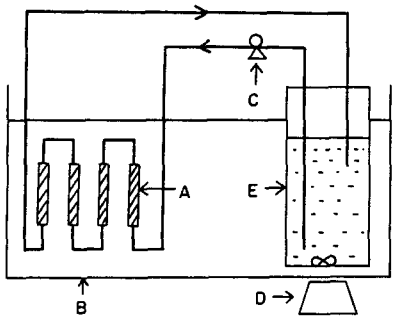

FIG. 1. Diagram for model extracorporeal shunt system using asparaginase tube: (A) asparaginase tube; (B) thermostatted bath; (C) peristaltic pump; (D) magnetic stirrer; (E) reservoir.

polymerization reaction medium. However, the contamination of this amino acid in the asparaginase tube prevented the microassay of L-asparagine or L-aspartic acid in blood plasma. The protective effect of amino acids other than sodium L-aspartate was therefore investigated. As shown in Table 2, the asparaginase tube could be prepared with the addition of L-serine giving the same yield of activity as with sodium L-aspartate.

Effect of Thickness of Gel Layer and Inside Diameter of Asparaginase Tube. The effect on enzyme activity of the thickness of the gel layer and the inside diameter of the asparaginase tube was investigated. The results are shown in Table 3. The most active asparaginase tube was obtained when the

TABLE 1. **Effect of Concentration of Monomers on Enzyme Activity and Physical Properties of Asparaginase Tube**[a]

Acrylamide (mg)	*N,N'*-methylene-bisacrylamide (mg)	Asparaginase tube		Physical properties
		Activity (μmol/h)	Yield[b] (%)	
150	4	190	18.6	Soft, elastic
	12	200	19.6	Rigid, elastic
	20	130	12.7	Soft, fragile
200	4	142	13.9	Soft, elastic
	12	102	10.0	Rigid, elastic
	20	102	10.0	Rigid, fragile
250	4	96	9.4	Rigid, elastic
	12	124	12.2	Rigid, fragile
	20	70	6.9	Rigid, fragile

[a] Asparaginase tubes were prepared under the standard conditions using 0.2 mg (1,020 μmol/h) asparaginase. The thickness of the gel layer and the inside diameter of the tube were 0.8 mm and 3 mm, respectively. The activity of the asparaginase tube was measured under the conditions of standard enzyme assay.
[b] The total activity of asparaginase used was taken as 100%.

TABLE 2. **Protective Effect of Amino Acids on Immobilization of Asparaginase**[a]

Amino acid added	Asparaginase tube	
	Activity (μmol/h)	Yield[b] (%)
None	54	5.3
L-Alanine	165	16.2
L-Na-aspartate	214	21.0
L-Na-glutamate	150	14.7
L-Lysine-HCl	180	17.6
D-Lysine-HCl	171	16.7
L-Serine	218	21.4
D-Serine	205	20.1

[a] Asparaginase tubes were prepared under the optimal conditions given in Table I (acrylamide, 150 mg; *N,N'*-methylenebisacrylamide, 12 mg), except for added amino acids (300 mg).
[b] The total activity of asparaginase used was taken as 100%.

TABLE 3. **Effect of Thickness of Gel Layer and Inside Diameter of Asparaginase Tube on Enzyme Activity**[a]

Thickness of gel layer (mm)	Inside diameter (mm)	Tube length (cm)	Inner surface area of gel (cm^2)	Activity of asparaginase tube (μmol/h/tube)	
				Tube	Crushed
2.3	3	2.61	2.46	51	556
1.8	3	3.69	3.48	77	533
1.4	3	5.17	4.87	101	541
0.8	3	10.48	9.87	216	582
0.8	4	8.29	10.41	195	540
0.8	5	6.86	10.77	216	564
0.8	6	5.85	11.02	178	552

[a] Asparaginase tubes were prepared under the same conditions as given in Table II, except for varying the thickness of the gel layer and the inside diameter of the tube. L-Serine was used as a protective substance. The activity of the asparaginase tube was measured under the conditions of the standard enzyme assay. The asparaginase gel was removed from the glass tube and crushed to 9-mesh size. Crushed gel was suspended in 20 ml 0.05 M L-asparagine solution (pH 8.0), and incubated for 10 min at 37°C with shaking. The reaction was stopped by the addition of trichloroacetic acid, and the liberated ammonia was measured.

thickness of the gel layer and the inside diameter were 0.8 mm and 3–5 mm, respectively.

Effect of Amount of Asparaginase on Enzyme Activity. For preparation of the asparaginase tube having higher activity, the amount of asparaginase that can be entrapped into polyacrylamide gel–lattice was studied. Table 4 shows that the activity of the asparaginase tube becomes higher when increasing amounts of asparaginase are used, but the total yield of activity decreases. When more than 12 mg asparaginase was used, leakage of asparaginase from the gel–lattice was detected.

Effect of Shape of Immobilized Asparaginase. To obtain information on the intrinsic yield of activity after immobilization of asparaginase into a polyacrylamide gel–lattice, the effect of the shape of the immobilized asparaginase on the enzyme activity was studied.

As shown in Table 5, the activity increases with increasing catalytic area. That is, the apparent yield of activity for the asparaginase tube is 20% compared with the native asparaginase used, but almost all enzyme used for immobilization is entrapped into the gel–lattice.

Characteristics and Application of Asparaginase Tube

Relationship between Flow Rate of Substrate Solution and Reaction Rate. The effect of the flow rate of the substrate solution on the reaction rate

TABLE 4. **Effect of Amount of Asparaginase on Enzyme Activity**[a]

Asparaginase used		Asparaginase tube			
		Activity (μmol/h/tube)		Yield[b] (%)	
Amount (mg)	Activity (μmol/h)	Tube	Crushed	Tube	Crushed
0.12	1,032	240	923	23.2	89.4
0.24	2,064	345	1,965	16.8	95.2
0.60	5,160	450	4,275	8.7	82.8
1.20	10,320	532	8,550	5.2	82.8
3.00	25,800	787	23,625	3.1	91.6
6.00	51,600	915	45,000	1.8	87.4
12.00	103,200	1,335	75,000	1.3	72.7

[a]Asparaginase tubes were prepared under the optimal conditions given in Table 3 (thickness of gel layer, 0.8 mm; inside diameter, 3 mm), except for varying the amount of asparaginase. The activity of the asparaginase used in this experiment was 8,600 μmol/h/mg of the preparation.
[b]The total activity of asparaginase used was taken as 100%.

TABLE 5. **Effect of Shape of Immobilized Asparaginase on Enzyme Activity**[a]

Shape of immobilized asparaginase	Immobilized asparaginase	
	Activity (μmol/h per tube)	Yield[b] (%)
Tube	199	19.5
Crushed gel (9-mesh)	538	52.7
Homogenized gel	940	92.2

[a] Asparaginase tubes were prepared under the same conditions as given in Table 2. L-Serine was used as a protective substance. Asparaginase gel was removed from the glass tube and crushed to 9-mesh size or homogenized with mortar. Crushed or homogenized gel was suspended in 20 ml 0.05 M L-asparagine solution (pH 8.0) and incubated for 10 min at 37°C with shaking. The reaction was stopped by the addition of trichloroacetic acid, and the liberated ammonia was measured by the colorimetric method, using Nessler's reagent.

[b] The total activity of asparaginase used was taken as 100%.

was investigated in order to clarify whether the relationship between flow rate and reaction rate in the case of an asparaginase tube is similar to that for the granular type of immobilized asparaginase. The results are shown in Fig. 2. From the data, it is clear that reaction rate is inversely related to flow rate at both substrate concentrations.

Effect of pH. The pH dependence of the initial rate of hydrolysis of L-asparagine by an asparaginase tube and native asparaginase is shown in Fig. 3. The asparaginase tube shows the highest activity at pH 7.0, whereas the native enzyme is most active at pH 8.0.

Comparison of Michaelis Constants. The effect of substrate concentration on the enzyme activity was investigated, and the results obtained were plotted by the method of Lineweaver and Burk (17) for the estimation of the Michaelis constant (K_m). The K_m values for native asparaginase and the asparaginase tube were calculated to be 1.8×10^{-5} M and 4.0×10^{-3} M, respectively.

Operational Stability. The stability of the asparaginase tube during continuous operation was studied. The results are shown in Fig. 4. From this figure, the half-life of the asparaginase tube was calculated to be 16 days.

Effect of Chymotrypsin on Enzyme Activity. To obtain further information on the stability of the asparaginase tube, the effect of chymotrypsin on asparaginase activity was investigated and compared with that on the native enzyme. As shown in Table 6, native asparaginase was almost inactivated by incubation with chymotrypsin for 10 min, but the asparaginase tube was not affected by treatment for 60 min.

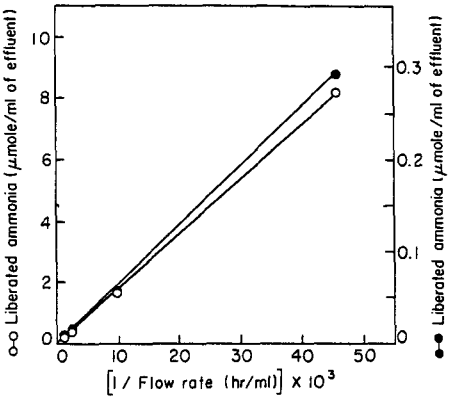

FIG. 2. Relationship between flow rate of asparagine solution and reaction rate. A specified concentration of asparagine solution (pH 8.0) was passed through the asparaginase tube at 37°C at a specified flow rate. The liberated ammonia in the effluent was measured by the colorimetric method, using Nessler's reagent. O—O: 50 mM L-asparagine; ●—●: 0.5 mM L-asparagine.

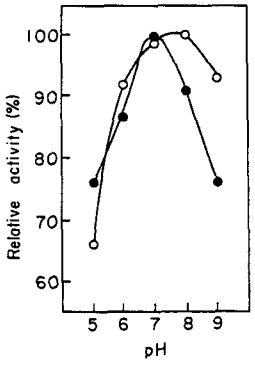

FIG. 3. Effect of pH on the reaction rate. The enzyme reaction was carried out under standard assay conditions except for the buffers employed. At pH 5.0–6.0, pH 6.0–8.0, and pH 8.0–9.0, 0.05 M acetate buffer, 0.05 M phosphate buffer, and 0.05 M borate buffer, respectively, were employed. In the case of the asparaginase tube, substrate solution was passed through the tube at a flow rate of 30 ml/h. The reaction rates obtained at optimum pH were taken as 100% in each case. O—O: native asparaginase; ●—●: asparaginase tube.

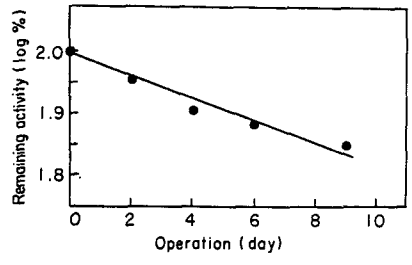

FIG. 4. Operational stability of asparaginase tube. A solution of 0.05 M L-asparagine dissolved in physiological saline was passed through the asparaginase tube at 37°C at a flow rate of 6 ml/h. After appropriate operation, the activity of the tube was measured under the conditions of standard enzyme assay.

TABLE 6. **Effect of Chymotrypsin on Enzyme Activity**[a]

Enzyme preparation	Remaining activity[b] (%)
Native asparaginase	2
Asparaginase tube	96

[a] In the case of native asparaginase, 2 ml 0.05 M borate buffer (pH 8.0) containing 200 μg asparaginase and 20 μg chymotrypsin was incubated at 37°C for 10 min. In the case of the asparaginase tube, 10 ml 0.05 M borate buffer (pH 8.0) containing 20 μg chymotrypsin was circulated through the tube at a flow rate of 30 ml/h at 37°C for 60 min. After these treatments, the remaining enzyme activity was immediately determined by the standard assay system.
[b] The activity before treatment was taken as 100% in each case.

Model Extracorporeal Shunt System Using Asparaginase Tube. As the preliminary test for the effectiveness of the asparaginase tube in vitro, clearance of L-asparagine was investigated in a model extracoporeal shunt system using the asparaginase tube. As shown in Table 7, L-asparagine was completely decomposed after circulation for 3 h.

Animal Experiment for Extracorporeal Shunt Using Asparaginase Tube. After the model experiment described above, to clarify the effectiveness of the asparaginase tube in vivo, amino acid levels in the blood plasma of a dog were determined before and after treatment with an extracorporeal shunt using the asparaginase tube. The results are shown in Table 8. The L-asparagine level in the blood plasma decreased to about 1/4 after circulation for 1 h. Circulation through the shunt was continued, but the L-asparagine level did not further decrease.

TABLE 7. **Clearance of L-Asparagine in Model Extracorporeal Shunt Using Asparaginase Tube**[a]

Circulation time (min)	L-Asparagine (nmol/ml)	L-Aspartic acid (nmol/ml)
0	32	0
20	22	10
40	17	15
60	12	20
120	2	30
180	0	32

[a] Experimental details are given in the text.

TABLE 8. **Animal Experiment for Extracorporeal Shunt Using Asparaginase Tube**[a]

Circulation time (min)	L-Asparagine (nmol/ml)	L-Aspartic acid (nmol/ml)
0	40	17
30	19	34
60	9	42
120	9	42

[a]Experimental details are given in the text.

DISCUSSION

We attempted to prepare the asparaginase tube by using polyacrylamide gel, for the purpose of therapeutic use in an extracorporeal shunt system. As shown in Table I, a physically durable and active asparaginase tube was obtained. Further, to obtain an even more active tube, the effect on enzyme activity of gel-layer thickness and inside diameter of the asparaginase tube was investigated. It was found that the activity of the asparaginase tube was dependent on the inner-surface area when a constant amount of asparaginase was used for the preparation of the tube. This finding fact indicates that the enzyme reaction occurs near the surface region of gel, and the diffusion of substrate or product in the gel is the limiting step for enzyme reaction rate. Accordingly, the asparaginase tube is inferior to the granular type of immobilized asparaginase in enzyme activity. The tube is superior to the granules, however, in physical properties.

The enzymatic properties of the asparaginase tube were compared with those of the native enzyme. The optimum pH of the asparaginase tube was about 1 pH unit more acid than that of native enzyme. This shift in optimum pH is also observed in other immobilized enzymes prepared by using polyacrylamide gel (18–20). The reason for the shift of optimum pH of the tube is, however, not clear.

The effect of substrate concentration on the enzyme activity was also investigated. The apparent K_m (Michaelis constant) value of the asparaginase tube was about 200 times higher than that of the native enzyme. The same phenomena were observed with the enzyme covalently bound to a nylon tube (6) or Dacron vascular prostheses (13) and in the microencapsulated enzyme (8). The reason for the increase in K_m value is the limitation of permeation rate of substrate and product through the polymer gel lattice.

In order to clarify the effectiveness of the asparaginase tube, both model and animal experiments of the extracorporeal shunt using the

asparaginase tube were performed. In the model experiment, L-asparagine was completely decomposed after circulation for 3 h. In the animal experiment, however, the concentration of L-asparagine in the blood plasma of the dog decreased to 25% of the initial concentration after circulation for 2 h, but reached a plateau even if circulation was continued. It is possible that when the L-asparagine level in the blood is lowered, inductive formation of L-asparagine synthetase in vivo may occur to maintain the L-asparagine level in a normal state.

Asparaginase entrapped into a tube is resistant to the attack of proteolytic enzymes, and is not inactivated by specific antibody. In the case of the immobilized asparaginase attached to a nylon tube (6,9) or Dacron vascular prostheses (13), the enzyme directly contacts the proteolytic enzymes or antibodies in the blood, and may be inactivated. Further, with an extracorporeal shunt, no formation of thrombus in the asparaginase tube or other side effects were observed. These excellent properties indicate that the asparaginase tube is promising for clinical use in the therapy of lymphosarcoma.

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