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PRODUCTION OF L-ASPARTIC ACID BY *E. coli* ASPARTASE IMMOBILIZED ON PHENOL-FORMALDEHYDE RESIN

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Aspartase was extracted from *E. coli* cells by autolysis in the presence of the substrate. The enzyme could be conveniently immobilized to a weakly basic anion exchange resin Duolite A7 by adsorption. Enzymatic properties of the immobilized aspartase were found to be fit for industrial purposes. Consequently, L-aspartic acid has been industrially produced by this immobilized-enzyme process since 1974.

INTRODUCTION

L-Aspartic acid has a wide and steady demand both in pharmaceutical use and as a seasoning ingredient in food and in sour beverages. In 1958, Kitahara et al. exploited an enzymatic process for the production of L-aspartic acid, using aspartase from *E. coli* to convert fumaric acid and ammonia to L-aspartic acid (1). The process had been practiced on an industrial scale for more than 15 years until Tosa et al. introduced the use of polyacrylamide-gel entrapped microorganisms in 1973 (2).

On the other hand, the present authors have employed ion-exchange resins as suitable carriers to immobilize various enzymes for industrial purposes (3,4). Because a single enzymatic process is involved in the production of L-aspartic acid, the immobilization of the enzyme and not the whole cell appeared interesting if the cell-bound aspartase could be efficiently extracted. The present paper deals with extraction and immobilization of the enzyme to the weakly basic anion-exchange resin Duolite A7 and also with the results of industrial application of the immobilized enzyme.

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MATERIALS AND METHODS

The Microorganism Used and the Method of Culture

Escherichia coli IAM 1062 (ATCC 21026) was aerobically grown for 20 h at 37°C in a medium of pH 7.2 containing 2% glucose, 1% meat extract, 1% peptone, 0.3% NaCl, and 1% $CaCO_3$, according to the Kitahara's method (1).

Method of Extraction of Aspartase from the Cells

The microbial cells were collected from the culture broth by filtration and were not suitable for homogenization by a Manton Gaulin homogenizer. Instead, one of the following methods were applied: (1) freezing and thawing of the cells; (2) autolysis of the cells at room temperature for 20 h; (3) the same as (2) at 5°C; (4) extraction of the enzyme from the cells with the addition of a cationic surface active agent, Nimeen S-25 (product of Nihon Yushi, Tokyo) at 200 μg/ml; (5) autolysis of the cells for 20 h after the addition of the surface active agent as described above; (6) autolysis of the cells for 20 h after the addition of M/10 sodium aspartate; (7) 10 KC sonication of the cells for 5 min.

Ion-Exchange Resins and Adsorption-Type Resins Used as Carriers of the Enzyme

The ion-exchange resins and tne adsorption-type resins listed in Table 1 were used for the selection of the carriers of the enzymes. All of them are commercially available. Diaion WA 20 from Mitsubishi Kasei Co., Ltd. (Japan), Duolite A2, A4, A6, A7, A41, A57, ES109, ES104, A102D, S30, ES33, and S37 from Diamond Shamrock Chemical Co., Ltd. (U.S.A.), and Amberlite XAD7 from Rohm and Haas Co., Ltd. (U.S.A.).

Method of Immobilization of Aspartase

Selection of Resins. Ion-exchange resins and adsorption-type resins were washed with 2N HCl and water. Each resin was then immersed in a saturated solution of fumaric acid and the suspension solution was neutralized by the addition of NaOH. The resins were thoroughly washed with water and suspended in M/10 sodium aspartate solution of pH 7.5 to 8.0. Five milliliters of each resin was mixed with 15 ml of the enzyme solution containing 5.0 mg/ml protein and kept at 5°C for 18 h with intermittent shaking. The supernatant was discarded and the resin was thoroughly washed with a phosphate buffer.

TABLE 1. **Comparison of Methods of Extraction of Aspartase from *E. coli* Cells**

		Type of resin			
	Resin	Functional group[a]	Pore	Matrix[b]	Residual fumarate[c] (%)
Diaion	WA20	W.B.	High porous	S	100
Duolite	A2	W.B.	Macro porous	P	0
	A4				5.2
	A6				20.2
	A7				3.9
	A41	M.B.	Transparent gel	E	100
	A57				93
	ES109	S.B.		S	97
	ES104		Homo porous		100
	A102D				108
	S30	ADS	Macro porous		20.4
	ES33				83
	S37				6.7
Amberlite	XAD7			AE	62

[a] W.B., weakly basic; S.B., strongly basic; M.B., moderately basic; ADS, adsorption.
[b] S, polystylene; P, phenol-formaldehyde; E, epoxy; AE, acrylic ester.
[c] Five milliliters of each enzyme-adsorbed carrier were incubated with 110 ml of ammonium fumarate solution (pH 8.2, containing 1 mM $MnCl_2$) at 37°C for 6 h and the amounts of residual fumarate were determined.

Determination of the Optimal Conditions for Adsorption of the Enzyme. For the determination of the optimal pH for adsorption of the enzyme, Duolite A7 was prepared in the OH form and each 5 ml of the resin was immersed for 20 h in 20 ml of 1 M phosphate buffer of various pH values from 6.0 to 9.0. Each 5 ml of the resin was recovered by decantation, mixed with 15 ml of the enzyme solution (protein 4.5 mg/ml, aspartase activity 55 U/ml) and kept at 5°C for 22 h.

For the determination of the optimal temperature, the enzyme solution (protein 3.8 mg/ml, aspartase 34 U/ml) in 6 void volumes was passed through the column packed with the resin at a space velocity of 0.5 at the temperatures of 0, 27, and 37°C.

For the determination of the optimal amount of the enzyme solution to charge on the resin, both batch-type and column-type experiments were carried out. In the batch-type experiment, 5 ml of Duolite A7 (pH 8.0) was mixed with various amounts of the enzyme solution (protein 6.3 mg/ml, aspartase 49 U/ml). In the column-type experiment, the enzyme solution (protein 2.9 mg/ml, aspartase 34 U/ml) in 3 to 9 void volumes was passed through the column packed with resin.

For the determination of the effect of the purity of the enzyme, a crude extract of the enzyme (specific activity 17.3 U/ml) and an enzyme

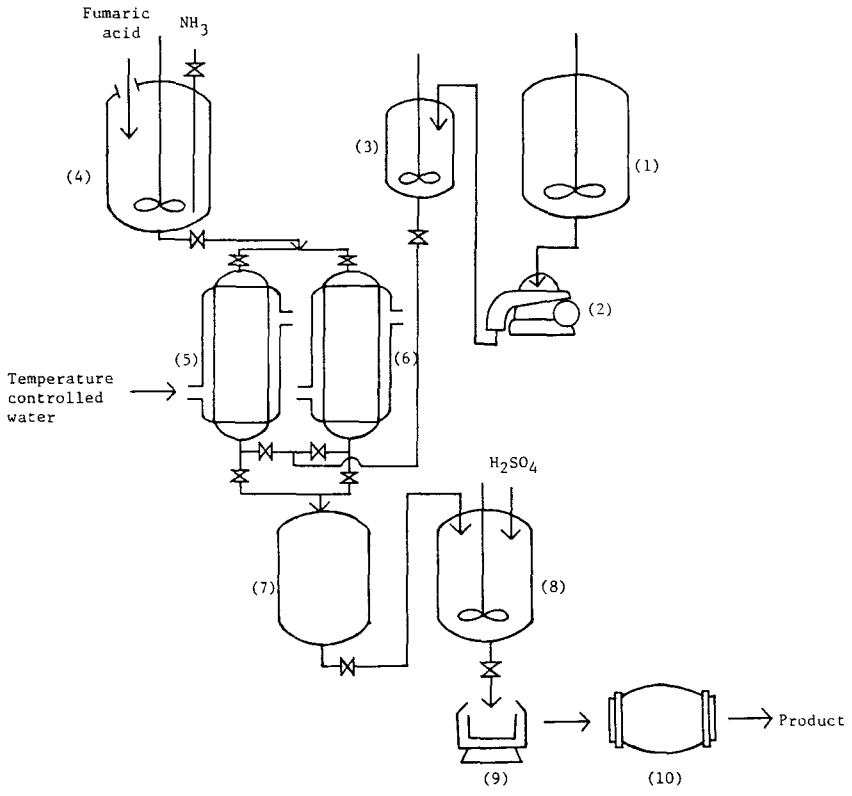

FIG. 1. Flow of immobilized aspartase process. (1) Fermenter for *E. coli* cells, (2) centrifuge, (3) enzyme extractor, (4) substrate solution tank, (5,6) immobilized enzyme column, (7) product solution reservoir, (8) crystallizer of aspartic acid, (9) centrifuge, (10) dryer.

preparation partially purified by ammonium sulfate fractionation and DEAE-Sephadex chromatography (specific activity 126 U/mg) were examined for immobilization by adsorption.

For the immobilization of the enzyme without an extraction process of the enzyme from the cells, 3, 5, and 10 g of the cells, respectively, were mixed directly with 5 ml of Duolite A7 in 0.1 M phosphate buffer at pH 7.5 and kept at 5°C for 18 h.

For the immobilization of the enzyme by covalent binding to the carriers, the following three methods were applied: immobilization to Duolite A7 through triazine derivative according to M. D. Lilly's method (5), immobilization to arylamino porous glass by diazo coupling, and immobilization to alkylamino porous glass by Schiff-base formation with glutaraldehyde according to H. H. Weetall's method (6,7).

The Apparatus for the Continuous Production of L-Aspartic Acid

The apparatus of the immobilized aspartase process used for the continuous production of L-aspartic acid is shown in Fig. 1. It consisted of a tank to extract the enzyme from the cells, a tank to dissolve and neutralize the substrate solution, two columns of immobilized aspartase (400 l in each column), a tank to store the reaction product solution, a crystallizer, a centrifuge, and a dryer.

Analysis

Fumaric Acid. Fumaric acid was determined titrimetrically by using M/10 $KMnO_4$ solution, which was consumed in proportion to the amount of fumaric acid.

L-Aspartic Acid. After paper chromatography of the sample in a developing solvent (*n*-butanol, acetic acid, water: 120, 30, 50), the spots corresponding to L-aspartic acid were colored with 0.2% ninhydrin reagent in acetone, extracted with methanol containing 0.4 ml of saturated aqueous $Cu(NO_3)_2$ solution in 1000 ml methanol, and assayed at 505–510 nm.

Protein. Proteins were determined according to the Folin–Lowry method (8).

Aspartase. Aspartase activity of the sample was determined as follows. Ammonium fumarate solution of pH 8.7, containing 200 mg fumaric acid per ml, was prepared. Eight milliliters of this solution were mixed with 2 ml of the enzyme solution, incubated at 37°C for 60 min, and the fumaric acid consumed was determined. One unit of the enzyme activity was defined as the amount of the enzyme that consumed 1 μmol of fumaric acid in 1 min. The activity of the immobilized aspartase was then determined. The substrate solution with pH adjusted to 8.7 by ammonia containing 120 g/l of fumaric acid and 1 mM $MnCl_2$, was passed down through the column packed with the immobilized enzyme and was temperature controlled to 37°C in a space velocity of 10. The activity was expressed as fumaric acid consumed (μmoles) by 1 ml of the immobilized enzyme in one minute.

RESULTS AND DISCUSSION

A Comparison of the Methods of Extraction of Aspartase from E. coli *Cells*

Various methods described under Materials and Methods above were examined to extract aspartase from the *E. coli* cells, and the results are summarized in Table 2. Any noticeable superiority was not found among

TABLE 2. **Selection of Resins for Adsorption of Aspartase**

Procedure	Supernatant[a]		
	Volume[b] (ml)	Activity[c]	Protein (mg/ml)
Freezing and thawing	6.1	19.0	4.7
Autolysis at room temperature for 20 h	5.9	21.2	4.8
Autolysis at 5°C for 20 h	6.0	19.0	5.2
Addition of Nimeen S-25, 200 μg/ml	6.0	19.0	5.6
Autolysis for 20 h after addition of Nimeen S-25, 200 μg/ml	6.0	22.4	5.6
Autolysis for 20 h after addition of sodium aspartate	6.1	19.0	5.6
Sonication for 5 min	6.0	19.5	6.0

[a] Obtained by centrifugation for 20 min at 11,000 g.
[b] Volume of supernatants obtained.
[c] Amount of ammonium fumarate consumed (mg/ml/h).

these methods. Hereafter the autolysis procedure detailed below was chosen for the extraction of aspartase. The effect of pH on the extraction by autolysis was examined, and the results are shown in Fig. 2. The enzyme activity was extracted maximally at pH 8.0 together with other proteins. A pH of 7 was more suitable for extraction because the specific activity of the extract prepared at this pH was the highest, and so the prepared enzyme was more stable.

In practice, 100 g of frozen cells was suspended in 200 ml of 0.1 M phosphate buffer of pH 7.0 containing 0.5 mM $MnCl_2$, was thoroughly mixed, kept at 5°C for 18 h, and centrifuged. The cell debris was washed by a small volume of the same buffer and the washed solution was combined with the previous supernatant, making the total volume of the solution 200 ml.

Immobilization of Aspartase

Selection of Resins. For the purification of aspartase, DEAE-Sephadex has been used by many researchers. Therefore, selection of carriers was made mostly among anion-exchange resins together with some adsorption-type resins. The results are shown in Table 1. Duolite A2, A4, A7, and S37 were found to be good carriers of aspartase. Hereafter, Duolite A7 was chosen with due consideration to the price and the physical properties of the resin.

The effect of pH on adsorption of aspartase on Duolite A7 was examined and the results obtained are shown in Fig. 3. As shown in the figure, there was no significant difference in the amount of protein adsorbed,

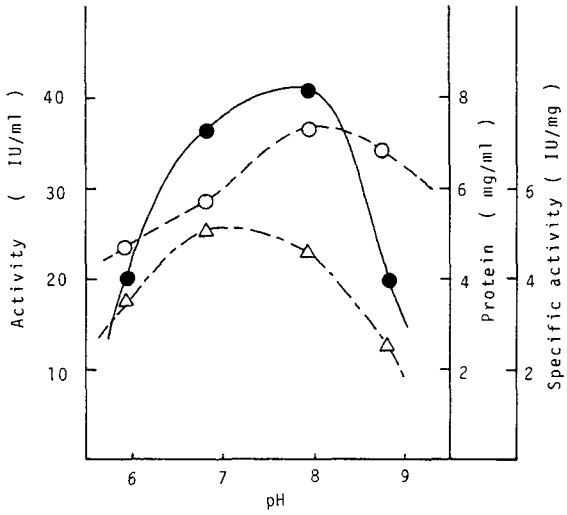

FIG. 2. Effect of pH on extraction of aspartase from *E. coli* cells. ●——●, Activity; ○---○, protein; △---△, specific activity.

but the activity of aspartase adsorbed at pH 8.0 was the highest. As for the effect of temperature (Fig. 4), the amount of protein adsorbed increased with the increasing temperature, but the activity at 27°C was the highest, the activity at 37°C being much less.

The optimal amounts of the enzyme to charge on the resins were determined in both batch-type and column-type experiments. As shown in Fig. 5, both protein and activity were bound to the resin to the maximum levels at a charge of 30 mg protein per ml of the resin in the batch-type

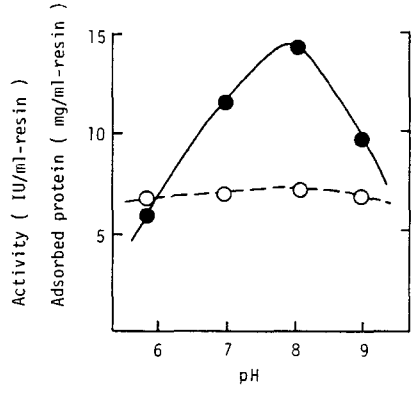

FIG. 3. Effect of pH on adsorption of aspartase to Duolite A7. ●——●, Activity; ○---○, adsorbed protein.

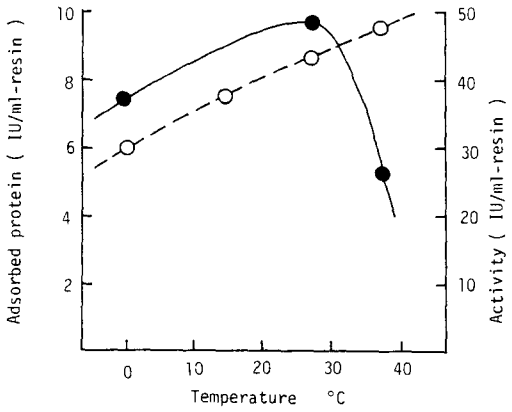

FIG. 4. Effect of temperature on adsorption of aspartase to Duolite A7. ●——●, Activity (IU/ml resin); ○- - -○, adsorbed protein (mg/ml resin).

operation. On the other hand, in the column-type operation, more protein could be bound by a further charge of the enzyme, but the activity was at a maximum at a charge of 12 mg protein per ml of the resin (Fig. 6).

In Table 3, the results are shown of the effect of purity of the enzyme charged on the resin. Comparable amounts of activity of both crude extract and partially purified enzyme were charged on the resins, respectively. If the

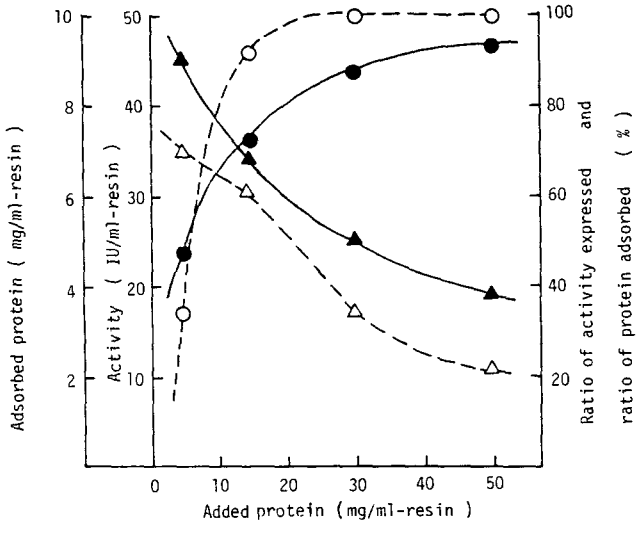

FIG. 5. Effect of the amounts of the enzyme protein to be charged on Duolite A7 on immobilization in a batch system. ●——●, Activity; ○- - -○, adsorbed protein; ▲——▲, ratio of activity expressed; △- - -△, ratio of protein adsorbed.

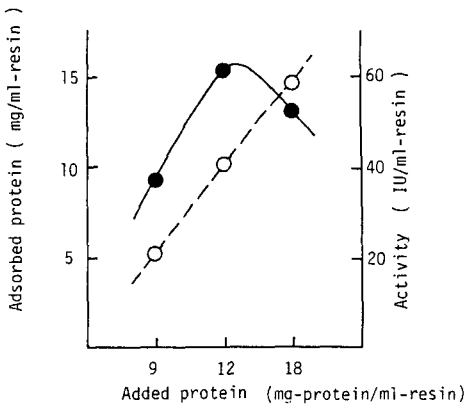

FIG. 6. Effect of the amounts of the enzyme protein to be charged on Duolite A7 on immobilization in a column system. ●——●, Activity; ○- - -○, adsorbed protein.

amount of charge was increased, the partially purified enzyme could undoubtedly be bound more to the resin, but such a procedure seemed impractical with respect to the lower recovery of the enzyme in purification.

In the following experiment, it was found that the step of enzyme separation could be simplified. As shown in Table 4, when the whole autolyzed cells were mixed with the resin, immobilized aspartase more active than that prepared with the supernatant of the extract could be obtained, presumably by accelerating the extraction of the enzyme from the cells.

Comparison of the Methods of Immobilization. For the comparison of the methods of immobilization, covalent binding of aspartase was examined. Among the four methods examined, covalent binding to porous glass through the Schiff base gave a preparation of the highest activity. A triazinyl

TABLE 3. **Effect of Purity of Aspartase on the Preparation of Immobilized Enzymes**

Added enzyme			Immobilized enzyme		
Specific activity (IU/mg protein)	Protein (mg/ml resin)	Total activity (IU/ml resin)	Protein (mg/ml resin)	Column activity (IU/ml resin)	Ratio of activity expressed (%)
17.3	22.2	384	7.5	51	27
126.0	3.0	380	2.7	74	21

TABLE 4. **Preparation of Duolite A7-ADS-Aspartase Using Whole Cells or Supernatant of the Extract of Cells of *E. coli***

Cell weight (g)	Enzyme used	Adsorbed protein (mg/ml resin)	Activity of immobilized enzyme (IU/ml resin)
3		8.2	82
5	Whole cell	9.8	125
10		12.0	145
3		4.5	40
5	Supernatant	8.2	71
10		10.3	118

derivative of Duolite A7 could bind more protein than unmodified Duolite A7, as shown in Table 5. However, there were no significant differences among the four preparations in stability of enzyme activity. The simplest and most practical procedure, designated as Duolite A7-ADS-aspartase, which is adopted hereafter, is schematically depicted in Fig. 7.

Properties of Immobilized Aspartase

Optimal pH. The effect of pH on the activity of Duolite A7-ADS-aspartase is shown in Fig. 8. The optimal pH was found to be in the range 8.5–8.8, similar to that of the native enzyme.

Thermal Stability. A 1 M solution of L-aspartate was passed through the column of Duolite A7-ADS-aspartase at temperatures of 37, 45, and 55°C at SV 0.5 for 100 h. As shown in Fig. 9, the immobilized aspartase was stable at 37°C but rather unstable at 45°C. However, the immobilized enzyme was more thermostable than the native enzyme.

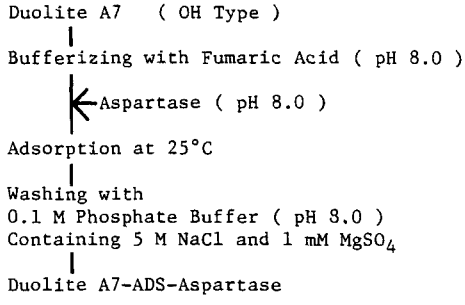

FIG. 7. Preparation flow of immobilized aspartase.

TABLE 5. **Comparison of Various Types of Immobilized Aspartase**

Carrier	Method of immobilization	Immobilized enzyme[b]			
		Protein (mg/ml resin)	Activity (IU/ml resin)	Ratio of activity expressed (%)	Half-life (days)
Duolite A7	ADS[a]	9.4	67	48.5	18
Duolite A7	CVB,[a] by triazine	14.7	65	30.3	20
Arylamino-porous glass	CVB, by diazo coupling	26.5	115	29.7	20
Alkylamino-porous glass	CVB, by Schiff-base formation	25.7	120	32.5	22

[a] ADS, adsorption; CVB, covalent binding.
[b] Enzyme added: protein, 4.5 mg/ml; activity, 66 IU/ml; specific activity, 14.6 IU/mg protein.

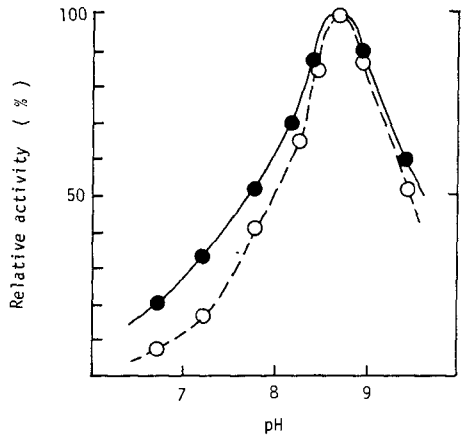

FIG. 8. Effect of pH on activity of Duolite A7-ADS-aspartase and native aspartase. ●——●, Immobilized enzyme; ○- - -○, native enzyme.

Effect of Mn^{++}. It is known that divalent metal ions such as Mg^{++} and Mn^{++} activate aspartase of *E. coli* (9). As shown in Fig. 10, the Duolite A7-ADS-aspartase was found to be effectively activated by 1 mM Mn^{++}, an amount far less than that required for the native enzyme.

Effect of the Concentration of the Substrate. The relationship between the flow rate of the substrate solution and the conversion rate to the product was studied in the range of fumaric acid, from 60 to 200 g/l. The results are shown in Fig. 11. The higher conversion ratio was attained with the lower

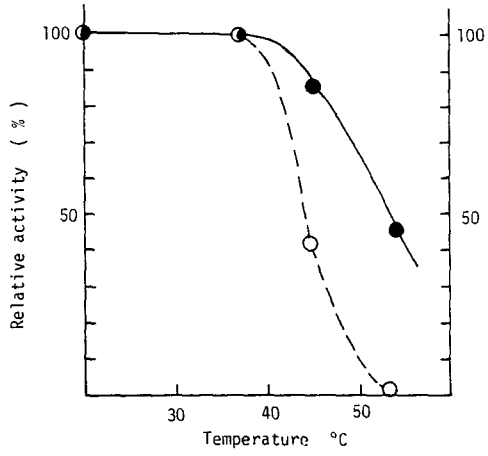

FIG. 9. Thermal stability of Duolite A7-ADS-aspartase and native aspartase. ●——●, Immobilized enzyme operated for 100 h; ○- - -○, native enzyme incubated for 5 h.

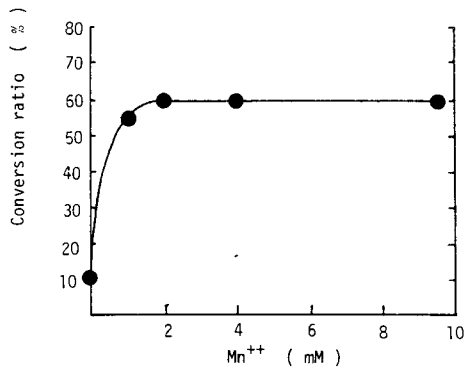

FIG. 10. Effect of the concentration of Mn^{++} on activity of Duolite A7-ADS-aspartase.

concentration of the substrate solution, whereas the total amount of the product increased with the increase of the concentration of the substrate. Conditions suitable for practical production were determined to be as follows: an ammonium fumarate solution (200–230 g of fumaric acid neutralized with ammonia and filled up to 1 l) is passed through the column in a space velocity around 0.75, avoiding the condition of crystallization of ammonium fumarate during the operation.

It is worthwhile to mention that aspartase immobilized to the resin by the present method did not release from the resin even by contact with 2 M substrate solution.

Continuous Production of L-Aspartic Acid with Duolite-ADS-Aspartase

Continuous production of L-aspartic acid was carried out on an industrial scale with the apparatus shown in Fig. 1. The substrate solution was composed of 220 g of fumaric acid neutralized to pH 8.7 with ammonia and 1mM $MnCl_2$, and was filled up to 1 l. The solution was passed downward through one of the columns at the rate of 300 l/h. The temperature was kept at 37°C at the outlet of the column by circulating cooling water into the jacket around the column since the enzyme reaction was exoergonic. The conversion ratio of the substrate was maintained at more than 99% by controlling the flow rate. When the flow rate decreased to 100–150 l/h, the substrate solution was shifted to pass through another column at a rate of 300 l/h.

The half-life of the activity of Duolite A7-ADS-aspartase was found to be 18 days in a laboratory experiment. A similar examination of half-life could not be performed on the factory scale. However, the conversion ratio of the substrate solution could be maintained at more than 99% during a

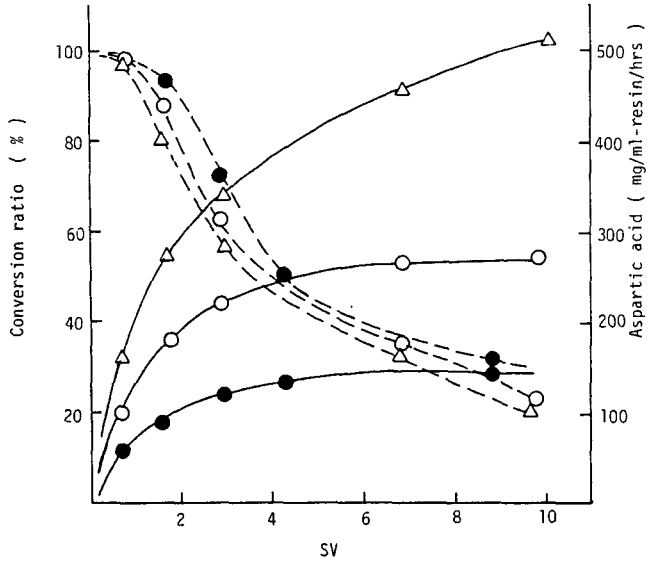

FIG. 11. Effect of flow rate (SV) and the concentration of fumaric acid on the conversion to aspartic acid by Duolite A7-ADS-aspartase in a column. —△—, Fumaric acid, 200 g/l; —○—, fumaric acid, 120 g/l; —●—, fumaric acid, 60 g/l; — — —, conversion ratio; ———, aspartic acid.

3-month continuous operation without decreasing the flow rate of the solution to less than SV = 0.75.

The product solution obtained from the column was so transparent that L-aspartic acid could be crystallized out simply by adjusting the pH of the solution to 2.8 and keeping it below 25°C for 3 h.

Economic Evaluation

As described above, a useful immobilized aspartase could be prepared by autolysis of *E. coli* cells in the presence of L-aspartate, mixing the autolysate directly with the resins, and removing the cell debris from the resins by washing with water. The resulting immobilized aspartase, Duolite A7-ADS-aspartase, was very stable and could convert 2 M ammonium fumarate solution almost completely to L-aspartate in a space velocity of 0.75, proving that the method of production of L-aspartic acid with this immobilized aspartase is an industrially practical process.

This immobilized aspartase process has made possible such innovations as reduction of the amount of enzyme consumed, compacting of the facilities, particularly that of the main reactor, automatic and continuous

operation of the enzyme reaction, simplification of the purification steps, and reduction of labor costs.

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