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REASON FOR HIGHER STABILITY OF ASPARTASE ACTIVITY OF IMMOBILIZED *ESCHERICHIA COLI* CELLS

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Summary

1. The reason for higher stability of the aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) activity of immobilized *Escherichia coli* cells in comparison with intact cells and immobilized aspartase was investigated.

2. *E. coli* cells were disrupted by sonication or autolysis, and fractionated into subcellular fractions by centrifugation. It was found that the aspartase of *E. coli* binds to cellular particles or membranes within cells.

3. These subcellular fractions were immobilized by the polyacrylamide gel method, and the operational stabilities of the immobilized preparations were compared. The aspartase activity of the immobilized precipitate fraction was more stable than that of the immobilized soluble fraction.

4. The aspartase activity of *E. coli* cells treated with detergents such as deoxycholate or Triton X-100 was unstable after immobilization by the polyacrylamide method.

5. Native aspartase was immobilized by covalent binding to CNBr-activated Sepharose, ionic binding to TEAE-cellulose and hydrophobic binding to aminopentyl-Sepharose. These immobilized aspartase preparations were then entrapped in the polyacrylamide gel lattice. Their operational stabilities were compared, and all immobilized aspartases prepared by binding native enzyme to water-insoluble carriers were found to be more stable than the aspartase entrapped in the polyacrylamide gel lattice.

6. From the above results, it is presumed that the stability of the aspartase may be enhanced by immobilization of whole cells, since the enzyme binds to cellular particles or membranes within cells.

Introduction

Recently, the immobilization of whole microbial cells and their applications have been the subject of increased interest.

In the previous papers [1—3], we reported the detailed conditions for the immobilization of whole cells of *Escherichia coli* having higher aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) activity, the enzymatic properties of the immobilized cells, and the basic studies on the engineering analysis of continuous production of L-aspartic acid from ammonium fumarate. On the basis of these studies, we, Tanabe Seiyaku Co., Ltd., have been operating this system industrially since 1973.

During these investigations we found that the aspartase of immobilized *E. coli* cells is very stable in comparison with the immobilized aspartase prepared by the polyacrylamide gel method [3,4]. The operational stability of enzyme activity of immobilized enzymes and immobilized microbial cells is one of the important factors influencing success of industrialization of the system. However, it has remained unknown why the aspartase of immobilized cells is very stable, so we carried out some experiments to solve these problems, and the results are presented in this paper.

Materials and Methods

Chemical compounds

Acrylamide monomer, potassium persulfate and deoxycholate were purchased from Katayama Chemical Industries Co., Ltd. (Osaka, Japan). *N,N'*-methylenebisacrylamide (BIS), β-dimethylaminopropionitrile (DMAPN) and Triton X-100 were purchased from Tokyo Kasei Kogyo Co., Ltd., (Tokyo, Japan). Sepharose 4B and 6B were purchased from Pharmacia Fine Chemical Co., (Uppsala, Sweden). TEAE-cellulose was purchased from Brown Co., (New Hampshire, U.S.A.). CNBr-activated Sepharose was prepared by the method of Axén et al. [5], and aminopentyl-Sepharose 4B (AP-Sepharose 4B) was prepared by the method of Kristiansen et al. [6].

E. coli cells

E. coli ATCC 11303 cells were obtained by the method previously reported [1] except for the type of corn steep liquor. In the case of ref. 1, the dried form was used, in this paper the liquid one.

Native aspartase

Native aspartase was prepared by the method previously reported [7], and 11.4 mg of partially purified preparation was obtained from 1 g (wet weight) of intact cells. The activity of this preparation was 1138 μmol/h per mg of protein (in the case of 1 M ammonium fumarate as a substrate) and 160 μmol/h per mg of protein (in the case of 0.05 M substrate) under standard assay conditions.

Preparation of various subcellular fractions

To obtain various subcellular fractions, *E. coli* cells were treated as follows.

(1) Autolysis and sonication of E. coli cells. E. coli cells were suspended (50% w/v) in 1 M ammonium fumarate (pH 8.5) containing 1 mM Mg^{2+}. The suspended cells were sonicated by an oscillator for 15 min at 9 kHz or autolyzed by incubation at 37°C for 24 h. After these treatments precipitates and soluble fractions were obtained by centrifugation (27 000 × *g*, 20 min).

(2) Treatment of E. coli cells with detergent. To 50% (w/v) *E. coli* cells suspended in 1 M ammonium fumarate (pH 8.5) containing 1 mM Mg^{2+}, deoxycholate or Triton X-100 were added to a final concentration of 0.25%, and then the suspension was incubated at 37°C for 24 h. After these treatments both precipitate and soluble fractions were obtained by centrifugation (27 000 $\times g$, 20 min).

Immobilization of intact cells and subcellular fractions

Intact cells and subcellular fractions were immobilized by polyacrylamide gel method as previously reported [1].

Immobilization of native aspartase

Immobilization of native aspartase was carried out as follows.

(1) Entrapment into polyacrylamide gel lattice. Native aspartase was immobilized by polyacrylamide gel method as previously reported [4].

(2) Covalent binding to CNBr-activated Sepharose 6B. To a mixture containing 24 ml of 50 mM sodium phosphate buffer (pH 8.0) and 6 ml of aspartase solution (protein: 67 mg), 6 g (packed weight) of CNBr-activated Sepharose 6B were added. The mixture was stirred for 16 h at 7°C and then filtered. The resultant immobilized aspartase was washed with the same buffer.

(3) Ionic binding to TEAE-cellulose. 2 ml of aspartase solution (protein; 32 mg) were added to 10 g (packed weight) of TEAE-cellulose suspended in 48 ml of 1 mM sodium phosphate buffer (pH 8.0). The mixture was stirred at 7°C for 14 h, and filtered. The resulted immobilized aspartase was washed with the same buffer.

(4) Hydrophobic binding to AP-Sepharose 4B. To 6 g (packed weight) of AP-Sepharose 4B suspended in 21 ml of 1 mM sodium phosphate buffer (pH 7.0), 20 ml of aspartase solution (protein: 320 mg) were added. Then the mixture was stirred at 7°C for 16 h, and filtered. The resulted immobilized aspartase was washed with the same buffer.

Entrapment of immobilized aspartase into polyacrylamide gel lattice

1 or 2 g (packed weight) of immobilized aspartase was suspended in 6 ml of distilled water. To the suspension, 2.25 g of acrylamide, 120 mg of BIS, 1.5 ml of 5% DMAPN and 1.5 ml of 1% $K_2S_2O_8$ were added, and the mixture was allowed to stand at below 40°C for 30 min. The resultant gel was made into particles of around 3 mm in diameter. The particles of gel were washed with 0.9% saline solution.

Standard assay of aspartase activity

(1) Native aspartase. A mixture of native aspartase obtained from 0.1 g (wet weight) of intact cells and 3 ml of 1 M or 0.05 M ammonium fumarate (pH 8.5) containing 1 mM Mg^{2+} was incubated at 37°C for 15 min. After the reaction had been stopped by immersion in a boling water bath for 5 min, the precipitates were removed by centrifugation. The L-aspartic acid formed in the supernatant was measured.

(2) Intact cells and subcellular fractions. The assay of intact cells was carried out by the method previously described [1]. The subcellular fractions were

assayed under the same conditions as for intact cells except for using the fractions corresponding to 1 g (wet weight) of intact cells.

(3) Immobilized preparations. The assay of immobilized cells and immobilized subcellular fractions was carried out by the method previously described [1] except for using immobilized preparations corresponding to 1 g (wet weight) of intact cells. Immobilized aspartases were assayed as follows. For the immobilized aspartases obtained by ionic and hydrophobic binding methods, a mixture of 1 g (wet weight) of the immobilized enzymes and 30 ml of 0.05 M ammonium fumarate (pH 8.5) containing 1 mM Mg^{2+} was incubated at 37°C for 60 min with shaking. After the reaction, the immobilized enzyme was filtered off, and the L-aspartic acid formed in the filtrate was measured. The other immobilized aspartases were assayed under the same conditions as the above-mentioned method except that 1 M ammonium fumarate (pH 8.5) containing 1 mM Mg^{2+} as a substrate solution was employed.

(4) Estimation of L-aspartic acid. L-Aspartic acid was measured by bioassay using *Leuconostoc mesenteroides* P-60 [8].

(5) Enzyme activity. Aspartase activity was expressed in terms of micromoles of L-aspartic acid formed per hour.

(6) Estimation of half-life of a column packed with immobilized preparations. For determination of aspartase activity of a column the effluent was collected as a sample at a flow rate giving between 20 and 30% of maximum conversion rate and the L-aspartic acid produced in the effluent was measured. The half-life was estimated by assuming exponential decay of activity vs. time.

Results

Stability of aspartase from intact cells, immobilized cells and immobilized enzyme

The operational stabilities of aspartase from intact cells, immobilized cells and immobilized enzyme were investigated by the column method, and their half-lives were estimated as shown in Table I. The stability of immobilized cells was higher than those of both intact cells and immobilized aspartase. These results suggest that the aspartase becomes unstable after being extracted from *E. coli* cells even if it is immobilized by polyacrylamide gel.

Relation between location and stability of aspartase in cells

In order to clarify the difference of stability between immobilized aspartase and immobilized cells, the location of aspartase protein in *E. coli* cells was investigated by fractionation of the cells as described below. Subcellular fractions thus obtained were immobilized by polyacrylamide gel and their operational stabilities were investigated.

Preparation of subcellular fractions and their immobilization. Intact cells of *E. coli* were autolyzed or sonicated in a solution of 1 M ammonium fumarate (pH 8.5) containing 1 mM Mg^{2+}, and their precipitate and soluble fractions were obtained from the autolysates or sonicates by centrifugation. The resultant subcellular fractions were immobilized by polyacrylamide gel and the aspartase activity of the obtained immobilized preparations were compared (Table II).

TABLE I

STABILITY OF IMMOBILIZED ASPARTASE, IMMOBILIZED CELLS AND INTACT CELLS

In the case of immobilized aspartase and immobilized cells, a solution of 1 M ammonium fumarate (pH 8.5, 1 mM Mg^{2+}) was passed through the column packed with 30 ml of immobilized preparations at a flow rate of 13.8 ml/h at 37°C. The half-life was estimated by the method described in the text. In the case of intact cells, 1 g of intact cells was suspended in 5 ml of 1 M ammonium fumarate (pH 8.5, 1 mM Mg^{2+}). The cell suspension was enclosed in a Visking tube, and the tube was immersed in 1 l of the substrate solution at 37°C. After appropriate incubation, a part of the intact cells was taken out from the tube, and the remaining aspartase activity was determined by the standard assay method. The half-life was estimated by extrapolation of the daily results.

Preparation	Stability (Half-life, days)
Immobilized aspartase *	20
Immobilized cells	120
Intact cells	10

* Prepared by polyacrylamide method

As shown in Table II, most of the aspartase was localized in the precipitate fraction of autolysate or the insoluble fraction of sonicate. The yield of aspartase activity of the immobilized precipitates was higher than those of the immobilized soluble fractions in both treatments for disruption of cells. Further, in the case of autolysed cells, the aspartase activities of autolysate and precipitate fraction were found to be increased by immobilization. This activation is considered to be caused by an increase in the permeability to substrate and/or product due to immobilization as in the case of activated immobilized cells [1].

Stability of immobilized subcellular fractions. The operational stabilities of aspartase activity of these immobilized subcellular fractions were investigated by a column method, as follows. A solution of 1 M ammonium fumarate (pH

TABLE II

IMMOBILIZATION OF *E. COLI* CELL FRACTIONS, AND THEIR ASPARTASE ACTIVITIES

Experimental conditions are described in the text.

Preparation	Aspartase activity (μmol/h *)		Yield of activity after immobilization (%)
	Native	Immobilized	
Intact cells	1 820	1 310	72
		18 400 **	1011
Sonicated cells			
sonicate	16 200	16 300	101
ppt. fraction	4 970	4 970	100
soluble fraction	13 240	7 520	57
Autolyzed cells			
autolysate	13 000	18 400	142
ppt. fraction	10 820	14 440	133
soluble fraction	2 420	460	19

* Corresponding to 1 g (wet weight) of intact cells.

** Immobilized cells after activation by incubating with substrate solution at 37°C for 24 h. On the activation see ref. 1.

TABLE III

OPERATIONAL STABILITY OF ASPARTASE ACTIVITY OF VARIOUS IMMOBILIZED PREPARATIONS

A solution of 1 M ammonium fumarate (pH 8.5, 1 mM Mg^{2+}) was passed through columns packed with 10 ml of immobilized preparations at 37°C at the flow rate of 9.6 ml/h. The half-life was estimated by the method described in the text.

Immobilized preparation	Stability (Half-life, days)
Cells	120
Sonicated cells	
sonicate	38
ppt. fraction	74
soluble fraction	6
Autolyzed cells	
autolysate	44
ppt. fraction	54
soluble fraction	6

8.5) containing 1 mM Mg^{2+} was passed through columns packed with immobilized preparations at 37°C for a long period, and the preparations' half-lives were estimated (Table III). The stabilities of the immobilized sonicate, autolysate and subcellular fractions were lower than that of the immobilized intact cells. However, the aspartase activities of the immobilized precipitate fractions were more stable than those of the immobilized soluble fractions.

Detergent treatment of cells and immobilization of subcellular fractions. To solubilize the aspartase bound to particles or membranes of intact cells, *E. coli* cells were autolyzed in the presence of detergents such as deoxycholate or Triton X-100, and then the treated cell suspensions were fractionated into subcellular by fractions centrifugation. The precipitate and soluble fractions thus obtained were immobilized by polyacrylamide gel, and their activities were compared with those of native fractions (Table IV).

TABLE IV

IMMOBILIZATION OF FRACTIONS OF *E. COLI* CELLS TREATED WITH DETERGENTS, AND THEIR ASPARTASE ACTIVITIES

Experimental conditions are described in the text.

Preparation	Aspartase activity (μmol/h *)		Yield of activity after immobilization (%)
	Native	Immobilized	
Deoxycholate-treated cells			
autolysate	25 680	20 180	79
ppt. fraction	11 080	15 220	137
soluble fraction	10 550	3 140	30
Triton-treated cells			
autolysate	24 420	21 840	89
ppt. fraction	12 050	16 970	141
soluble fraction	11 320	6 190	55

* Corresponding to 1 g (wet weight) of intact cells.

TABLE V

STABILITY OF ASPARTASE ACTIVITY OF VARIOUS IMMOBILIZED PREPARATIONS TREATED WITH DETERGENTS FOR A LONG INCUBATION PERIOD

Immobilized preparations were stood in a solution of 1 M ammonium fumarate (pH 8.5, 1 mM Mg^{2+}) at 25°C, and their remaning activities were determined every day by the method described in the text. The half-life was estimated by extrapolation of the daily results.

Immobilized preparation	Stability (Half-life, days)
Deoxychelate-treated cells	
autolysate	9
ppt. fraction	13
soluble fraction	8
Triton-treated cells	
autolysate	9
ppt. fraction	9
soluble fraction	5

As shown in Table IV, the activity of the precipitate fraction was increased by immobilization. The reason is considered to be the same as in the case of Table II. The activity of soluble fraction was decreased by immobilization.

Stability of detergent treated immobilized subcellular fractions. The stabilities of aspartase activity of the immobilized subcellular fractions treated with detergent were investigated by standing at 25°C for long periods, and their half-lives were estimated (Table V). The aspartase activities of the precipitate fraction and detergent-treated autolyzed cells were unstable in comparison with that of the cells without detergent treatment. From these results, it is suggested that the aspartase becomes unstable by solubilization from cellular particles or membranes.

Model experiment

From the results described above it was presumed that the aspartase is fixed to cellular particles or membranes within cells and that its stability may be enhanced by immobilization.

In order to clarify this presumption, the following model experiments were carried out.

Preparation of immobilized aspartases and entrapped-immobilized aspartases. Native aspartase was immobilized by covalent binding to CNBr-activated Sepharose 6B, ionic binding to TEAE-cellulose and hydrophobic binding to AP-Sepharose 4B. Further, these immobilized aspartase preparations were entrapped into a polyacrylamide gel lattice. Their aspartase activities were estimated, and the results are shown in Table VI. After entrapment in a polyacrylamide gel lattice, the activities of immobilized aspartases were significantly reduced.

Stability of immobilized aspartases and entrapped-immobilized aspartases. For three kinds of immobilized aspartases prepared by binding native enzyme to water-insoluble carriers and two kinds of entrapped-immobilized aspartases, stabilities were investigated by the same column method as described in Table III and compared with that of immobilized aspartase prepared by direct entrapment of native enzyme in a polyacrylamide gel lattice. The results were summarized with respect to half-life (Table VII).

TABLE VI

ASPARTASE ACTIVITIES OF VARIOUS IMMOBILIZED AND ENTRAPPED IMMOBILIZED ASPARTASE PREPARATIONS

Percentages in brackets indicate activity relative to native enzyme used. Assays were carried out as described in the text by using 1 M ammonium fumarate (pH 8.5, 1 mM Mg^{2+}) as substrate for covalent binding preparation and 0.05 M ammonium fumarate (pH 8.5, 1 mM Mg^{2+}) for ionic and hydrophobic binding preparations.

Preparation	Aspartase activity (μmol/h)		
	Native aspartase	Immobilized aspartase	Entrapped-immibilized aspartase
CNBr-activated Sepharose-aspartase (covalent binding)	75 790 (100%)	43 310 (57.1%)	1 814 (2.3%)
TEAE-cellulose-aspartase (ionic binding)	5 112 (100%)	1 500 (29.3%)	51 (1.0%)
AP-Sepharose-aspartase (hydrophobic binding)	51 430 (100%)	5 246 (10.2%)	645 (1.3%)

As shown in Table VII, the CNBr-activated Sepharose-aspartase complex immobilized by the covalent binding method and the entrapped-CNBr-activated Sepharose-aspartase complex were more stable than aspartase entrapped by a polyacrylamide gel lattice. A TEAE-cellulose-aspartase complex immobilized by the ionic binding method, an AP-Sepharose-aspartase complex immobilized by hydrophobic binding and an entrapped AP-Sepharose-aspartase complex were also more stable at a low concentration of dissolved substrate than enzyme entrapped by the polyacrylamide method. However, when a high concentration of substrate solution was passed through the column packed with the entrapped AP-Sepharose-aspartase complex, its stability was the same as that of the entrapped aspartase (Table VII). This result suggests that the aspartase may be released from AP-Sepharose within the gel lattice by high ionic strength of sub-

TABLE VII

OPERATIONAL STABILITY OF VARIOUS IMMOBILIZED ASPARTASE PREPARATIONS

A solution of 0.05 M or 1 M ammonium fumarate (pH 8.5, 1 mM Mg^{2+}) was passed through columns packed with 30 ml of immobilized aspartase preparations at flow rate of 13.8 ml/h at 37°C. The half-life was estimated by the method described in the text.

Immobilized aspartase preparation	Concentration of substrate (M)	Stability (Half-life, days)
CNBr-activated Sepharose-aspartase	1	131
Entrapped CNBr-activated Sepharose-aspartase	1	108
TEAE-cellulose-aspartase	0.05	46
AP-Sepharose-aspartase	0.05	34
Entrapped AP-Sepharose-aspartase	1	17
	0.05	34
Entrapped aspartase	1	19
	0.05	12

strate solution, and the stability is lowered. The stability experiment was not carried out for entrapped TEAE-cellulose-aspartase complex due to the low activity of this complex as shown in Table VI.

Discussion

We compared the stability of aspartase activity of immobilized cells, immobilized enzyme and intact (i.e. not immobilized) cells; the results are shown in Table I. The initial activities of immobilized cells and intact cells were almost the same, but the activity of intact cells rapidly decreased whereas that of immobilized cells was very stable. These results indicate that the immobilized cells are more advantageous for industrial production of L-aspartic acid than the intact cells and the immobilized enzyme.

As for the mechanism of this stabilization, we considered that the aspartase of immobilized cells is stable due to the binding of some cellular particles or membranes within the cells. To confirm this assumption, we carried out some experiments as follows. After sonication or autolysis of intact cells, both precipitate and soluble fractions were obtained, and they were immobilized separately by the polyacrylamide method (Table II). The stabilities of aspartase activity of these immobilized fractions were investigated, and it was found that the activity of the immobilized precipitate fraction is very stable, whereas that of immobilized soluble fraction is very unstable (Table III). After intact cells had been preliminarily treated with detergents and autolyzed, the precipitate and soluble fractions obtained were immobilized separately by the polyacrylamide method (Table IV), and the stabilities of these preparations were found to be low and almost the same (Table V). That is, the results indicate that the aspartase becomes unstable upon solubilization. In other words, the binding of aspartase to cellular particles and/or membranes may play an important role in stabilization of the enzyme.

For further confirmation of this assumption, we carried out model experiments. As shown in Table VII, all immobilized preparations obtained by binding native aspartase to water insoluble carriers were much stable than the aspartase entrapped into a polyacrylamide gel lattice. Further, it is well known that when an enzyme is attached to polymers such as cellulose, agarose, dextran etc., its stability is enhanced in comparison with native enzyme. From these results, the above assumption is considered to be correct. Therefore, it is clear that in some cases the technique of immobilation of microbial cells is better for a continuous enzyme reaction than that of enzyme immobilization.

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References

1 Chibata, I., Tosa, T. and Sato, T. (1974) Appl. Microbiol. 27, 878—885
2 Tosa, T., Sato, T., Mori, T. and Chibata, I. (1974) Appl. Microbiol. 27, 886—889

3 Sato, T., Mori, T., Tosa, T., Chibata, I., Furui, M., Yamashita, K. and Sumi, A. (1975) Biotech. Bioeng. 17, 1797—1804
4 Tosa, T., Sato, T., Mori, T., Matuo, Y. and Chibata, I. (1973) Biotech. Bioeng 15, 69—84
5 Axén, R., Porath, J. and Ernback, S. (1967) Nature, 214, 1302—1304
6 Kristiansen, T., Einarson, M., Sundberg, L. and Porath, J. (1970) FEBS Lett. 7, 264—296
7 Tosa, T., Sato, T., Sano, R., Yamamoto, K., Matuo, Y. and Chibata, I. (1974) Biochim. Biophys. Acta 334, 1—11
8 Henderson, L.M. and Snell, E.E. (1948) J. Biol. Chem. 172, 15—29