Biochimica et Biophysica Acta, 570 (1979) 179—186

BBA 68819

IMMOBILIZATION OF *ESCHERICHIA COLI* CELLS CONTAINING ASPARTASE ACTIVITY WITH κ-CARRAGEENAN

ENZYMIC PROPERTIES AND APPLICATION FOR L-ASPARTIC ACID PRODUCTION

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(Received February 5th, 1979)

Key words: κ-Carrageenan, Immobilized aspartase, L-Aspartic acid production; (E. coli)

Summary

Whole cells of *Escherichia coli* having high aspartase (L-asparate ammonia-lyase, EC 4.3.1.1) activity were immobilized by entrapping into a κ-carrageenan gel. The obtained immobilized cells were treated with glutaraldehyde or with glutaraldehyde and hexamethylenediamine.

The enzymic properties of three immobilized cell preparations were investigated, and compared with those of the soluble aspartate. The optimum pH of the aspartase reaction was 9.0 for the three immobilized cell preparations and 9.5 for the soluble enzyme. The optimum temperature for three immobilized cell preparations was 5—10°C higher than that for the soluble enzyme. The apparent K_m values of immobilized cell preparations were about five times higher than that of the soluble enzyme. The heat stability of intact cells was increased by immobilization.

The operational stability of the immobilized cell columns was higher at pH 8.5 than at optimum pH of the aspartase reaction. From the column effluents, L-aspartic acid was obtained in a good yield.

Abbreviations: cell type 1, *E. coli* cells immobilized with κ-carrageenan; cell type 2, *E. coli* cells immobilized with κ-carrageenan and then treated with glutaraldehyde; cell type 3, *E. coli* cells immobilized with κ-carrageenan and then treated with glutaraldehyde and hexamethylenediamine.

Introduction

In previous papers [1—3], we reported that *Escherichia coli* cells immobilized with polyacrylamide can be advantagiously used for continuous production of L-aspartic acid from ammonium fumarate by the action of aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1). In 1973, we succeeded in converting this to an industrial scale.

For further improvement of this system, we extensively investigated immobilization of *E. coli* cells by a new technique using κ-carrageenan (a polysaccharide prepared from seaweed) [4].

In order to clarify the conditions for continuous production of L-aspartic acid, the basic enzymic properties of the immobilized *E. coli* cells with κ-carrageenan, and the continuous method for the production of L-aspartic acid from ammonium fumarate were investigated by using columns packed with the immobilized cell preparations.

Materials and Methods

Materials

κ-Carrageenan was obtained from Sansyo Co., Ltd. (Osaka, Japan). Meast, autolyzate of brewer's yeast, was obtained from Ebios Yakuhin Kogyo, Co., Ltd. (Osaka, Japan). Glutaraldehyde was purchased from Katayama Chemical Industries Co., Ltd. (Osaka, Japan). Hexamethylenediamine was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Other reagents were obtained from Katayama Chemical Industries Co., Ltd. (Osaka, Japan). Soluble aspartase was prepared by the method previously reported [5], and 36.6 mg partially purified preparation was obtained from 1 g (wet weight) *E. coli* cells. The activity of this preparation was 1670 unit/mg protein under standard assay conditions.

Preparations of Immobilized E. coli cells

E. coli ATCC 11303 was aerobically grown in a medium (pH 7.0) containing 0.5% ammonium fumarate, 1.14% fumaric acid, 2% corn steep liquor, 2% Meast, 0.2% KH_2PO_4 and 0.05% $MgSO_4 \cdot 7H_2O$ at 37°C for 20 h with shaking. The cells were collected by centrifugation, and 8 g (wet weight) of the cells were suspended in 8 ml 0.9% NaCl at 40°C. 2.07 g κ-carrageenan were dissolved in 45 ml 0.9% NaCl at 80°C, and then the temperature of the solution was brought to 45°C. Both solutions were mixed, and mixture cooled at around 10°C for 30 min. In order to increase the gel strength, the gel was soaked in cold 0.3 M KCl. After this treatment, the resultant stiff gel was made to cubic gel of 3 × 3 × 3 mm. From 1 g (wet weight) of intact cells, 12 ml immobilized cells (cell type 1) were obtained and aspartase activity was 56 340 units/g cells (4700 units/ml gel) under standard assay conditions after activation by incubating with 1 M ammonium fumarate (pH 8.5) containing 1 mM Mg^{2+} at 37°C for 72 h.

Treatment of immobilized E. coli cells with bifunctional reagents

Treatment of the immobilized cells with glutaraldehyde (cell type 2) or with

glutaraldehyde and hexamethylenediamine (cell type 3) was carried out according to the procedure previously described [4]. The aspartase activity after activation was 37 460 units/g cells (3120 units/ml gel) for the former preparation and 49 400 units/g cells (4120 units/ml gel) for the latter.

Standard assay of aspartase activity

Unless otherwise noted, standard assay of aspartase activity was carried out as follows.

Native aspartase. 0.1 ml soluble aspartase (4.8 mg/ml) and 9.9 ml 1.5 M ammonium fumarate (pH 9.0), 1 mM Mg^{2+} were incubated at 37°C for 10 min. After the reaction was stopped by immersion in a boiling waterbath for 15 min, the precipitates were removed by centrifugation, and the L-aspartic acid formed in the supernatant was measured.

Intact cells. The assay of intact cells was carried out by the method previously described [4].

Immobilized cell preparations. A solution of 1.5 M ammonium fumarate (pH 9.0), 1 mM Mg^{2+} was applied to column packed with 6 ml (corresponding to 0.5 g wet intact cells) of the immobilized cell preparations at 37°C at flow rate of space velocity (=14—15 h^{-1}) *. The L-aspartic acid in the effluent was measured.

Estimation of L-aspartic acid. L-Aspartic acid was measured by bioassay using *Leuconostoc messenteroides* P-60 [6].

Enzyme activity. One unit of enzyme activity was defined as the amount of enzyme giving 1 μmol L-aspartic acid/h.

Estimation of half-life of column packed with immobilized cell preparations

The half-life of immobilized cell preparations was estimated by the method previously described [7].

Results

Enzymic properties of various immobilized E. coli cell preparations

In order to clarify the suitable conditions for continuous enzyme reaction, some basic enzymic properties of cells of type 1, 2, and 3 were investigated in comparison with those of soluble aspartase or intact cells.

Effect of pH on reaction rate. The effect on pH of the initial rate of formation of L-aspartic acid from ammonium fumarate by the three immobilized cell preparations was compared with that by the soluble aspartase. As shown in Fig. 1, the pH-activity curves on the aspartase reaction are approximately the same for the three immobilized cell preparations, but their curves shift about 0.5 pH unit toward acidic pH values in comparison with that of the soluble enzyme. The optimal pH was 9.0 for the three immobilized cell preparations and 9.5 for the soluble enzyme.

Effect of temperature on reaction rate. The effect of temperature on the aspartase reaction by the three immobilized cell preparations and the soluble

* Space velocity is the volume of liquid passing through a given volume of immobilized cell preparations in 1 h divided by the latter volume.

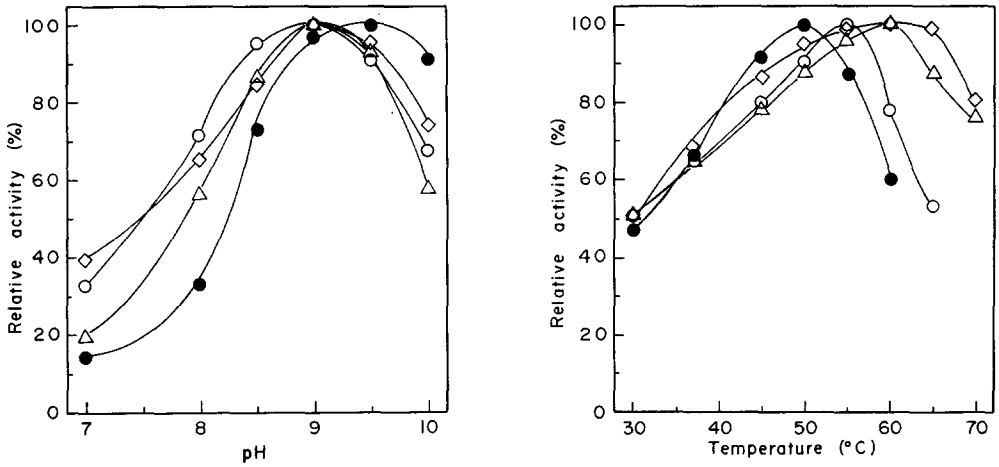

Fig. 1. Effect of pH on the rate of formation of L-aspartic acid. The pH of the substrate was varied. An 1 M ammonium fumarate/1 M NH_4Cl solution (adjusted to indicated pH with 5 N NaOH), 1 mM Mg^{2+} was used as substrate. The enzyme activities are expressed as percentage of the maximum activity attained at the appropriate optimum pH of enzyme preparations. ●———●, soluble aspartase; ○———○, cell type 1; △———△, cell type 2, ◇———◇, cell type 3.

Fig. 2. Effect of temperature on the rate of formation of L-aspartic acid. The temperature of the incubation was varied and the enzyme activities are expressed as percentage of the maximum activity attained at the appropriate optimum temperature of enzyme preparations. ●———●, soluble aspartase; ○———○, cell type 1; △———△, cell type 2; ◇———◇, cell type 3.

aspartase was investigated, and the results are shown in Fig. 2. The figure indicates that the optimum temperatures for the three immobilized cell preparations are 5—10°C higher than that for the soluble enzyme. For the estimation of the apparent activation energy of these preparations, the results shown in Fig. 2 were plotted by the method of Arrhenius. The apparent activation energy was calculated to be 5480 cal/mol for cell type 1, 5220 cal/mol for cell type 2, 5330 cal/mol for cell type 3 and 8290 cal/mol for the soluble enzyme, respectively.

Michaelis constants. The effect of substrate concentration on the aspartase reaction by the three immobilized cell preparations and the soluble aspartase was investigated, and the results obtained were plotted by the method of Lineweaver and Burk [8] for the estimation of the apparent Michaelis constant (K_m). From the results, the apparent K_m was calculated to be 0.85 M for cell type 1, 0.72 M for cell type 2, 0.71 M for cell type 3 and 0.15 M for the soluble enzyme, respectively.

Heat stability. The heat stability of cell type 1, cell type 2 and cell type 3 was compared with that of the soluble aspartase and of the intact cells at elevated temperature for 30 min. The results are shown in Fig. 3. After treatment at 60° C for 30 min, the aspartase activities of the soluble enzyme and the intact cells were decreased to below 10% of the respective initial activity, but the three immobilized cell preparations retained about 40% of their initial activities.

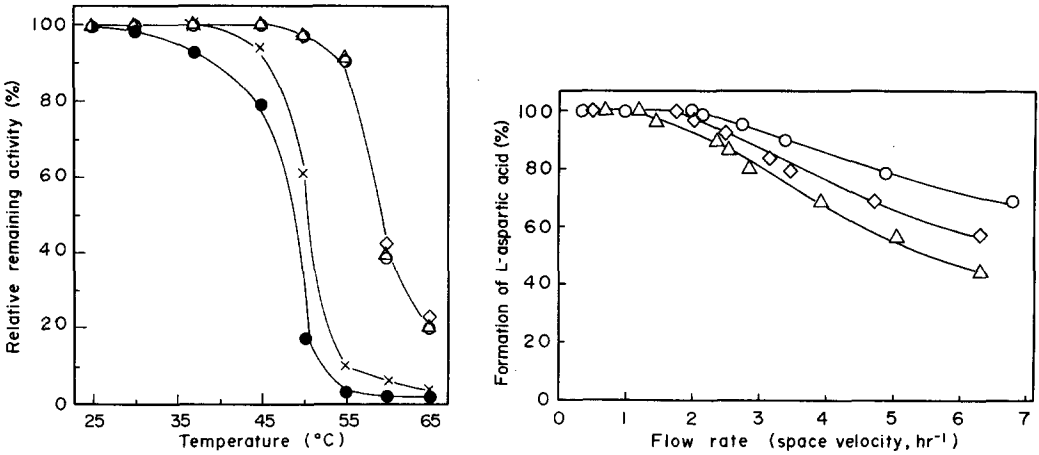

Fig. 3. Heat stability. In the case of the soluble aspartase 0.1 ml enzyme solution (4.8 mg/ml) and 0.1 ml of 0.1 M potassium phosphate buffer (pH 7.0) was treated at the indicated temperature for 30 min, rapidly cooled, and 9.8 ml 1.5 M ammonium fumarate (pH 9.0), 1 mM Mg^{2+} was added. In the case of the intact cells, 2 ml intact cell suspension (0.25 mg cells/ml of the same buffer) was treated at the indicated temperature for 30 min, rapidly cooled, and 30 ml 1.5 M ammonium fumarate (pH 9.0), 1 mM Mg^{2+} was added. This mixture was incubated under standard assay conditions, and the L-aspartic acid formed was measured. In the case of the immobilized cell preparations, 6 ml (corresponding to 0.5 g wet intact cells) of the immobilized cell preparations and 10 ml same buffer was treated at the indicated temperature for 30 min and rapidly cooled. The treated immobilized cells preparations were separately packed into columns, and the enzyme activities were measured. ●———●, soluble aspartase; X———X, intact cells; ○———○, cell type 1; △———△, cell type 2; ◇———◇, cell type 3.

Fig. 4. Relationship of flow rate of substrate and formation of L-aspartic acid from ammonium fumarate. A solution of 1 M ammonium fumarate (pH 8.5), 1 mM Mg^{2+} was applied to the column packed with 12 ml (corresponding to 1 g wet intact cells) immobilized cell preparation as 37° C at the indicated flow rates. The L-aspartic acid formed in the effluents was measured. ○———○, cell type 1; △———△, cell type 2; ◇———◇, cell type 3.

Continuous production of L-aspartic acid

The conditions for continuous production of L-aspartic acid were investigated by using columns packed with the immobilized cell preparations.

Effect of pH of substrate solution on operational stability. A substrate solution of varous pH was passed through the columns packed with the immobilized cell preparations at 37°C for long period, and the half-lives of these preparations were estimated as shown in Table I. These three immobilized preparations were very stable at below pH 8.5, and their stabilities were markedly reduced with increase of pH of the substrate solution.

Effect of flow rate of substrate solution on formation of L-aspartic acid. The typical data on the relation between the flow rate of the solution of 1 M ammonium fumarate (pH 8.5), 1 mM Mg^{2+} and the extents of the reaction are shown in Fig. 4. The figure shows that the flow rates of space velocity (SV) = 2.04 h^{-1} for cell type 1, SV = 1.20 h^{-1} for cell type 2 and SV = 1.75 h^{-1} for cell type 3 are the maximal for the complete conversion of 1 M ammonium fumarate to L-aspartic acid. If a solution of 1.5 M ammonium fumarate (pH 8.5), 1 mM Mg^{2+} was used as a substrate solution, the maximal flow rate enable the complete conversion was SV = 1.2 h^{-1} for cell type 1, SV = 0.8 h^{-1} for cell type 2 and SV = 1.1 h^{-1} for cell type 3, respectively.

TABLE I

EFFECT OF pH OF SUBSTRATE ON OPERATIONAL STABILITY

1.5 M ammonium fumarate (indicated pH, 1 mM Mg^{2+}) was passed through columns packed with 12 ml immobilized cell preparations at flow rate of $SV = 0.8\ h^{-1}$ at 37°C.

pH of substrate	Stability at 37°C (half-life, days)		
	cell type 1	cell type 2	cell type 3
7.0	95	238	693
8.0	93	240	675
8.5	92	237	682
9.0	54	132	391
9.5	27	71	136
10.0	11	21	48

Production of L-aspartic acid. A solution of 1.5 M ammonium fumarate (pH 8.5), 1 mM Mg^{2+} was passed through a column (2.5 × 30 cm) packed with the immobilized cell preparation (cell type 3) at a flow rate of 90 ml/h at 37°C. 1 l of the effluent was adjusted to pH 2.8 with 60% H_2SO_4 at 90°C and then cooled at 15°C. The L-aspartic acid crystallized out was collected by filtration and washed with water. The yield was 179.6 g (90% of theoretical), $[\alpha]_D^{20} = +25.5$ ($c = 8$ in 6 N HCl).

Discussion

In previous papers [9,10], we found that κ-carrageenan is a suitable polymer for immobilization of many kinds of enzymes and microbial cells. Further, for continuous production of L-aspartic acid from ammonium fumarate, we investigated immobilization of *E. coli* containing high aspartase activity by using κ-carrageenan and revealed the suitable conditions [4].

In addition, in order to clarify the most suitable conditions for continuous production of L-aspartic acid, the basic enzymic properties of the immobilized cell preparations with κ-carrageenan were investigated in comparison with those of the soluble enzyme or the intact cells. The results are presented in this paper.

The apparent K_m values of the three immobilized cell preparations were about five times higher than that of the soluble enzyme. This increase of K_m value may be due to the change of permeation rate of substrate or product through the carrageenan gel matrix. This is supported from the results shown in Fig. 5.

Other enzymic properties were also changes by immobilization. The optimum pH of the three immobilized cell preparations on enzyme reaction was slightly shifted (about 0.5 pH unit) toward the acidic pH value in comparison with that of the soluble enzyme (Fig. 1). The precise reason for this apparent pH shift toward the acidic side is not known.

Heat stability of immobilized cell preparations was markedly increased in comparison with that of the soluble enzyme or intact cells (Fig. 3). The reason of higher stability of the immobilized preparation with κ-carrageenan is not

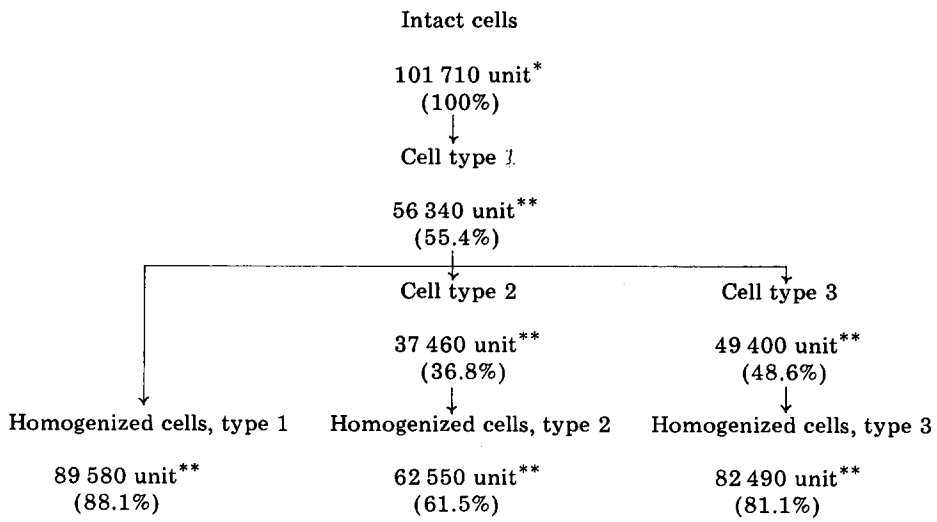

Fig. 5. Schematic comparison of aspartase activity of various enzyme preparations/unit weight of intact cells. Homogenization of immobilized cell preparations was carried out by using a mortar and pestle. Aspartase activities are values obtained from 1 g (wet weight) of intact cells. *, activity in the presence of Triton X-100; **, activity after activation.

clear, but by several experiments we found that 'κ-carrageenan in liquid state' does not show any stabilizing effect and 'κ-carrageenan in gel state' shows apparent stabilization of enzyme activity. This result suggests that gel matrix of κ-carrageenan may play an important role for this stabilization. These experiments will be presented elswhere.

Conditions for continuous production of L-aspartic acid using the column packed with the immobilized cell preparations were investigated, and the acid could be produced in a high yield. As shown in Table I, the immobilized cell column was more stable at pH 8.5 than at pH 9.0 (optimum for aspartase reaction), and their half-lives were calculated to be 92 days for cell type 1, 237 days for cell type 2 and 682 days for cell type 3 at 37°C, respectively. These results suggest that the operation at pH 8.5 is more advantageous for continuous production of L-aspartic acid than that at pH 9.0.

In previous paper [4], we compared the efficiency of *E. coli* cells immobilized with polyacrylamide and κ-carrageenan in producing of L-aspartic acid, and found that *E. coli* cells immobilized with κ-carrageenan and then treated with glutaraldehyde and hexamethylenediamine show the highest productivity. Therefore, we considered that this preparation is most advantageous for continuous of L-aspartic acid, and we changed conventional polyacrylamide method to this new carrageenan method for industrial production of L-aspartic acid from ammonium fumarate.

Acknowledgements

We are grateful to Dr. K. Yamamoto and Mr. I. Takata of this laboratory for their helpful discussion on this study and to Miss E. Yufune for her technical assistance.

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