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Purification of Aspartase by Aqueous Two-Phase System and Affinity Membrane Chromatography in Sequence

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ABSTRACT

A simple and rapid scheme which coupled aqueous two-phase extraction with affinity membrane chromatography for the recovery of aspartase from *Escherichia coli* was developed. The aspartase was recovered and purified from cell homogenate by three successive polyethylene glycol–phosphate aqueous two-phase extractions with high activity yield. During the extraction steps, cell debris, nucelic acids, and most contaminating proteins were removed. The aspartase was recovered in the phosphate-rich phase. The enzyme was further purified by affinity chromatography in which the regenerated microporous cellulose membrane and L-aspartate were used as support and ligand, respectively. The aspartase solution was forced to flow convectively through the pores in which ligand L-aspartate was immobilized on the surface. The affinity membrane chromatography carried out under a high flow rate resulted in a productivity of 17 L/L/h. The overall purification scheme yielded aspartase with a specific activity of 27.3 units/mg, a 32-fold increase in purity, and a 72% recovery yield. SDS-PAGE showed little contaminating proteins were presented in the purified aspartase.

Key Words. Aspartase; Aqueous two-phase system; Affinity membrane chromatography; L-Aspartate-linked membrane

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INTRODUCTION

The purification of protein from cell homogenate generally requires a sequence of isolation and purification operations. The aqueous two-phase system (ATPS) has been used in recent years as a mild, efficient, and scalable method for the isolation of proteins from cell homogenate (1). Affinity adsorption, on the other hand, is an attractive purification method by which a high degree of purification can be achieved by a simple procedure. However, scale-up of conventional affinity adsorption process, which uses porous beads as the adsorbent, has resulted in several difficulties, principally due to kinetic limitations. It has recently been demonstrated that a microfiltration membrane can be used as a robust and fast-operating affinity adsorbent (2–4). Since the pore diameters of microfiltration membranes are 0.1 to 3.0 μm, when a solution permeates through the pores the diffusion distance for the ligates to the affinity sites are markedly smaller than those of chromatographic beads. This leads to affinity membrane adsorption being a rapid, efficient, and scalable purification operation (2).

Aspartase (L-aspartate ammonia lyase, E.C. 4.3.1.1) catalyzes the reaction that converts L-aspartate into fumarate and ammonium. Aspartase purifications have been reported by several investigators (5–11). However, these purification processes involve so many unit operations (such as cell debris removal; nucleic acids removal, ammonium sulfate fractionation, ion exchanger, and affinity chromatography) that the recovery yield is low. In this work we report on the application of ATPS and affinity membrane chromatography for the fast purification of aspartase from *Escherichia coli* cell homogenate. The aspartase was recovered directly from the cell homogenate by the polyethylene glycol (PEG)–phosphate aqueous two-phase system. It was further purified by affinity membrane chromatography operating at a high flow rate. The affinity membrane was prepared by immobilizing affinity ligand L-aspartate to a regenerated cellulose microporous membrane. Also, the performance of this affinity membrane chromatography and the affinity binding behavior between aspartase and L-aspartate were studied.

MATERIAL AND METHODS

Aqueous Two-Phase System

E. coli (ATCC 11105) was grown aerobically at 37°C in a medium containing yeast extract, 48 g/L; fumaric acid, 40 g/L; K_2SO_4, 4 g/L; $MgSO_4$, 0.5 g/L; and NH_4OH added to establish pH 7.8. The cell mass was harvested by centrifugation at 13,000*g* for 20 minutes and stored at −20°C.

For cell disruption, the thawed cell mass was resuspended at 20% w/v in 50 mM, pH 7.0 phosphate buffer containing 1 mM EDTA and 1 mM mercaptoethanol and thoroughly homogenized in a ice bath by an ultrasonic cell disrupter (Heat System, Farmingdale, NY, USA). The aspartase in the cell homogenate was recovered by three consecutive PEG–phosphate two-phase extractions. The composition of the first extraction system was 12% K_2HPO_4, 11% PEG 1540, and 25% cell homogenate. After phase separation, the top PEG-rich phase was carefully collected and mixed with a phosphate buffer of pH 6.0 (K_2HPO_4/KH_2PO_4 molar ratio 0.53) for a second extraction. The second extraction system consisted of 60% of the top phase of the first extraction, 6.7% phosphate, and 3.5% NaCl. The third extraction was carried out by mixing the top phase of the second extraction with phosphate buffer of pH 6.0. PEG 35000 and NaCl were added to this extraction system to exclude aspartase from the PEG phase and into the phosphate phase. Composition of the third extraction system was 40% of the top phase of the second extraction, 6.7% phosphate, 4.85% PEG 35000, and 3.5% NaCl. All the composition used in the extractions were expressed in weight percent (w/w). At the end of the third extraction, the bottom phosphate phase was collected and dialyzed with 10 mM phosphate buffer of pH 7.0 overnight. The dialyzed solution was saved at 4°C as a crude aspartase solution for affinity membrane chromatography. The overall operation scheme of ATPS is shown in Fig. 1.

Affinity Membrane Preparation

Ligand L-aspartate was covalently linked to a microfiltration membrane via a spacer arm. The regenerated cellulose membrane with a pore size of 0.45 μm and a diameter of 25 mm was obtained from Sartorius (Gottingen, Germany). A filter holder made of nylon was used to hold a stack of membranes. Epichlorohydrin was used as an activation agent for the coupling L-aspartate to the regenerated cellulose membrane according to the method described by Porath (11). Activation was carried out at 60°C for 2 hours by circulating epichlorohydrin solution through a stack of 50 membranes. The epichlorohydrin solution was prepared by thoroughly mixing 10 mL epichlorohydrin in 80 mL of 1 N NaOH. The activated membrane was then carefully washed with deionized water until the reagent was completely removed.

Ethylenediamine was then coupled to the activated membrane by circulating 100 mL of 1 M ethylenediamine solution through the membrane at 60°C for 2 hours. After thorough wash with 0.1 M, pH 9.2 borate buffer, the immobilized amine was activated using 100 mL of 2.5% glutaraldehyde

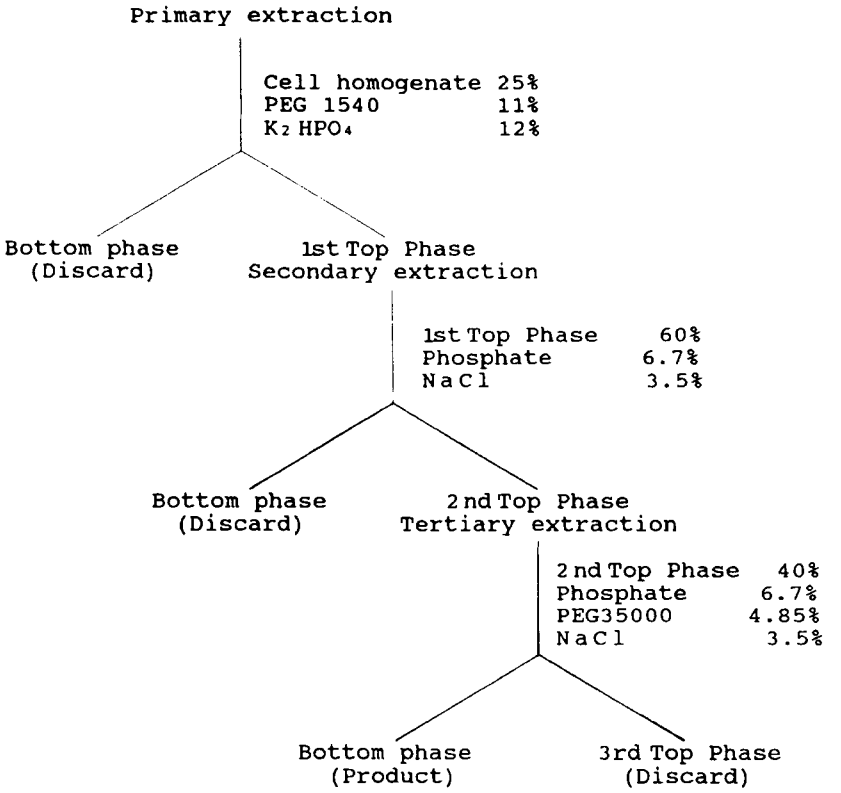

FIG. 1 Aqueous two-phase systems (ATPS) for the extraction of aspartase from *E. coli* cell homogenate.

at room temperature for 1 hour. The activated membrane was thoroughly washed with borate buffer until the residue glutaraldehyde was completely removed. The affinity ligand, L-aspartate acid, was then coupled to the glutaraldehyde activated membrane by circulating 100 mL of 1 M L-aspartate solution through the membrane stack at room temperature for 12 hours. The L-aspartate solution was prepared by dissolving L-aspartic acid in 0.1 M, pH 9.2 borate buffer. The so obtained L-aspartate-linked membrane was washed thoroughly and stored at 4°C in 50 mM, pH 7 phosphate buffer.

Affinity Membrane Chromatography

The affinity chromatography system consisted of a stack of 50 L-aspartate-linked membranes (volume ca. 1.7 mL), FMI pump, Gilson UV detec-

tor and fraction collector. Initially, phosphate buffer (0.05 M, pH 7.0) was pumped through the membranes to equilibrate the membranes. The flow was then switched to load 5 mL aspartase solution as prepared from the ATPS. After sample loading, phosphate buffer was pumped through the membranes until the absorbance at 280 nm of the effluent returned to its baseline value. A 0.2 M NaCl solution was then pumped through the membrane module to elute the bound aspartase. The pumping flow rate in each phase of the experiment was kept at 2 mL/min. The effluent from the membrane stack was collected by a fraction collector at 1 mL per tube. The purified aspartase obtained from the affinity chromatography was employed to study the affinity binding behavior of aspartase to the ligand L-aspartate at room temperature. Breakthrough curves were obtained by pumping aspartase solution through the affinity membranes at different flow rates to study the effect of flow rate on binding capacity. The stack of affinity membranes was regenerated using 0.2 M NaCl solution and equilibrated with phosphate buffer for repetitive affinity binding.

Analytical Methods

Protein concentration was measured based on the Bradford assay (12). Aspartase activity was measured by following the formation of fumarate at 240 nm in a Shimadzu UV-160A spectrophotometer. The standard aspartase assay was carried out at 30°C with an assay mixture containing 100 mM Tris, pH 7.4, 100 mM L-aspartic acid, and 2 mM $CaCl_2$. One activity unit (U) is defined as the amount of enzyme producing 1 μmole of fumarate per minute. Aspartase purity was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis according to the method of Blackshear (13).

RESULTS AND DISCUSSION

Aqueous Two-Phase System

An aqueous two-phase system (ATPS) can be created by mixing solutions of PEG and phosphate. Protein and cell debris will partition between the two phases. The exact location of a desired protein depends on such parameters as the concentration and molecular weight of PEG, pH, and ionic strength of the mixture. ATPS can be applied to remove cell debris effectively from cell homogenate and at same time achieve a certain degree of purification. Several extractions in ATPS are usually performed to purify a desired protein. As shown in the purification table (Table 1), the first extraction completely recover the separated aspartase in the PEG-rich top phase. The extraction also shows some selectivity for the aspartase, resulting in about a threefold increase in the specific activity compared

TABLE 1
Purification of Aspartase from *E. coli*

Step	Total activity (units)	Specific activity (units/mg)	Protein concentration (mg/mL)	Yield (%)	Fold purification
Cell homogenate	1366	0.85	19.30	100	1.0
ATPS:					
First top phase	1353	2.37	4.80	99	2.8
Second top phase	1104	4.95	1.77	81	5.8
Third bottom phase	1161	5.87	1.57	85	6.9
Affinity membrane	983	27.3	0.2	72	32.1

to the cell homogenate. A further purification based on partition was carried out by adding phosphate buffer of pH 6.0 and NaCl to the top phase of first extraction to perform a secondary two-phase extraction. During this step, selective removal of contaminating proteins such as fumarase was achieved (data not shown). The specific activity increased sixfold at the expense of 80% recovery of aspartase activity in the top phase. Since the recovery of protein from the PEG phase is more difficult than from the phosphate phase, a tertiary extraction was performed to extract the aspartase into the phosphate-rich bottom phase by adding phosphate and high molecular weight PEG 35000 to the top phase of the second extraction. As shown in Table 1, most of the aspartase in the PEG-rich phase can be recovered in the bottom phase by the addition of PEG 35000 to the system; the purity of aspartase is enriched about 7-fold. Once the separated aspartase is in the phosphate-rich phase, further purification of the aspartase by affinity chromatography is easily carried out since the high concentration of phosphate in the recovered aspartase solution can be easily removed by dialysis.

Affinity Membrane Chromatography

The aspartase obtained in ATPS was further purified by affinity chromatography. A stack of 50 L-aspartate-linked membranes was used as an affinity adsorbent. A flow rate of 2 mL/min, which yields a mean residence time of 50 seconds, was employed for the chromatography. As shown in Fig. 2, all of the aspartase activity was bound to the ligand L-aspartate during the loading step. Washing the membranes with 0.05 M, pH 7.0 phosphate buffer only removed the contaminating proteins. For the elution of aspartase, 0.2 M NaCl was sufficient to remove all the bound aspartase. Collected fractions 19 to 23 were pooled as the final purified aspartase.

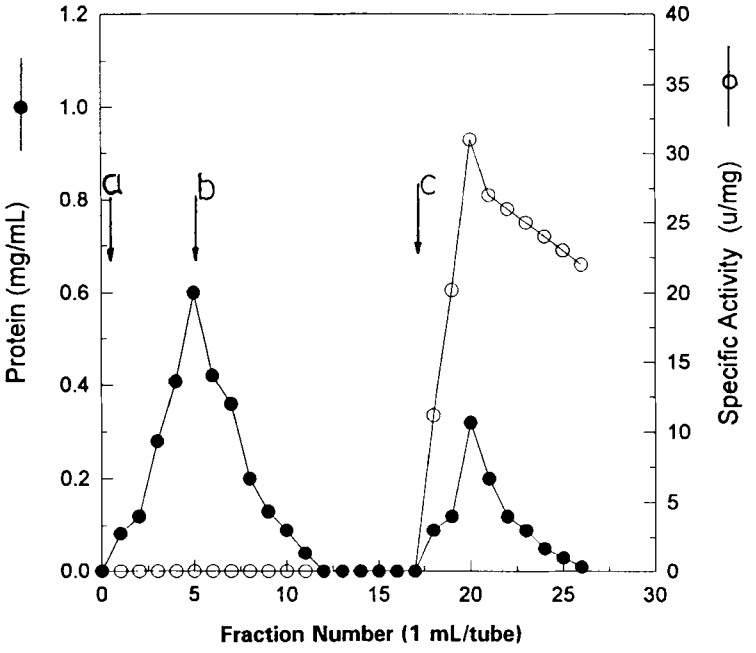

FIG. 2 Affinity membrane chromatography of the aspartase solution obtained from ATPS. (a) Loading 5 mL aspartase solution; (b) washing with 0.05 M, pH 7.0 phosphate buffer; (c) elution with 0.2 M NaCl.

The specific activity was 27.3 U/mg, and there was about a 32-fold increase in purity. After equilibration with phosphate buffer, the affinity membrane stack was used for the next cycle of aspartase purification. It took about 15 minutes for one cycle of operation. The 36 mL of aspartase solution from ATPS was purified by five successive cycles of affinity membrane chromatography. Based upon these experiment results, the productivity of this affinity membrane chromatography was calculated to be 17 L/L/h, i.e., 17 L aspartase solution could be purified by 1 L affinity membrane in an hour.

The fractions obtained in each purification step were subjected to SDS-PAGE, and the results are shown in Fig. 3. Besides the aspartase, the aspartase solution obtained from affinity membrane chromatography presents a small amount of contaminating proteins. The presence of contaminating proteins is probably due to the ion-exchange effect of the ligand L-aspartate. Since the ligand L-aspartate was covalently immobilized to

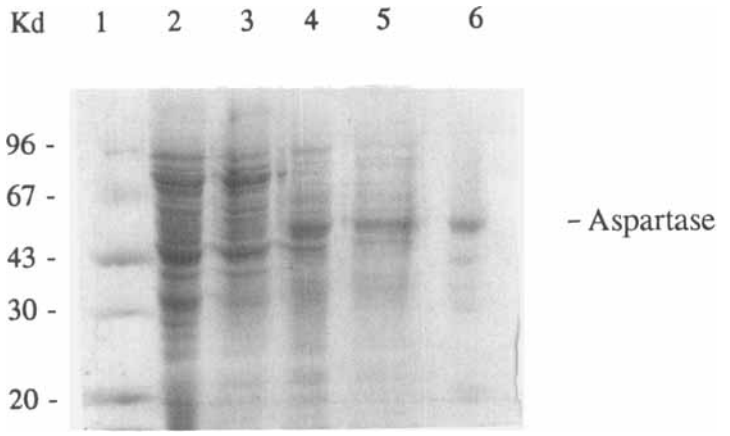

FIG. 3 SDS-PAGE of the fractions obtained during the purification of aspartase from *E. coli*. Lane 1, molecular marker: phosphorylase b (96 K_d); albumin (67 K_d); ovalbumin (43 K_d); carbonic anhydrase (30 K_d); trypsin inhibitor (20 K_d). Lane 2, supernatant of cell homogenate. Lane 3, top phase of 1st extraction. Lane 4, top phase of 2nd extraction. Lane 5, bottom phase of 3rd extraction. Lane 6, product from affinity membrane chromatography.

the cellulose membrane through its amino group, the two remaining carboxyl groups of aspartate behaved like a cation exchanger at pH 7. The positively charged proteins will bind to the L-aspartate nonspecifically, while the negatively charged aspartase (pI 4.8) binds to the L-aspartate through affinity interactions. The affinity binding behavior was studied in a batch mode by circulating the purified aspartase (27.3 U/mg) of different concentrations through a stack of L-aspartate-linked membranes. Figure 4 shows the equilibrium binding isotherm of L-aspartate-linked membrane for the enzyme aspartase. As shown in the inset of Fig. 4, the binding isotherm can be replotted according to the method of Scatchard:

$$Q^* = Q_{max} - K_d \cdot Q^*/C^*$$

where Q^* is the amount of aspartase binding to ligand L-aspartate and C^* is the free aspartase concentration. The linear plot indicates that aspartase binding to the ligand aspartate can be described as a Langmuir-type adsorption. Based on this linear plot, the maximal amount of aspartase binding to the aspartate, Q_{max}, was calculated from the intercept to be 1.25 mg/g of the affinity membrane. The dissociation constant, K_d, was obtained from the slope to be 0.0149 mg/mL. K_d can be converted to 8.3×10^{-8} mol/L based on the molecular weight of aspartase (ca. 180,000 dal-

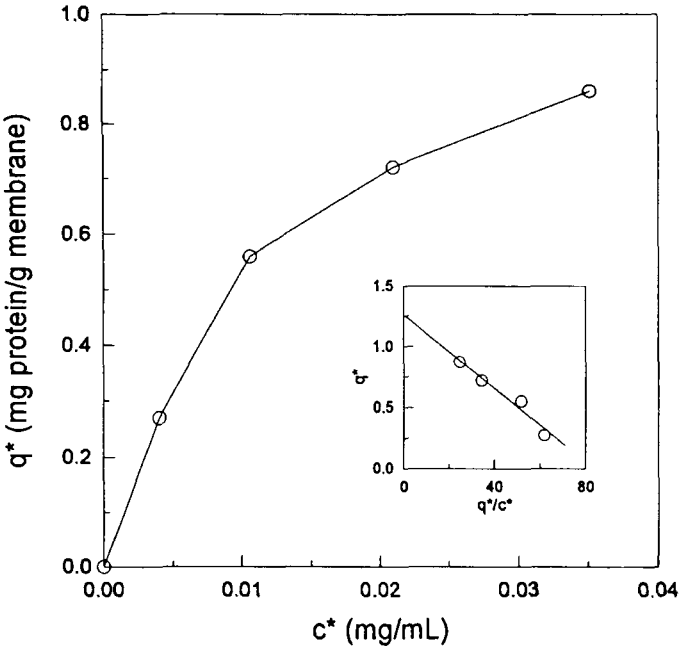

FIG. 4 Equilibrium affinity binding isotherm of aspartase for the L-aspartate linked membrane. The inset is the Scatchard plot of binding isotherm.

ton). This small dissociation constant implies a strong affinity interaction between aspartase and ligand L-aspartate.

Figure 5 shows the breakthrough curves of aspartase adsorption to the L-aspartate-linked membranes and unmodified membranes. The stack of unmodified membranes shows no appreciable aspartase binding capacity, since a sharp breakthrough curve was obtained immediately after aspartase solution was loaded. On the other hand, aspartase solution (27.3 U/mg) at flow rates of 2 and 0.5 mL/min demonstrates almost the same breakthrough curve in the adsorption using aspartate-linked membranes. The binding capacity of the affinity membrane was estimated by calculating the area behind the breakthrough curve to be about 0.9 mg/g of membrane. This indicates that the flow rate can be increased to at least 2 mL/min without losing the dynamic binding capacity for aspartase. Since a high flow rate can be employed without reducing the binding capacity, a higher throughput of purified aspatase can be expected with affinity membrane chromatography compared to conventional chromatography.

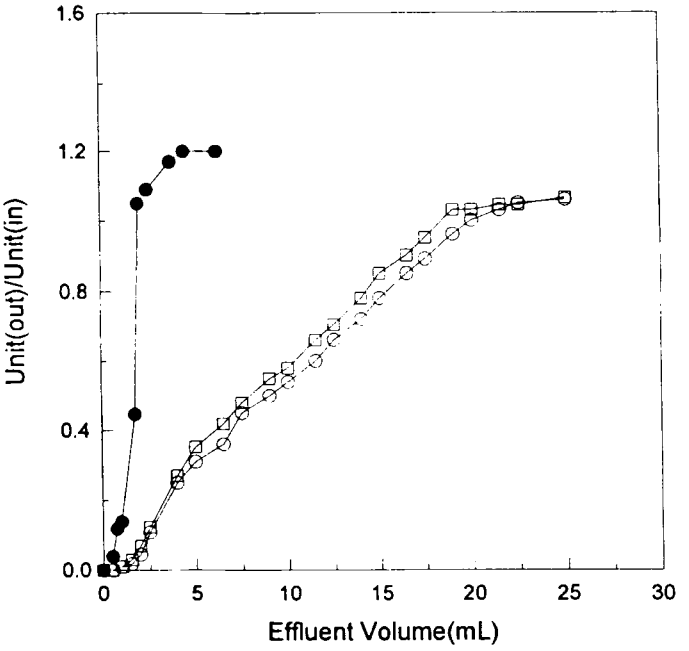

FIG. 5 Effect of flow rate on adsorption breakthrough curve of purified aspartase (27.3 U/mg) using a stack of 50 membranes. Flow rate, (●) 2 mL/min with unmodified cellulose membranes; (□) 2 mL/min and (○) 0.5 mL/min with L-aspartate-linked membranes.

CONCLUSION

By taking into account the number of unit operations employed for purification and their feasibility for scaling up, an ATPS followed by affinity membrane chromatography is a valuable scheme for the fast purification of intracellular proteins. Enzyme aspartase has a strong affinity for the ligand L-aspartate, and the affinity constant is in the order of 10^{-8} mol/L. However, L-aspartate is not a good ligand candidate for aspartase since the ion-exchange effect of its carboxyl groups may cause nonspecific binding. Affinity membrane has a low binding capacity, but the chromatograph can be operated at a high flow rate without losing its binding capacity. The high flow rate will reduce the cycle time for affinity chromatography purification. As a consequence, high productivity can be expected for affinity membrane chromatography.

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