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# CHARACTERISTICS AND APPLICATIONS OF *N*-(ω-AMINOHEXYL)-L-ASPARTIC ACID–SEPHAROSE AS AN AFFINITY ADSORBENT*

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## SUMMARY

1. The affinity adsorbent, *N*-(ω-aminohexyl)-L-aspartic acid–Sepharose 6B (AHA–Sepharose), was prepared by covalently linking AHA to Sepharose 6B previously activated with CNBr.

2. The enzymes relating to the metabolism of L-aspartic acid showed a group-specific adsorption to AHA–Sepharose column. Asparaginase, aspartase, aspartate-β-decarboxylase and modified asparaginase with tetranitromethane were adsorbed to AHA–Sepharose column and eluted by increasing the ionic strength with NaCl. Asparaginase and aspartase were eluted with the respective substrate or product of lower ionic strength, whereas holo- and apo-enzymes of aspartate β-decarboxylase and the modified asparaginase could not be eluted with the respective substrate or product of lower ionic strength.

3. Fumarase was not adsorbed to this column.

4. By the affinity chromatography employing the AHA–Sepharose column, asparaginase from *Proteus vulgaris* was purified easily and in a high yield.

## INTRODUCTION

It is well known that affinity chromatography is an advantageous technique for enzyme purification [1, 2], and this advantage is due to the biological specificity in contrast to other chromatographic methods. It is indicated that the enzyme adsorbed to the affinity adsorbent can be selectively purified by elution with its substrate [3], or its inhibitor [4].

Recently, it has been found that an affinity chromatography is useful not only for enzyme purification but also for obtaining the enzymes of the analogous function.

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Abbreviations: AHA, *N*-(ω-aminohexyl)-L-aspartic acid.

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Lowe and Dean [5] reported that one group of dehydrogenases was specifically adsorbed to the NAD- or NADP-cellulose column. Further, Uren [6] reported that one group of proteolytic enzymes showed an affinity to a column of *N*-(2-ethyl-cellulose-glycyl-D-phenylalanine. It is expected, from these results, that when one of the metabolites is immobilized, one group of enzymes relating to the metabolite shows an affinity to the insoluble metabolite.

In this presentation, *N*-($\omega$-aminohexyl)-L-aspartic acid (AHA) was covalently linked to Sepharose 6B previously activated with CNBr. The behaviors of the enzymes relating to the metabolism of L-aspartic acid to the AHA–Sepharose were investigated. The applications of the adsorbent were also investigated.

## MATERIALS AND METHODS

### *Materials*

Crystalline pure asparaginase from *Proteus vulgaris* was prepared according to the procedure described by Tosa et al. [7]. Crystalline asparaginase from *Escherichia coli* was obtained from Kyowa Hakko Kogyo Co., Tokyo, Japan. Crystalline pure asparatate $\beta$-decarboxylase from *Pseudomonas dacunhae* was prepared in our Research Laboratory [8]. L-Aspartic acid and L-alanine were the products of Tanabe Seiyaku Co., Osaka, Japan. D-Aspartic acid was obtained from Sigma Chemical Co., St. Louis, U.S.A. Sephadex G-25 and Sepharose 6B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Ampholine carrier ampholytes were purchased from LKB-Produkter AB, Stockholm-Bromma, Sweden. All other chemicals used in this experiment were reagent grade.

### *Preparation of apoenzyme of aspartate $\beta$-decarboxylase*

The apoenzyme of aspartate $\beta$-decarboxylase was prepared by dialyzing the holoenzyme against 1 M sodium acetate buffer (pH 5.0) containing 0.1 M L-aspartic acid according to the method of Tate and Meister [9].

### *Preparation of crude aspartase*

Crude aspartase was prepared from *E. coli* Ki 1023 [10] as follows. The cells were grown at 37 °C for 24 h in a medium (pH 7.0) containing 3% ammonium fumarate, 0.2% $K_2HPO_4$, 0.05% $MgSO_4 \cdot 7\,H_2O$, 2% corn-steep liquor and 0.05% $CaCO_3$. The cells harvested were washed with physiological saline and lyophilized. Lyophilized cells were suspended in 0.1 M sodium phosphate buffer (pH 8.5) at a concentration of 10 mg/ml, and sonicated at 10 kcycles for 40 min in an ice bath. After removal of the debris by centrifugation, cell extracts were lyophilized. Lyophilized cell extract (3 g) was dissolved in 200 ml of 0.01 M sodium phosphate buffer (pH 7.0), and solid $(NH_4)_2SO_4$ was added to 30% satn at 0 °C. After removal of precipitate by centrifugation, solid $(NH_4)_2SO_4$ was added to 50% satn at 0 °C to the supernatant. The precipitate obtained was dissolved in a small volume of 0.01 M sodium phosphate buffer (pH 7.0), and the enzyme solution was used as a crude sample.

### *Modification of asparaginase by tetranitromethane*

Modification of tyrosyl residues in the asparaginase molecule was performed by tetranitromethane according to the method of Solokovsky et al. [11]. Crystalline

asparaginase (10 mg) from *P. vulgaris* was dissolved in 1.0 ml of 0.05 M Tris–HCl buffer (pH 8.0). To the solution, 10 $\mu$l of tetranitromethane was slowly added with stirring while adjusting the pH to 8 with dilute NaOH at 25 °C. After stirring for 1 h, the mixture was passed through the column of Sephadex G-25 equilibrated with 0.01 M sodium phosphate buffer (pH 7.0). The resulting effluent was collected and the absorbance was measured at 428 nm. The modified asparaginase obtained contained 1.194 $\mu$moles of nitrotyrosine calculated from the molar extinction coefficient of nitrotyrosine. The modification ratio of the tyrosyl residue was 42% calculated from the result that asparaginase contains 2.86 $\mu$moles of tyrosine per 10 mg of enzyme [12]. The activity of the modified asparaginase was 8% of the native enzyme.

*Preparation of AHA–Sepharose*

AHA–Sepharose was prepared according to the method of Kristiansen et al. [3] with some modifications as follows.

(1) *Preparation of* L*(+)-monochlorosuccinic acid.* 6.7 g of D(−)-aspartic acid and 20 g of NaCl were dissolved in 50 ml of 1 M HCl, and to the solution 50 ml of 4% $NaNO_2$ was added dropwise with stirring at 0–5 °C. After further stirring for 20 h, 50 ml of conc. HCl was added, and the reaction product was extracted 3 times with diethyl ether. By evaporation of the resulting diethyl ether layer, 2.6 g of monochlorosuccinic acid was obtained in crystalline form.

(2) *Preparation of AHA.* 2.6 g of monochlorosuccinic acid was dissolved in 50 ml of absolute ethanol. To the solution 50 ml of ethanol solution containing 7.5 g hexamethylene diamine was slowly added, and the mixture was refluxed with stirring at 65 °C for 30 h. Then, 100 ml of water was added and the oily product was obtained by concentration under vacuum at 50 °C. The product was applied on a column of Amberlite IRA 401 ($OH^-$ type). The column was thoroughly washed with water, and was developed with 14% $NH_4OH$. The eluate was evaporated to dryness under vacuum at 50 °C, and the residue was dissolved in a small volume of water. By adding 200 ml of methanol to 10 ml of the solution, 0.5 g of AHA was obtained in the crystalline form (m.p. 191 °C).

(3) *Coupling of AHA to Sepharose 6B.* 0.5 g of AHA was dissolved in 100 ml of 0.1 M $NaHCO_3$, and to the solution was added 6 g (packed weight) of Sepharose 6B previously activated by CNBr according to the procedure described by Axén et al. [13]. The suspension was gently stirred for 24 h at 25 °C and then washed thoroughly with 0.1 M $NaHCO_3$ and 1 M NaCl.

*Assay of asparaginase activity*

Asparaginase activity was assayed as described previously [14]. One asparaginase unit (I.U.) is defined as the amount of enzyme that hydrolyzes 1 $\mu$mole of substrate per min under the standard assay conditions.

*Assay of aspartase activity*

Aspartase activity was assayed as follows. The reaction mixture composed of 1.6 ml of sodium borate buffer (pH 8.5), 1.3 ml of 3% ammonium fumarate containing $MnCl_2$ at the concentration of 3 mM and 0.1 ml of enzyme solution was incubated for 30 min. The reaction was stopped by heating for 5 min at 95 °C. The formation of L-aspartic acid was estimated by bioassay using *Leuconostoc mesenteroides* P-60

according to the method of Henderson and Snell [15]. The amount of ammonium fumarate consumed in the reaction mixture was spectrophotometrically measured at 240 nm. These amounts were expressed as $\mu$mole/h.

*Assay of aspartate $\beta$-decarboxylase activity*

Aspartate $\beta$-decarboxylase activity was assayed by manometric measurements of $CO_2$ liberated from L-aspartic acid in the presence of 5 $\mu$moles of $\alpha$-ketoglutarate according to the method of Kakimoto et al. [8]. One decarboxylase unit is defined as the amount of enzyme forming 1 $\mu$mole of $CO_2$ per min under the standard assay conditions.

*Determination of* L-*aspartic acid*

L-Aspartic acid in the effluent was determined by the ninhydrin colorimetric method [16] using a Technicon auto-analyzer.

*Determination of protein*

Protein was determined by the method of Lowry et al. [17], using crystalline bovine albumin as a standard, or by measurement of the absorbance at 280 nm. Absorbance was measured at 20–25 °C by a Hitachi–Perkin–Elmer 139 spectrophotometer, using cuvettes of 1-cm optical path.

*Isoelectric focusing*

Isoelectric focusing was carried out by the method of Vesterberg and Svensson [18]. Ampholine carrier ampholytes used in this study were selected to give a gradient of pH 3–10, and the density gradient was made up with sucrose. Enzyme (10 mg) was applied and focused at 0–1 °C for 48 h with a final potential of 700 V and 1.3 mA. The pH values of the fractions were measured at 0–1 °C with an accuracy of $\pm$0.02.

*Column chromatography with AHA–Sepharose*

AHA–Sepharose was packed into a column (0.6 cm $\times$ 9 cm) and equilibrated with 0.01 M sodium acetate buffer (pH 7.0) unless otherwise noticed. Samples were applied to the column and the elution from the column was carried out by the gradient method with NaCl or L-aspartic acid, or by the stepwise method with the buffer containing various concentrations of NaCl, substrate or product. The flow rate was adjusted by a Technicon proportioning pump. The ionic strength was determined by its conductivity using the NaCl solution as a standard. Conductivity was measured at 20–25 °C by a Radiometer Type CDM 2d conductivity meter and a cell of Type CDC 114.

## RESULTS

*Affinity chromatography of asparaginase and modified asparaginase*

To examine the adsorption of asparaginase to AHA–Sepharose, pure enzyme preparations were applied to AHA–Sepharose and unsubstituted Sepharose 6B columns. Both asparaginase from *P. vulgaris* and from *E. coli* were not adsorbed to the unsubstituted Sepharose 6B column, whereas they were adsorbed to the AHA–

Sepharose column and could be eluted by increasing the ionic strength with NaCl (Figs 1A and 2A). The ionic strength of the fraction that showed the maximum enzyme activity was measured to be 0.060 for the *P. vulgaris* enzyme and 0.062 for the *E. coli* enzyme. The proteins were recovered in the yield of 92% for the *P. vulgaris* enzyme and 82% for the *E. coli* enzyme, but the recoveries of the enzyme activity were 28% for the *P. vulgaris* enzyme and 37% for the *E. coli* enzyme. As shown in Figs 1B and 2B, both adsorbed enzymes could be eluted with the buffer of lower ionic strength containing L-aspartic acid without significant loss of the enzyme activity. The ionic strength and concentration of L-aspartic acid of the fraction having the maximum

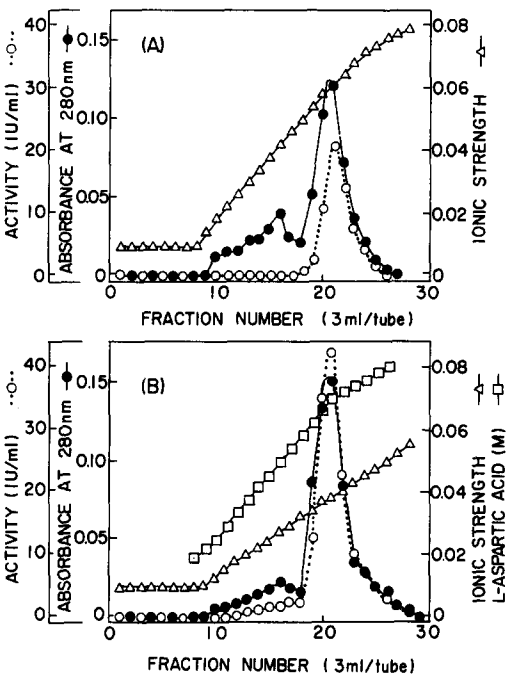


Fig. 1. Chromatography of *P. vulgaris* asparaginase on an AHA–Sepharose column. The enzyme (3.18 mg, 800 I.U.) was dissolved in 1.0 ml of 0.01 M sodium acetate buffer (pH 7.0; $I = 0.008$), and applied to the column. Elution was carried out with linear gradients from 0 to 0.1 M NaCl in (A) and to 0.1 M L-aspartic acid (pH 7.0) in (B) in the sodium acetate buffer. Fractions of 3 ml were collected. Other conditions are given in the text.

enzyme activity were measured to be 0.040 and 70 mM, respectively, for both enzymes (Figs 1B and 2B). The recoveries of protein and enzyme activity were 94% and 54% for the *P. vulgaris* enzyme and 94% and 76% for the *E. coli* enzyme, respectively.

The modified asparaginase was not adsorbed to the unsubstituted Sepharose 6B column, but adsorbed to the AHA–Sepharose column. The adsorbed modified enzyme was not eluted with 0.15 M L-aspartic acid (pH 7.0) containing 0.01 M NaCl ($I = 0.133$), but eluted with 0.01 M sodium acetate buffer (pH 7.0) containing 0.5 M NaCl ($I = 0.510$) (Fig. 3), and the recovery of protein was 95%.

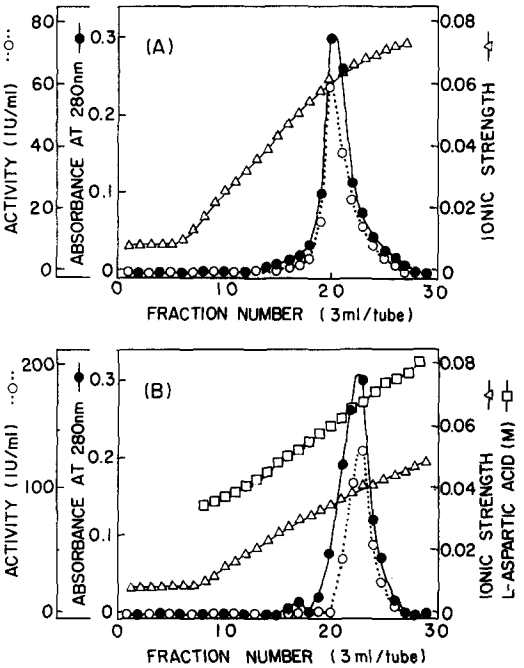


Fig. 2. Chromatography of *E. coli* asparaginase on an AHA–Sepharose column. The enzyme (5.1 mg, 1240 I.U.) was dissolved in 0.5 ml of 0.01 M sodium acetate buffer (pH 7.0; $I = 0.008$), and applied to the column. Other legends are the same as in Fig. 1.

*Affinity chromatography of aspartate β-decarboxylase*

Pure enzyme preparations were chromatographed on columns of AHA–Sepharose and unsubstituted Sepharose 6B. Both preparations of holo- and apo-enzymes were not adsorbed to unsubstituted Sepharose 6B column, whereas they were

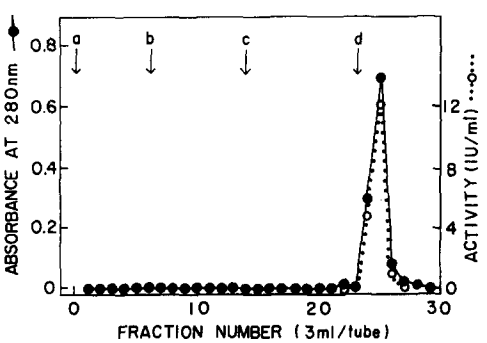


Fig. 3. Chromatography of *P. vulgaris* asparaginase modified by tetranitromethane on an AHA–Sepharose column. The modified enzyme (2.3 mg) was dissolved in 0.5 ml of 0.01 M sodium acetate buffer (pH 7.0, $I = 0.008$), and applied to the column. (a), 0.01 M sodium acetate buffer (pH 7.0); $I$ 0.008). Stepwise elution was carried out by using the same buffer containing the following concentration of NaCl: (b), 0.1 M ($I = 0.113$); (c), 0.2 M ($I = 0.210$); (d), 0.5 M ($I = 0.512$). Other legends are the same as in Fig. 1.

adsorbed to the AHA-Sepharose column and could be eluted by increasing the ionic strength with NaCl (Fig. 4). The ionic strength of the fraction having the maximum enzyme activity or protein content were measured to be 0.228 for the holoenzyme and 0.233 for the apoenzyme, respectively. The recoveries of protein were 95% for the holoenzyme and 84% for the apoenzyme, and that of the enzyme activity was 55% for the holoenzyme. The enzyme adsorbed was not eluted with 0.15 M L-aspartic acid (pH 7.0) containing 0.1 M NaCl ($I = 0.113$) or 0.5 M L-alanine containing 0.1 M NaCl (pH 7.0) ($I = 0.103$).

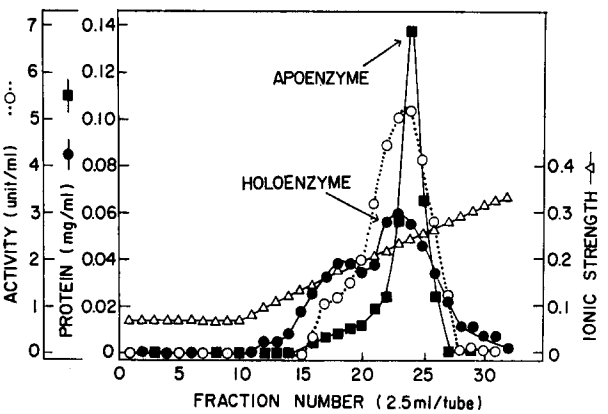


Fig. 4. Chromatography of aspartate $\beta$-decarboxylase on an AHA-Sepharose column. The holoenzyme (1.38 mg, 144.4 units) was dissolved in 0.5 ml of 0.01 M sodium acetate buffer (pH 7.0; $I = 0.008$), and the apoenzyme (1.15 mg) was dissolved in 1.0 ml of the same buffer. Both enzyme samples were separately applied to the column. Elution was carried out with linear gradients from 0 to 0.5 M NaCl in 0.01 M sodium acetate buffer (pH 7.0). Fractons of 2.5 ml were collected. Other conditions are given in the text.

*Affinity chromatography of crude aspartase containing fumarase*

When a crude preparation of aspartase was subjected to isoelectric focusing, aspartase and fumarase activities were separated; the p$I$ values were measured to be 5.65 for aspartase and 6.25 for fumarase, respectively. The result indicates that the crude preparation of aspartase contained fumarase. The adsorption of the crude preparation of aspartase to the AHA-Sepharose column was examined by applying the sample at $I = 0.008$ and 0.275. As shown in Fig. 5, only aspartase was adsorbed to the column, and contaminating fumarase was not adsorbed. The adsorbed aspartase was eluted by increasing $I$ to over 0.275 with NaCl (Fig. 5A), and the recoveries were 90% in the protein and 95% in the enzyme activity. The adsorbed aspartase was also eluted with 0.1 M sodium acetate buffer (pH 7.0) containing 0.1 M sodium fumarate ($I = 0.175$) (Fig. 5B), and the recoveries were 90% in the protein and 99% in the enzyme activity.

*Purification procedure of asparaginase from P. vulgaris*

Asparaginase from *P. vulgaris* was purified by the following steps employing a AHA-Sepharose column. To 42 ml of partially purified preparation (spec. act. 13.2

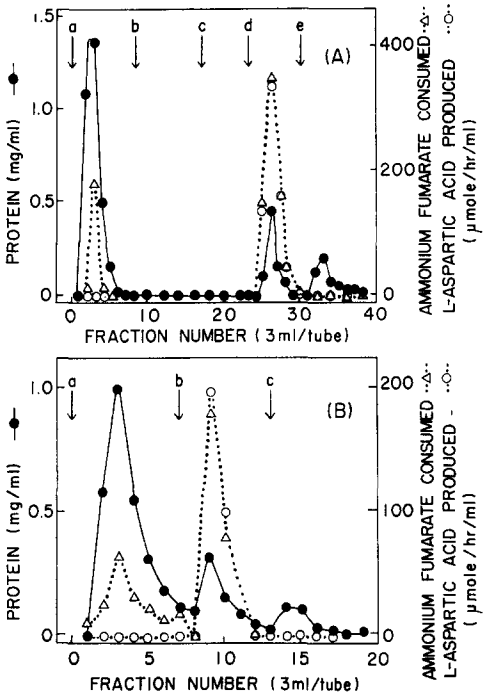


Fig. 5. Chromatography of the crude aspartase preparation containing fumarase on an AHA–Sepharose column. The enzyme preparation was dialyzed against 0.01 M sodium acetate buffer (pH 7.0; $I = 0.008$) (A) or 0.1 M sodium acetate buffer containing 0.2 M NaCl (pH 7.0, $I = 0.275$) (B), and 1.0 ml of the dialyzed sample (15 mg, enzyme activity = 1900 μmoles of L-aspartic acid/h) was applied to the column. Fractions of 3 ml were collected. Other conditions are given in the text. (A). (a), 0.01 M sodium acetate buffer (pH 7.0; $I = 0.008$). The column was washed with 0.1 M sodium acetate buffer containing 0.1 M NaCl ((b), $I = 0.110$) until none of protein was detected in the effluent. Stepwise elution was carried out by using 0.1 M sodium acetate buffer containing the following concentration of NaCl: (c), 0.2 M ($I = 0.275$); (d), 0.3 M ($I = 0.379$); (e), 0.5 M ($I = 0.581$). (B). The column was washed with 0.1 M sodium acetate buffer containing 0.2 M NaCl ((a), ($I = 0.275$) until none of protein was detected in the effluent. Stepwise elution was carried out by using the same buffer containing the following concentration of sodium fumarate (pH 7.0): (b), 0.1 M ($I = 0.175$); (c), 0.2 M ($I = 0.281$).

I.U./mg of protein) obtained from Steps I, II and III previously described [7] 350 mg of sodium L-aspartate was added. Then 21 ml of ethanol was added dropwise at 0 °C, and the mixture was allowed to stand for 1 h. The precipitate was discarded after centrifugation. To the supernatant 21 ml of ethanol was added dropwise at 0 °C. The resulting precipitate was collected by centrifugation, and dissolved in 50 ml of 0.01 M sodium acetate buffer (pH 7.0). The solution was applied to an AHA–Sepharose column (2 cm × 20 cm) equilibrated with 0.01 M sodium acetate buffer containing 0.04 M NaCl (pH 7.0). After the column was thoroughly washed with the equilibrating buffer, the enzyme was eluted with a 0.01 M sodium acetate buffer containing 0.07 M L-aspartic acid (pH 7.0). The elution pattern is shown in Fig. 6. The active fractions were collected and saturated with $(NH_4)_2SO_4$. The resulting precipitate was collected by centrifugation and dissolved in 0.01 M sodium phosphate buffer (pH 6.8) at the concentration of 2%. The crystallization was carried out by the method previously described [7]. A summary of this procedure is presented in Table I.

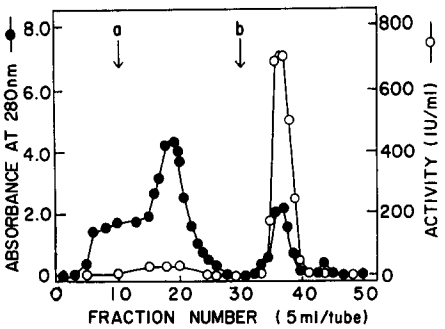


Fig. 6. Chromatography of *P. vulgaris* asparaginase from the ethanol fraction (33–50 vol. %) on an AHA–Sepharose column. Elution was carried out by using 0.01 M sodium acetate buffer containing 0.04 M NaCl (pH 7.0) at Position (a) and the buffer containing 0.07 M L-aspartic acid (pH 7.0) at Position (b). Other conditions are given in the text.

TABLE I

PURIFICATION OF ASPARAGINASE FROM *P. VULGARIS*

| Steps | Volume (ml) | Total protein (mg) | Spec. act. (I.U./mg) | Total activity (I.U.) | Yield (%) |
|---|---|---|---|---|---|
| I. Crude extract | 1050 | 19 820 | 1.1 | 21 800 | 100 |
| II. pH 4.5 supernatant | 930 | 9 420 | 1.9 | 17 900 | 82 |
| III. $(NH_4)_2SO_4$ (50–90%) | 42 | 1 020 | 13.2 | 13 500 | 62 |
| IV. Ethanol (33–50 vol %) | 50 | 149 | 70 | 10 460 | 48 |
| V. AHA–Sepharose | 24 | 30 | 310 | 9 160 | 42 |
| VI. Crystals | 1 | 27 | 300 | 8 080 | 37 |

## DISCUSSION

A group-specific adsorption in an affinity chromatography was first shown by Aspberg and Porath [19] with glycoproteins, and also shown by Uren [6] and Lowe and Dean [5] with some enzymes having analogous functions. In this paper, we investigated the affinity binding of enzymes relating to the metabolism of L-aspartic acid towards an adsorbent having aspartic acid as a ligand (AHA–Sepharose). It was found that enzymes directly related to the metabolism of L-aspartic acid, asparaginase, aspartase and aspartate $\beta$-decarboxylase, were adsorbed to the adsorbent, whereas fumarase was not adsorbed. That is, AHA–Sepharose has a characteristic of group-specific adsorption for enzymes having different functions. The affinity of these enzymes into the adsorbent are summarized in Table II.

From the difference in the elution behaviors with substrate or product of the respective enzyme, it is possible to classify the degree of binding specificity into higher and lower types. The degree of the specificity of asparaginase and aspartase to AHA-Sepharose is higher than that of aspartate $\beta$-decarboxylase and modified asparaginase, because the former two enzymes were eluted with substrate or product of lower ionic strength than in the case of NaCl (Figs 1, 2 and 5).

We previously reported that asparaginase from *P. vulgaris* has an antileukemic

TABLE II

AFFINITY OF ENZYMES ON AHA–SEPHAROSE

| Enzymes | Affinity (ionic strength to be eluted by NaCl) |
|---|---|
| Fumarase | Not adsorbed |
| Asparaginase: | |
| *P. vulgaris* | 0.060 |
| *E. coli* | 0.062 |
| Modified asparaginase | >0.210 |
| Aspartate β-decarboxylase: | |
| Holoenzyme | 0.228 |
| Apoenzyme | 0.233 |
| Aspartase | >0.275 |

activity as well as the enzyme from *E. coli*, and the two enzymes are enzymatically and physiologically almost the same, but differ immunologically from each other [7, 12, 14]. As shown in Figs 1 and 2, the two enzymes resembled each other in their behavior on affinity chromatography.

Asparaginase adsorbed to AHA–Sepharose was eluted with L-aspartic acid, the reaction product, of lower ionic strength than in the case of NaCl (Figs 1 and 2), indicating that the binding specificity of the enzyme to the adsorbent is of the higher type. Therefore, homogeneous asparaginase could be obtained in a higher yield from *P. vulgaris* by more simple purification procedures (Table I) compared with the method previously reported [7]. Kristiansen et al. [3] previously described the purification of asparaginase by the procedure of affinity chromatography using L-asparagine as a ligand. However, detailed data of the yield of enzyme activity and purification were not cited in their paper, so we cannot compare which adsorbent is superior for the purpose of purification of asparaginase.

The modified asparaginase was adsorbed onto an AHA–Sepharose column with a lower specificity (Fig. 3 and Table II). These results indicate that the adsorbent is applicable for the separation of native and modified asparaginase, in addition to the purification of asparaginase.

When crude aspartase containing fumarase was subjected to isoelectric focusing, aspartase (p*I* 5.65) was separated from fumarase (p*I* 6.25). Aspartase was adsorbed onto the AHA–Sepharose column, and could be eluted with sodium fumarate, the substrate, in a higher specificity, whereas fumarase was not adsorbed. Therefore, the adsorbent is useful for the separation of aspartase and fumarase.

In the case of aspartate β-decarboxylase, the holo- and apo-enzymes adsorbed onto the AHA–Sepharose column with the same affinity with lower specificity (Fig. 4 and Table II). The reason may be explained as follows from the result of Meister et al. [20], that is, for the appearance of the enzyme activity a Schiff base should be formed between the amino group of L-aspartic acid and the aldehyde group of pyridoxal phosphate. In the case of AHA–Sepharose a Schiff base cannot be formed, because the amino group of the aspartic acid of the adsorbent is not free. Therefore, it may be considered that the enzyme is adsorbed to AHA–Sepharose via the binding site of the enzyme except for pyridoxal phosphate.

These results described in this paper indicate that affinity chromatography can be used as an advantageous technique for the purification of enzyme protein and for the elucidation of the reaction mechanism of a biologically active substance.

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