Agricultural and Biological Chemistry

ISSN: 0002-1369 (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tbbb19

# Affinity Chromatography of a Group Specific Adsorbent for Pyridoxal Phosphate Dependent Enzymes

Tsutomu Masuda, Hiroshi Ozaki & Akira Tai

**To cite this article:** Tsutomu Masuda, Hiroshi Ozaki & Akira Tai (1986) Affinity Chromatography of a Group Specific Adsorbent for Pyridoxal Phosphate Dependent Enzymes, Agricultural and Biological Chemistry, 50:11, 2763-2769, DOI: 10.1080/00021369.1986.10867822

**To link to this article:** https://doi.org/10.1080/00021369.1986.10867822

# Affinity Chromatography of a Group Specific Adsorbent for Pyridoxal Phosphate Dependent Enzymes

Tsutomu MASUDA, Hiroshi OZAKI* and Akira TAI*,†

*Shitennoji International Buddhist University, Hanyuno, Habikino 583, Japan*
**Institute for Protein Research, Osaka University, Suita, Osaka 565, Japan*

Received April 16, 1986

Sepharose gels with L-amino carboxylic acid residues as a ligand (L-adsorbent) or with D-amino carboxylic acid residues (D-adsorbent) were prepared and used in affinity chromatography. Among enzymes involved in the metabolism of glutamic acid, a group of pyridoxal phosphate dependent enzymes such as aspartate aminotransferase, alanine aminotransferase, and glutamate decarboxylase had significant affinity for the L-adsorbent, while glutamate dehydrogenase (an NADP dependent enzyme) had no detectable affinity for either the L- or the D-adsorbent. The affinity chromatography with L-adsorbent was used for the purification of serine hydroxymethyltransferase; a pyridoxal phosphate dependent enzyme.

Enzymes involved in the metabolism of α-amino acids differentiate the chirality of chiral center carbons as well as the sort of α-substituent of the substrate. If the differentiation of chirality is brought about by the attractive force between amino carboxylic acid residues ($-CH(NH_2)COOH$) and the corresponding recognition sites in the enzyme, the enzyme is expected to have some affinity for adsorbents with either L- or D-amino acid residues as ligands. We have prepared L- and D-adsorbents (Fig. 1) by the reaction between BrCN activated Sepharose 4B and ε-amino groups of L- and D-lysine under controlled conditions, and we have examined their performance as an adsorbent for affinity chromatography using a series of enzymes involved in the metabolism of L-glutamic acid, a key com-

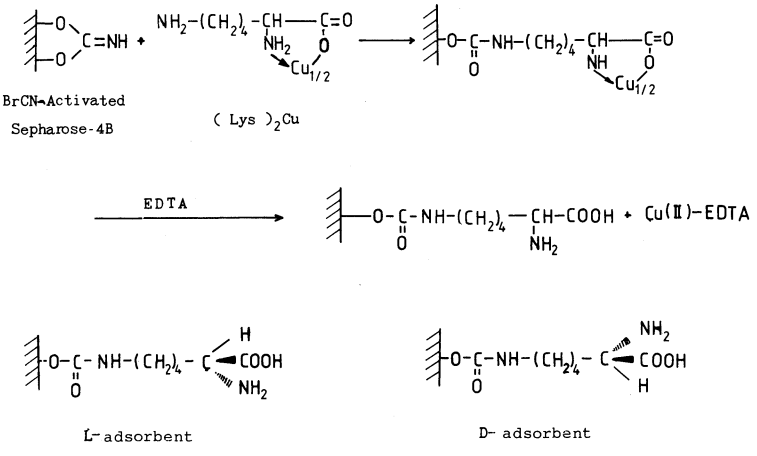


FIG. 1. Adsorbents of Affinity Chromatography.

† Present address: *Himeji Institute of Technology, 2176 Shosha Himeji 671-22, Japan.*

pound of amino acid metabolism. In this report, the features of the adsorbent and the application of the adsorbent for the purification of pyridoxal phosphate dependent enzymes will be described.

## EXPERIMENTAL

*Materials.* All chemicals used in this study were purchased from Wako Pure Chemical Co. and used without further purification unless otherwise noticed. L-Allothreonine was supplied by courtesy of Ajinomoto Co.. BrCN-activated Sepharose-4B was purchased from Pharmacia Fine Chemical Co. Maize seeds (*Zea mays* L.) were purchased from the Takii Seed Co., Kyoto. Aspartate aminotransferase (EC 2.6.1.1) from porcine heart, alanine aminotransferase (EC 2.6.1.2) from porcine heart, glutamate decarboxylase (EC 4.1.1.15) from *E. coli*, and glutamate dehydrogenase (EC 1.4.1.3) from bovine liver were purchased from the Sigma Chemical Co., and used without further purification. Partly purified serine hydroxymethyltransferase (EC 2.1.2.1) was obtained from 500 g of maize seedlings harvested 5 days after germination, homogenized with 1 liter of 0.1 M aqueous $K_2HPO_4$ containing 20 $\mu$M pyridoxal phosphate and 0.2 mM EDTA in a Waring blender. The homogenate was centrifuged (12,000 × *g*) for 15 min, and the supernatant was dialyzed against 20 mM phosphate buffer (pH 7.2, containing 20 $\mu$M pyridoxal phosphate and 0.2 mM EDTA) (Step 1). The ammonium sulfate precipitated fraction (35 to 55% saturation) of the solution of Step 1 was dissolved in 50 ml of 20 mM phosphate buffer and dialyzed against to the same buffer (Step 2). The enzyme solution of Step 2 was put on a column of DEAE-cellulose (2.3 × 40 cm), which had been equilibrated with 20 mM phosphate buffer (pH 7.2, containing 20 $\mu$M pyridoxal phosphate). The serine hydroxymethyltransferase on the column was eluted with 500 ml of 20 mM phosphate buffer (pH 7.2, containing 50 mM KCl and 20 $\mu$M pyridoxal phosphate). The combined active fractions were dialyzed against 20 mM phosphate buffer

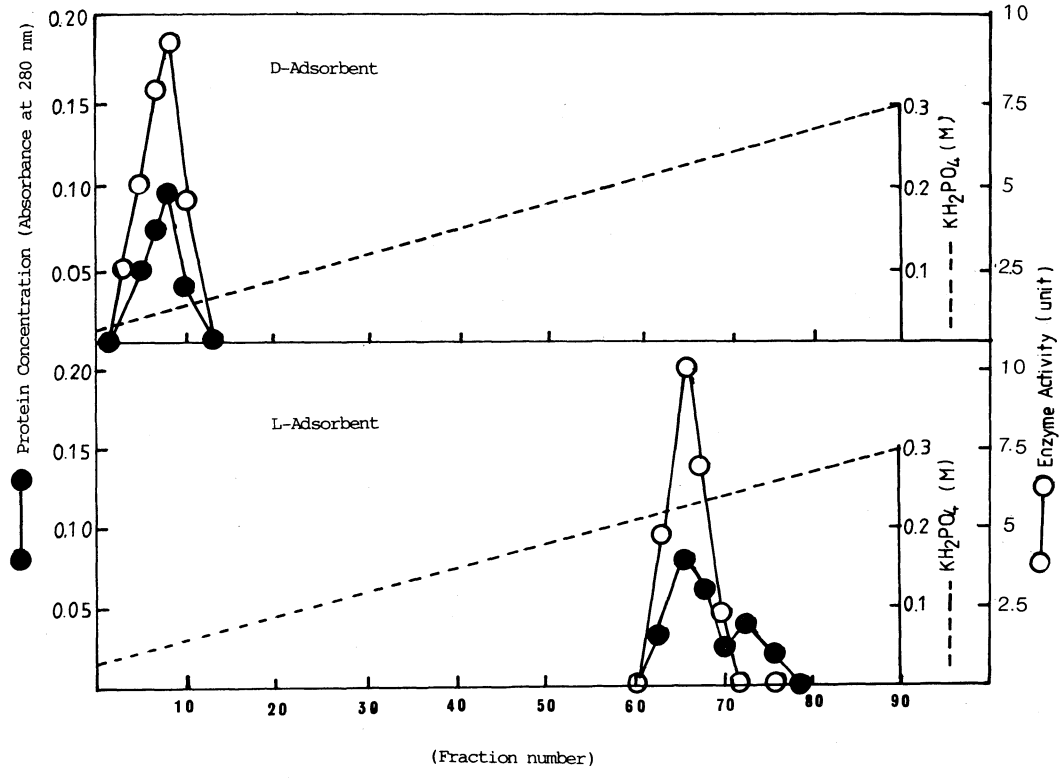


FIG. 2. Affinity Chromatography of Alanine Aminotransferase on L- and D-Adsorbent.

Alanine aminotransferase (0.85 mg of protein) dissolved in 1 ml of 20 mM phosphate buffer (pH 6.8) containing 20 $\mu$M pyridoxal phosphate and 0.2 mM EDTA was put on a column of L- or D-adsorbent (1 × 30 cm), previously equilibrated with the same buffer and eluted with a linear gradient of $KH_2PO_4$ in phosphate buffer from 0.02 to 0.3 M in a total volume of 300 ml. The eluate was collected as 3.3-ml fractions and for each fraction the protein concentration, the enzymatic activity, and $KH_2PO_4$ concentration were measured. ●, absorbance at 280 nm; ○, enzyme units; ----, concentration of $KH_2PO_4$.

(pH 6.8, containing 20 $\mu$M pyridoxal phosphate), and then were concentrated to 10 ml using a UK 10 filter (Step 3). The enzyme solution of Step 3 was chromatographed on the L-adsorbent.

*Adsorbents.* The copper(II) salt of L-lysine (28.9 mg) was dissolved in 100 ml of 0.5 M aqueous NaCl solution then the solution was adjusted to pH 8. Three grams of BrCN-activated Sepharose 4B was added to the solution and the mixture was stirred for 5 hr at room temperature. The resulting gel was separated by filtration and washed with 0.5 M aqueous NaCl solution until no copper(II) salt was detected in the washings. The gel was then transferred into a column and was treated with 250 ml of 0.05 M aqueous solution of EDTA to remove copper(II) which is bound to the $\alpha$-amino carboxyl terminals of the gel as complexes. The eluate was adjusted to pH 8 with aqueous NaOH and made up to 300 ml with water. The Cu(II)–EDTA complexes were measured colorimetrically at 730 nm. From the amount of copper(II) eluted by this treatment, the amount of lysine bound to the gel was estimated to 2 ~ 4 $\mu$mol/ml of gel. The gel in the column was further washed with 500 ml of a 0.5 M aqueous solution of ethanolamine and 500 ml of a 0.5 M aqueous solution of NaCl. The gel (L-adsorbent) thus obtained was suspended in 0.1 M NaCl solution and stored in a refrigerator. D-Adsorbent was prepared in the same way except for the use of D-lysine instead of L-lysine.

*Chromatography.* The D- or L-adsorbent (24 ml) was packed into a column of 1 cm ID. For the chromatography of pyridoxal phosphate dependent enzymes, the column was equilibrated with 20 mM phosphate buffer (pH 6.8) containing 0.2 mM EDTA and 20 $\mu$M pyridoxal phosphate. The conditions of chromatography for pyridoxal phosphate dependent enzymes are described in the text (Figs. 2, 3, and 4). The elution of the enzyme was monitored by the absorbance at 280 nm and by the enzyme activity of the eluent. The recoveries of enzyme activity were 85% for alanine aminotransferase, 89% for aspartate aminotrans-

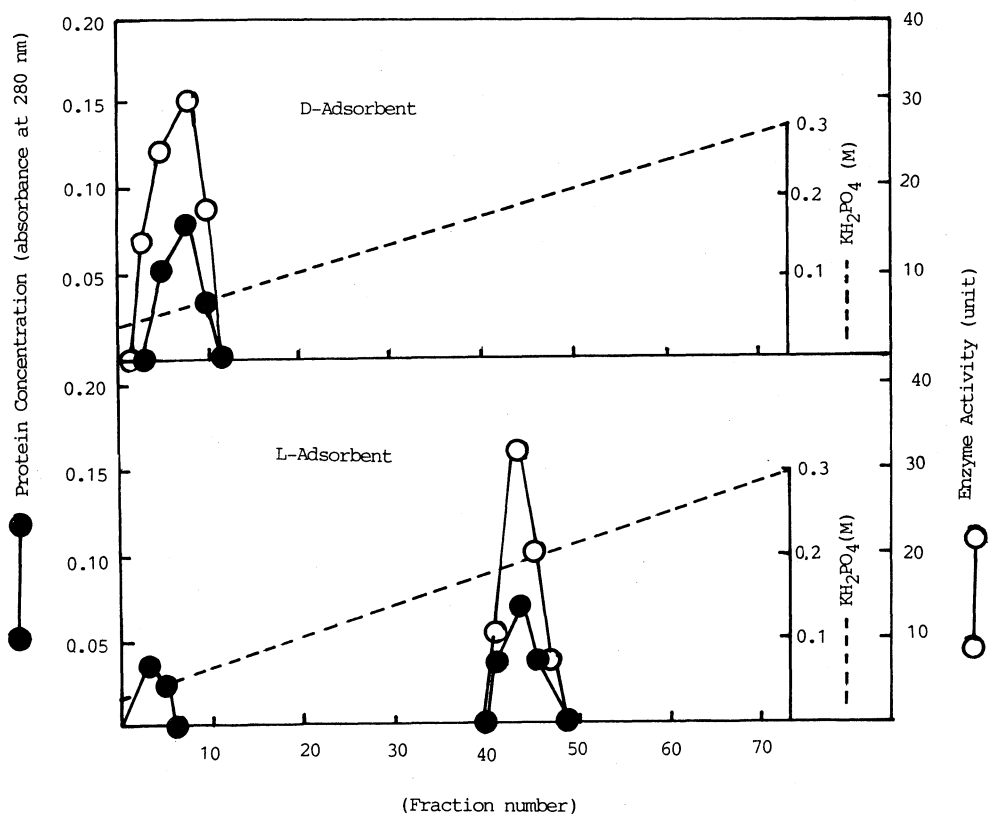


FIG. 3. Affinity Chromatography of Aspartate Aminotransferase on L- and D-Adsorbent.

Aspartate aminotransferase (0.80 mg of protein) dissolved in 1 ml of 20 mM phosphate buffer (pH 6.8) containing 0.2 mM EDTA and 20 $\mu$M pyridoxal phosphate was put on a column of L- or D-adsorbent (1 × 30 cm), previously equilibrated with the same buffer. The elution was done with a linear gradient of $KH_2PO_4$ in phosphate buffer from 0.02 to 0.3 M in a total volume of 300 ml. The eluate was fractionated and for each 4.3 ml fraction the protein concentration, enzymatic activity, and $KH_2PO_4$ concentration were measured. ●, absorbance at 280 nm; ○, enzyme units; ----, concentration of $KH_2PO_4$.

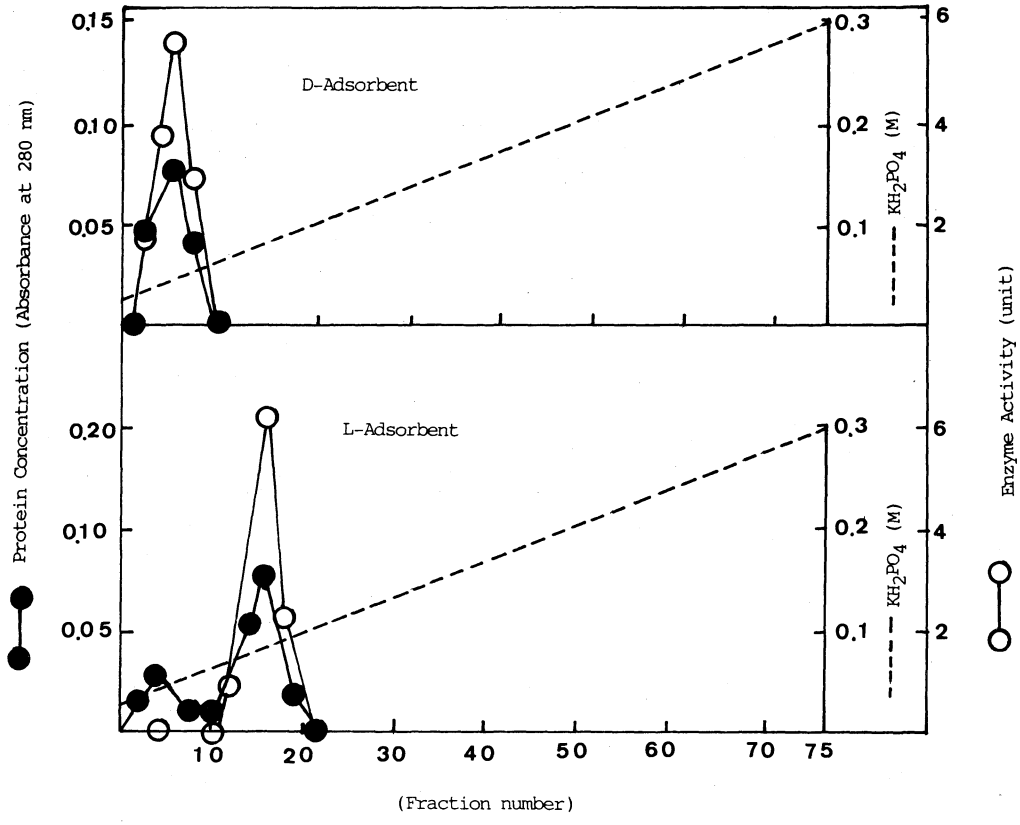


FIG. 4. Affinity Chromatography of Glutamate Decarboxylase on L- and D-Adsorbent.

Glutamate decarboxylase (0.90 mg of protein) dissolved in 1 ml of 20 mM phosphate buffer (pH 6.8) containing 0.2 mM EDTA and 20 $\mu$M pyridoxal phosphate, was put on a column of L- or D-adsorbent (1 × 30 cm), previously equilibrated with the same buffer. The elution was done with a linear gradient of $KH_2PO_4$ in phosphate buffer from 0.02 to 0.3 M in a total volume of 300 ml. The eluate was fractionated and for each 4.3 ml fraction the protein concentration, the enzymatic activity, and $KH_2PO_4$ concentration were measured. ●, absorbance at 280 nm; ○, enzyme units; ----, concentration of $KH_2PO_4$.

ferase, and 82% for glutamate decarboxylase. For the chromatography of glutamate dehydrogenase (NADP dependent enzymes), the column was equilibrated with 20 mM phosphate buffer (pH 6.8) containing 0.2 mM EDTA. Glutamate dehydrogenase (1.1 mg of protein dissolved in 1 ml of the buffer) was put on a column of L- or D-adsorbent (1 × 30 cm) and eluted with a linear gradient of concentrations of $KH_2PO_4$ (0.02 to 0.3 M) in 300 ml of phosphate buffer (pH 6.8).

*Enzyme assay.* The activities of aspartate aminotransferase and alanine aminotransferase were measured by the method of Okawa and coworkers[1] using a commercially available reagent kit (Wako Pure Chemical Co., Ltd.). The activity of glutamate decarboxylase was measured by the amount of L-4-amino butyric acid produced from glutamate by the method of Cizzani.[2] The activity of glutamate dehydrogenase was measured by the amount of NADPH formed, by the method of Anderson and coworkers.[3] The activity of serine hydroxymethyltransferase in the eluent of chromatography was measured by the formation of acetaldehyde from L-allothreonine by the method described in our previous report.[4]

## RESULTS AND DISCUSSION

The procedures for the preparation of L- and D-adsorbent are shown in Fig. 1. The reaction between BrCN-activated Sepharose 4B and the ε-amino groups of lysine was selectively proceeded when α-amino group was protected as a copper(II) complex.

The affinity of a series of enzymes involved in the metabolism of L-glutamic acid for D- and L-adsorbent was evaluated from the re-

tention volume of each enzyme in the ionic strength linear gradient chromatography.

Figure 2 shows the elution patterns of alanine aminotransferase from L- and D-adsorbent. With L-adsorbent, the enzyme was eluted when the eluting solution reached to an appreciably high ionic strength, while with D-adsorbent, the elution of enzyme started immediately after a void volume of solvent was eluted. This clearly showed the presence of stereospecific affinity between alanine aminotransferase and L-adsorbent. The other pyridoxal phosphate dependent enzymes involved in the metabolism of glutamic acid, aspartate aminotransferase and glutamate decarboxylase, also showed a significant affinity for L-adsorbent, as was found from the comparison of elution patterns on L- and D-adsorbents (Figs. 3 and 4). On the other hand, glutamate dehydrogenase was eluted from both L- and D-adsorbent immediately after a void volume of solvent was eluted. That is, this enzyme showed no affinity for either L- or D-adsorbent.

The prosthetic group of glutamate dehydrogenase is NADP. Our results indicated that the affinity for L-adsorbent was not common to all enzymes involved in the metabolism of α-amino acids but is limited to the pyridoxal phosphate dependent enzymes.

The differentiation of a chiral adsorbent by an enzyme implies that at least three of the groups surrounding the chiral center must interact with the active cavity of the enzyme.

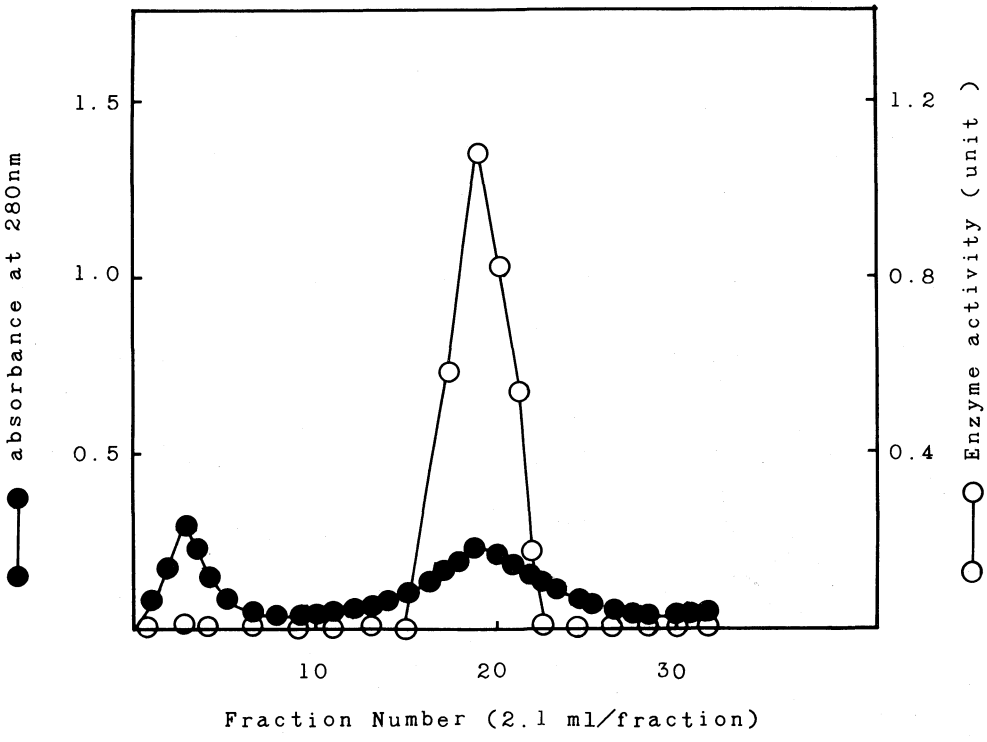


FIG. 5. Affinity Chromatography of the Crude Serine Hydroxymethyltransferase Preparation on the L-Adsorbent Column.

The crude serine hydroxymethyltransferase preparation (21 mg of protein) was put on the column (1 × 35 cm) of L-adsorbent equilibrated with 20 mM phosphate buffer (pH 6.8) containing 0.2 mM EDTA and 20 μM pyridoxal phosphate. After the column was thoroughly washed with the same buffer, the enzyme was eluted with the same buffer containing 50 mM KCl. ●, absorbance at 280 nm; ○, enzyme activity. Fractions 17 to 21 were combined and dialyzed against 20 mM phosphate buffer (pH 6.8 containing 0.2 mM EDTA and 20 μM pyridoxal phosphate).

In this case, the chiral terminal on the adsorbent should enter the cavity directing the methylene group to the inlet and the amino carboxyl terminal to the inside of the cavity. Thus, the position of the methylene group in the cavity was *a priori* determined. The other two interactions that recognize the stereoarrangement of the groups on the chiral center should constitute an attractive force leading to the affinity between L-adsorbent and enzymes. The pyridoxal phosphate dependent enzymes were expected to recognize the L-adsorbent in such a way that the cationic and anionic site spatially arranged in the active cavity face the carboxyl and amino group of the adsorbent by making two pairs of hydrogen bondings, when L-adsorbent is introduced into the cavity. The D-adsorbent cannot interact in this way. This mode of enantiomer differentiation is compatible with the mode of interaction between aspartate and the active cavity of aspartate aminotransferase proposed by Kirsch and

TABLE I. SUMMARY OF PURIFICATION OF SERINE HYDROXYMETHYLTRANSFERASE FROM MAIZE SEEDLINGS

| Steps | Total protein (mg) | Total units | Specific activity (units/mg protein) | Yield (%) |
|---|---|---|---|---|
| 1. Crude extract | 2,123 | 10.7 | 0.005 | 100 |
| 2. $(NH_4)_2SO_4$ (35~55%) | 370 | 9.1 | 0.024 | 86 |
| 3. DEAE-cellulose | 11.7 | 5.5 | 0.47 | 52 |
| 4. L-Adsorbent | 0.5 | 4.6 | 9.2 | 42 |

The activity of serine hydroxymethyltransferase in each purification step was measured from the amount of formaldehyde produced by the cleavage of L-serine by the method of Fujioka.[6]

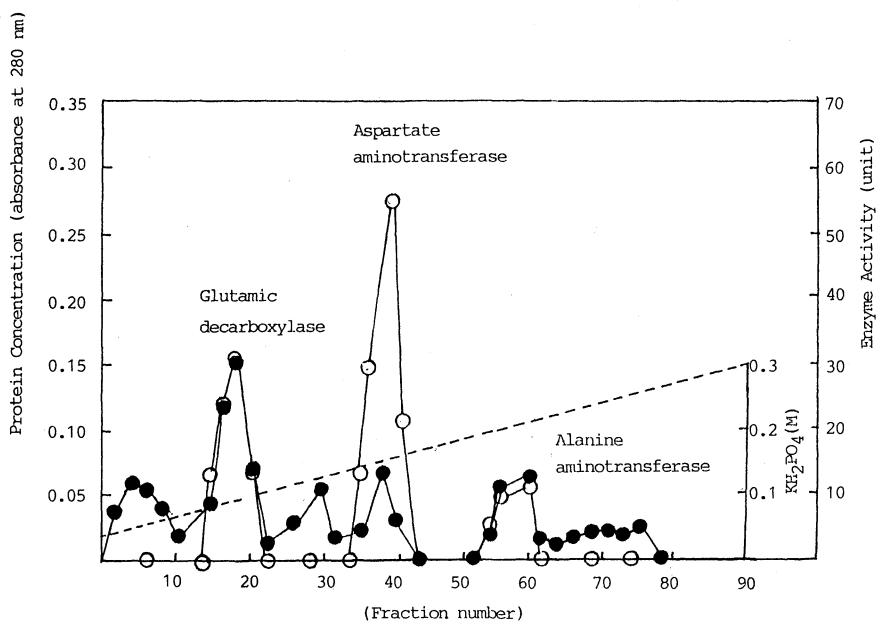


FIG. 6. Affinity Chromatography of a Mixture of Aspartate Aminotransferase, Alanine Aminotransferase, and Glutamate Decarboxylase on L-Adsorbent.

Aspartate aminotransferase (0.96 mg of protein), alanine aminotransferase (0.81 mg of protein), and glutamate decarboxylase (2.81 mg of protein) dissolved in 1 ml of 20 mM phosphate buffer (pH 6.8) containing 0.2 mM EDTA and 20 $\mu$M pyridoxal phosphate were put on a column of L-adsorbent (1 × 30 cm), previously equilibrated with the same buffer. The elution was done with a linear gradient of $KH_2PO_4$ in phosphate buffer from 0.02 to 0.3 M in a total volume of 300 ml. The eluate was fractionated and for each 3.3-ml fraction the protein concentration, the enzymatic activities of aspartate aminotransferase, alanine aminotransferase, and glutamate decarboxylase, and the $KH_2PO_4$ concentration were measured. ●, absorbance at 280 nm; ○, enzyme units; ----, concentration of $KH_2PO_4$.

coworkers[5] based on the results of X-ray crystallography. From molecular evolution, similar modes of interaction are expected in the series of pyridoxal phosphate dependent enzymes. Thus, we expected that L-adsorbent would be a useful adsorbent for the chromatographic purification of pyridoxal phosphate dependent enzymes.

Affinity chromatography with L-adsorbent was excellent for the purification of serine hydroxymethyl transferase, a pyridoxal phosphate dependent enzyme not involved in the metabolism of glutamate. The conditions of chromatography and the elution pattern are shown in Fig. 5. The recovery of the enzyme in the chromatography was 83.6% and the eluted enzyme was essentially pure by polyacrylamide gel electrophoresis. The purification efficiency of the enzyme from the crude extract of maize seedlings is summarized in Table I. The activity of the enzyme was amplified 1840-fold with overall recovery of 42%.

L-Adsorbent is also an effective adsorbent for the group separation of pyridoxal phosphate dependent enzymes from a crude extract of biomaterial. Furthermore, the mixture of the enzymes fixed on the adsorbent can be fractionated into each component by ion-strength linear gradient elution. As an example, the separation of an authentic mixture of alanine aminotransferase, asparate aminotransferase, and glutamate decarboxylase is shown in Fig. 6.

## REFERENCES

1) N. Okawa, Y. Miyoshi, S. Matuura and H. Misaki, *Rinshobyori*, **28**, 70 (1978).
2) I. Cozzani, *Analytical Biochemistry*, **33**, 125 (1970).
3) B. M. Anderson, C. D. Anderson, and J. E. Churchich, *Biochemistry*, **5**, 2893 (1966).
4) T. Masuda, M. Yoshino, A. Tai and H. Ozaki, *Agric. Biol. Chem.*, **44**, 2109 (1980).
5) J. F. Kirsch, G. Eichele, G. C. Ford, M. G. Vincent and J. N. Jansonius, *J. Mol. Biol.*, **174**, 497 (1984).
6) H. Ogawa and M. Fujioka, *J. Biochem.*, **90**, 381 (1981).