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SEPARATION OF ALANINE AMINOTRANSFERASE AND ALBUMIN ON VARIOUS SUBSTITUTED AGAROSES

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A systematic study was made to find an affinity chromatographic purification method for alanine aminotransferase (AAT). Sepharose 4B was derivatized with various substrate-like ligands and the separation of AAT and albumin was tested on gels. The largest separation of the proteins was achieved on an amino ethyl-agarose to which aminooxy alanine was coupled via the peptide bond. The center points of albumin and AAT peaks were 42 and 112 ml, respectively. Aminooxy alanine was the strongest inhibitor of AAT of the ligands tested. Some weak inhibitors, however, were able to separate the proteins, too. It is suggested, based on the results, that the purification mechanism is a combination of hydrophobic, electrostatic, and biospecific affinities.

INTRODUCTION

Affinity chromatographic methods used for pyridoxal phosphate (PLP) enzymes have mainly been based on the low K_m value between the apoenzyme and the coenzyme. In these systems an apoenzyme is first prepared and the protein is eluted through a column in which PLP or pyridoxamine phosphate is bound (1,2). The methods are often tedious to carry out and there is a risk of denaturation during the long purification procedure (3). Another type of affinity chromatography is based on the use of substrates, modifiers, or inhibitors of the enzyme bound to a support.

The purpose of our study was to find a purification method of the latter type for alanine aminotransferase (AAT). We succeeded earlier in getting a good purification with a gel to which cycloserine, a strong inhibitor of AAT, was bound (4). In the present study various substituted agaroses were prepared to find the best gels for the purification of AAT, and to elucidate the mechanism by which the purification takes place. Gels other than cycloserine derivative of agarose used in our previous study were obtained for the purification of AAT. It is suggested that the mechanism is related to "hydrophobic chromatography," in which bioaffinity also plays a part.

MATERIALS AND METHODS

L-alanine aminotransferase, EC 2.6.1.2. from pig heart in a 1.8 M $(NH_4)_2SO_4$ solution containing 74 or 115 units/mg protein, bovine albumin fraction *V* (96–99% albumin), Sepharose 4B 200, D-cycloserine, and homocysteine thiolactone were purchased from the Sigma Chemical Co., St. Louis, Missouri. Cyanogen bromide was obtained from the Eastman Kodak Co., Rochester, New York; and *N*-ethyl-*N'*-(3-dimethyl amino propyl)-carbodiimide, tetramethylene diamine, hexamethylene diamine, and 6-aminocaproic acid from E. Merck AG., Darmstadt, Germany. Lactate dehydrogenase and reduced β-nicotinamide adenine dinucleotide were purchased from Boehringer GmbH, Mannheim, Germany, and 2-aminohexane from K & K Laboratories, Plainview, New York. β-Aminooxy alanine was prepared from D-cycloserine as follows: 1 g of D-cycloserine was dissolved in 20 ml of water and the pH was adjusted to 0.5 with 6 M HCl. After 24 h at 40°C, water and the excess HCl were evaporated off with a rotatory evaporator. Ascending paper chromatography from the very hygroscopic aminooxy alanine dihydrochloride resulted in one ninhydrin positive spot ($R_f = 0.6$ in butanone:propionic acid:water = 75:25:30). Paper electrophoresis in 0.1 M sodium citrate, pH 3.3, also gave one band and its mobility, compared with that of cycloserine, was 0.7.

Preparation of the Agarose Derivatives

The gels were prepared from cyanogen bromide–activated Sepharose 4B using 0.1 g CNBr/ml gel. The amino ligands were coupled to the activated agarose at pH 9.5 (5). The ligand concentration was 0.25 M, except that it was 0.5 M for diamines. Dimethylformamide was added to give 20% final concentration to the reaction mixture of the gels presented in Table 2. Water-soluble carbodiimide was employed in the preparation of gels Nos. 2–4 in Table 1 when the carboxyl group was coupled to AE-agarose (5).

D-cycloserine and L-alanine in gels number 6 and number 7 in Table 1 were bound to butyraldehyde-agarose (gel No. 5; its preparation is in ref. 6) via the Schiff base reaction. The subsequent sodium borohydride reduction was carried out as presented elsewhere, except that a water–methanol mixture was used (7). A small water content favors Schiff base formation; thus, a smaller amount of amino compound can be used. The amino ligand (40 μmol/ml gel) was dissolved in a minimum volume of water, then methanol was added to give a 0.04 M ligand concentration, avoiding the precipitation of the amino compound. The water was gradually exchanged from the butyraldehyde-agarose with methanol.

Quantitative Determination of Amino Ligands Bound to Agarose

Ninhydrin reactive ligands were determined after acid hydrolysis of the gels by a Perkin–Elmer KLA-5 amino acid analyzer. The liberation of the ligands was carried out in ampuls containing 2 ml of packed gel and 2 ml of concentrated HCl. The solution was incubated for 12 h at 95°C. The standards were made in the same way in the ampuls containing 2 ml Sepharose and a known amount of added ligand. Cycloserine and aminooxy alanine were determined from the peak of serine to which they decompose in strong acid hydrolysis. The reported quantities of the ligands in Table 1 are determined by the method described above, except for gel number 3, which was estimated by a 5,5′-dithiobis(2-nitrobenzoic acid) reaction (5).

Protein Determination

Absorbance at 280 nm was measured by a Beckman DU-equipped Gilford Model 2220 spectrophotometer.

Determination of Activity of Alanine Aminotransferase

One milliliter of substrate solution containing 40 μmol of L-alanine, 20 μmol of disodium 2-oxoglutarate, 0.3 μmol of $NADH_2$, and 0.2 μl of lactate dehydrogenase in 0.1 M sodium-phosphate buffer, pH 7.6, was incubated with a 0.1 ml sample of AAT at 37°C for 30 min. The change in absorbance at 340 nm was measured in the linear part of the reaction curve.

Chromatographic Runs

The runs were carried out at 8°C using glass columns of 15 or 12 mm diameter for 50 or 25 ml gel volumes, respectively. The gels were equilibrated before runs with the elution buffers that are mentioned in the tables. Five microliters of commercial enzyme and about 2 mg of bovine albumin in 0.5 ml of the elution buffer were applied to the gel. The elution rate was 30–40 ml/h. The fractions were collected by an ISCO model 820 fraction collector. Between runs the gels were washed in the column with 1 M NaCl. The center points of the albumin and AAT peaks in Tables 1 and 2 represent the values when half of the total amount was eluted out of the column, as calculated from integrated area of the peaks.

RESULTS

In the beginning the salt concentration and pH value of the elution buffer were optimized to separate albumin and AAT on cycloserine-agarose

(CS-agarose). Sodium chloride at the concentration of 25–100 mM, in 25 mM sodium phosphate buffer, pH 7.6, gave best separation of the proteins. Increase of the salt concentration to 0.5 M caused a gradual equalization of the elution volumes. Decrease of pH from 8 to 6 in 25 mM phosphate containing 0.1 M NaCl increased the elution volume of AAT from 36 to 48 ml, while the elution volume of albumin was almost constant (28–30 ml). Therefore, 0.1 M NaCl in 25 mM sodium phosphate, pH 6.0, was selected for subsequent experiments.

The main results of our study are presented in Tables 1 and 2. As can be seen from the column headed v_{alb} in Table 1, AE-agarose (number 1) and, to a lesser extent, its derivatives (aminooxy alanine-, homocysteine-, and 2-oxoglutaric-AE-agarose; numbers 2–4) had the highest ability to retard albumin. The gels (numbers 6 and 7) synthetized via butyraldehyde-agarose (number 5) had low albumin elution volumes, probably because they are less ionic than AE-agaroses. The unreacted aldehydic ligands apparently are reduced to *n*-butanol ligands during the borohydride reduction.

The elution volumes of AAT are dependent on the ionic nature of the gel, as well (see column v_{AAT}). Even a 5–10% substitution (it can be figured out from the column quantity of ligand in Table 1) of the AE groups with additional ligands greatly enhanced the affinity of AAT to the gel. The largest relative difference in elution volumes of albumin and AAT (column v_{AAT}/v_{alb}) was on aminooxy alanine- and homocysteine-AE-agaroses (numbers 2 and 3). They both have free positively charged amino groups.

Figure 1 shows typical elution curves for the two proteins on aminooxy alanine-AE-agarose gel. As can be seen from this figure, the separation is complete. In all experiments reported in the tables and in the figures, 90–100% of the enzyme activity and albumin were recovered in the fractions after chromatographic runs.

As can be seen in Table 2, some experiments were done to test the effect of chain length and charge of the ligands. Because neither albumin nor AAT came out of the column totally on AH-agarose with the elution buffer used before, the salt concentration was raised to 0.5 M. Table 2 shows that the elution volume of albumin and particularly that of AAT increased with elongation of the carbon chain. Hydrophobic pentyl-agarose (number 4, in Table 2) had a slightly stronger affinity toward albumin and AAT than amino alkyl-agarose with similar ligand length (AB-agarose). Negatively charged carboxyl groups at the end of the hydrocarbon chain (gel number 5) possessed a low affinity to the proteins, and practically no separation was achieved on the gel.

The separation of albumin and AAT was studied more thoroughly on CS-agarose (number 8 in Table 1). Figure 2 shows an elution pattern that was obtained using the enzyme preparation containing 74 units/mg of

TABLE 1. **Chromatographic Values of the Elution of Pig Heart Alanine Aminotransferase (AAT) and Bovine Albumin on Various Derivatized Agaroses**[a]

Gel number	Structure of the gel	v_{alb} (ml)	v_{AAT} (ml)	v_{AAT}/v_{alb}	Abbreviation used in the text	Quantity of the ligand bound (μmol/ml of packed gel)
1	III-$NHCH_2CH_2NH_3^+$	47	67	1,4	AE-agarose	10
2	III-$NHCH_2CH_2NHCOCHCH_2-O-NH_2$ (with NH_3^+)	42	112	2,7	aminooxy alanine-AE-agarose	1
3	III-$NHCH_2CH_2NHCOCHCH_2CH_2SH$ (with NH_3^+)	44	105	2,4	homocysteine-AE-agarose	0.5
4	III-$NHCH_2CH_2NHCOCH_2CH_2COCOO^-$	46	69	1,5	2-oxoglutaric-AE-agarose	not measured
5	III-$NHCH_2CH_2CH_2CH_2CHO$	—	—	—	butyraldehyde-agarose	9
6	III-$NHCH_2CH_2CH_2CH_2CH_2NH-CH(-CH_2-O-C(=O)-NH)$	31	58	1,9	CS-pentyl-agarose	5
7	III-$NHCH_2CH_2CH_2CH_2CH_2NH-CH(CH_3)-COO^-$	36	49	1,4	alanine-pentyl-agarose	2
8	III-$NH-CH(-CH_2-O-C(=O)-NH)$	28	48	1,7	CS-agarose	10

[a] Fifty milliliters of the gel were packed into a column of 15 mm diameter. The elutions were carried out with a 25 mM sodium phosphate buffer, pH 6.0, containing 0.1 M NaCl. The abbreviations v_{alb} and v_{AAT} indicate the center points of albumin and AAT peaks, respectively. The value v_{AAT}/v_{alb} describes the ability of the support to separate the proteins. The sample was about 2 mg of albumin and 5 μl of the enzyme (specific activity 115 units/mg) in 0.5 ml of the elution buffer. AE = amino ethyl; CS = D-cycloserine.

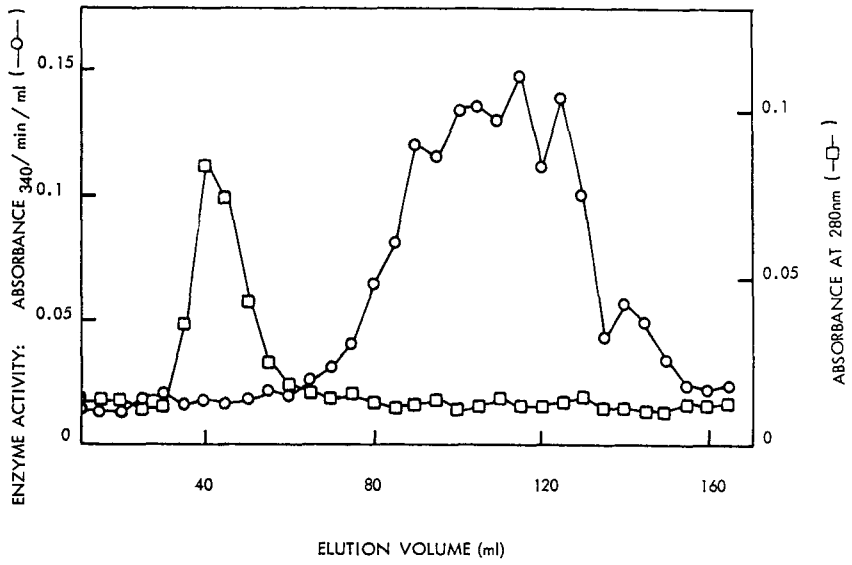

FIG. 1. Chromatography of pig heart alanine aminotransferase and bovine albumin on aminooxy alanine-AE-agarose. See Table 1 for the elution conditions and abbreviation of the gel.

TABLE 2. **Elution Values of Pig Heart Alanine Aminotransferase and Bovine Albumin on Some Derivatized Agaroses Having Ligands of Different Chain Length and Charge**[a]

Gel number	Structure of the gel	v_{alb} (ml)	v_{AAT} (ml)	v_{AAT}/v_{alb}	Abbreviations used in the text
1	III-$NHCH_2CH_2NH_3^+$	25	29	1.2	AE-agarose
2	III-$NHCH_2CH_2CH_2CH_2NH_3^+$	26	31	1.3	AB-agarose
3	III-$NHCH_2CH_2CH_2CH_2CH_2CH_2NH_3^+$	31	48	1.5	AH-agarose
4	III-$NH\underset{\displaystyle CH_3}{\underset{\vert}{C}}HCH_2CH_2CH_3$	26	38	1.5	pentyl-agarose
5	III-$NHCH_2CH_2CH_2CH_2CH_2COO^-$	27	28	1.0	caproic-agarose

[a] The gel volumes were 25 ml (diameter, 12 mm). Elutions were carried out with a 25 mM sodium phosphate buffer, pH 6.0, containing 0.5 M NaCl. The other conditions are the same as in Table 1. AE = amino ethyl; AB = amino butyl; AH = amino hexyl.

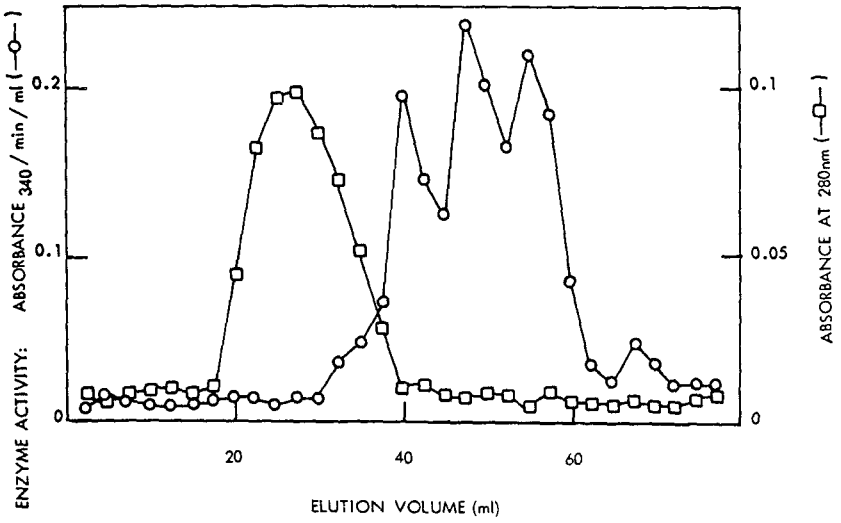

FIG. 2. Chromatography of alanine aminotransferase (from pig heart) and bovine albumin on CS-agarose. The conditions were the same as in Fig. 1 and in Table 1, except that the specific activity of the enzyme was 74 units/mg.

protein. One can see from this figure that the enzyme activity was partly divided into subfractions. An elution pattern of the same shape was difficult to reproduce, but three main activity peaks were usually found. An enzyme preparation with higher specific activity (115 units/mg) did not give more than one peak. Temperature denaturation of this enzyme leading to 50% inactivation (the sample was kept for 5 min at 70°C) gave only one peak, as well. It might be that in this preparation some subforms of the native enzyme are absent (8). The CS-agarose was found good enough to purify AAT from human serum without prepurification, as will be published later. Multiple subfractions were also observed in the serum.

In some experiments on CS-agarose the PLP coenzyme of the commercial AAT was changed to a pyridoxamine phosphate form (9) to investigate whether an interference between the active site and the ligand occurs in the chromatography. However, the enzyme activity was not found in the fractions after normal chromatographic runs or even after eluting with 1 M NaCl. Pyruvate or L-alanine at 1 mM in the elution buffer did not influence the purification system on CS-agarose.

DISCUSSION

The results indicate that the mechanism of separation of the proteins is not based on "pure" affinity chromatography, since the gels having only

weak inhibitory effect—e.g., AE-agarose (see Table 1)—could also separate the proteins. On the other hand, the transamination reaction involves two substrates, and all the functional groups of amino and keto acids have an influence on the binding of the substrates (3). Thus, any of the gels studied could, in principle, weakly affect AAT in a biospecific manner.

There is evidence that at least some of the gels do function biospecifically toward AAT: (a) because the PLP coenzyme of AAT was in an aldehydic form and because an amino group is essential in the Schiff base reaction, it is reasonable to assume that a free amino group in the ligand is important for good separation; (b) because the substitution of 5–10% of the total amino groups in the AE-agarose with amino acid inhibitors (gels number 2 and number 3 in Table 1) greatly increased the affinity of AAT to the gels as opposed to albumin; and (c) because aminooxy alanine-AE-agarose best separated the proteins, and because the ligand was the most potent inhibitor of AAT used (K_i of the order of 10^{-5} M).

It has been stated that "a nonbiospecific adsorption does not become obtrusive unless the enzyme is brought into a close contact with the spacer arm by prior biospecific adsorption on the attached ligand" (10). This model could explain the potency of some "hydrophobic" supports presented in the literature. The biospecific part of the ligand would thus act as a "hydrophobic, or electrical interaction catalyte," although the biospecific affinity would be weak. If the mechanism is catalytic, decreasing the elution rate of a protein mixture in a weakly biospecific column ought to enhance the nonbiospecific binding as well; in the same way as raising the temperature would do. Living cells may have a similar mechanism in the compartmentation of macromolecules.

Owing to the complex mechanism of transaminases and the limited amount of conclusive knowledge accumulated from "hydrophobic chromatography," it is difficult to draw exact conclusions about the purification mechanism of AAT. We suggest that the mechanism is based on a combination of hydrophobic, electrostatic, and biospecific affinities.

The gels that were best able to separate the proteins in our study are convenient in the purification of AAT and there is evidence that subforms of AAT can be recognized.

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