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SEPARATION OF ASPARTATE AMINOTRANSFERASE FROM ALBUMIN ON SUBSTITUTED AGAROSES

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The affinity of aspartate aminotransferase to its inhibitors coupled to Sepharose 4 B was tested. The affinity was measured as retardation of the enzyme compared to "inert" bovine serum albumin. Carboxylic ligands of citrate and 2-oxoglutarate bound to aminoethyl-Sepharose were the best of those tested in separation of the proteins. Because the ligands were not essentially hydrophobic and because it was shown that ion-exchange is not significant in the elution conditions used, it was suggested that the separation is based on the recognition of substrate or effector by the enzyme.

INTRODUCTION

Affinity methods dealing with pyridoxal-5'-phosphate (PLP)-dependent enzymes (1,2) are mainly based on what has been described as "general ligand affinity chromatography," in the context of adenine nucleotide-dependent enzymes (3). Schell, Cotariu, and Gozia presented a specific system for aspartate aminotransferase (AspAT, EC 2.6.1.1) using aspartate and 2-oxoglutarate bound to agarose via a six-carbon spacer (4). Our previous papers (5,6) described an affinity method for alanine aminotransferase (AlaAT, EC 2.6.1.2).

The purpose of the present work was to investigate the behavior of AspAT on differently substituted agaroses to elucidate the affinity mechanism and compare it with the results obtained with AlaAT.

The results indicate that the mechanism is biospecific but that the gels that best purify the enzymes are different.

MATERIALS AND METHODS

Pig heart cytosolic AspAT (230 units/mg of protein) in 3.0 M $(NH_4)_2SO_4$ solution, alkaline phosphatase (EC 3.1.3.1) from *Escherichia*

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coli, type III-S, acid phosphatase (EC 3.1.3.2) from wheat germ, and cytochrome *c* from horse heart, type II-A (practical grade, 75%) were purchased from Sigma Chem. Co. St. Louis, Missouri. Technical grade alkaline phosphatase from calf intestine was from Fluka AG., Buchs.

Preparation of the Agarose Derivatives

Citric acid was coupled with aminoethyl-Sepharose 4 B 200 with water soluble carbodiimide (7) using 70 mg of citric acid/ml of gel. The 2-oxoglutarate and citrate concentration of the gels was estimated using radioactive compounds to be about 1 μmol/ml. Synthetic and analytical methods for the other gels were the same as were used earlier (5).

Enzyme Activity and Protein Determinations

Asparate aminotransferase activity was determined using dinitrophenyl hydrazine (8). Alkaline and acid phosphatases were measured with the usual methods using 4-nitrophenylphosphate as the substrate. Absorbance at 410 nm indicated cytochrome *c*, while absorbance at 280 nm indicated protein.

RESULTS

Table 1 shows the elution volumes of AspAT and albumin on seven differently substituted agaroses. Gels containing carboxylic substituents attached to AE-agarose (numbers 1 and 2 in Table 1) separated the proteins best and resulted in a 10 to 20-fold purification with a yield of 85%, as can be estimated from Fig. 1. The optimum pH of the separation appeared to be at about 6, which was indicated by the fact that the elution volume of albumin was almost independent of the pH while AspAT retarded maximally at pH 6 (Fig. 2). The pH optimum might be due to interactions between carboxylic ligands and a histidyl residue of the active center of AspAT (9). It is noteworthy that AlaAT was also best separated at about pH 6, but essentially on gels containing free amino ligands (numbers 3–7 in Table 1, ref. 5).

Since the gels of Table 1 are not easily classified as hydrophobic, other reference proteins having different isoelectric points (IP) were introduced to test the ion-exchange properties. Table 2 shows their isoelectric points and elution volumes on 2-oxoglutaric-AE-agarose. All reference proteins other than cytochrome *c*, which have a high positive net charge at pH 6.0, had elution volumes very similar to albumin. Technical grade alkaline phosphatase was purified 20-fold with a yield of 80% in the run. The enzyme

TABLE 1. **Elution Volumes of Pig Heart Cytosolic Aspartate Aminotransferase and Bovine Albumin on Derivatized Agaroses**[a]

Gel number	Structure of the gel	v_{alb} (ml)	v_{AspAT} (ml)	v_{AspAT}/v_{alb}	Abbreviation used in the text
1	II—$NHCH_2CH_2NHCOCH_2CH_2COCOO^-$	46	66	1.4	2-oxoglutaric-AE-agarose
2	II—$NHCH_2CH_2NHCOC(CH_2COO^-)_2$—OH	40	56	1.4	Citrate-AE-agarose
3	II—NH—CH(CH_2)—C(=O)—NH—O	30	36	1.2	CS-agarose
4	II—$NHCH_2CH_2CH_2CH_2NH$—CH(CH_2)—C(=O)—NH—O	34	37	1.1	CS-butyl-agarose
5	II—$NHCH_2CH_2NHCOCH(NH_3^+)CH_2CH_2$—SH	44	52	1.2	Homocysteine-AE-agarose
6	II—$NHCH_2CH_2NHCOCH(NH_3^+)CH_2$—O—$NH_2$	43	46	1.1	Aminooxyalanine-AE-agarose
7	II—$NHCH_2CH_2NH_3^+$	44	45	1.0	AE-agarose

[a] Fifty milliliters of the gel were packed into a column with a 15-mm internal diameter. The elution was carried out at 8°C with 25 mM sodium phosphate buffer, pH 6.0, containing 0.1 M NaCl. The abbreviations v_{alb} and v_{AspAT} indicate the area-weighted center points of the albumin and AspAT elution curves, respectively. The samples contained about 2 mg of albumin and 5 μl of AspAT solution in 0.5 ml of the elution buffer.

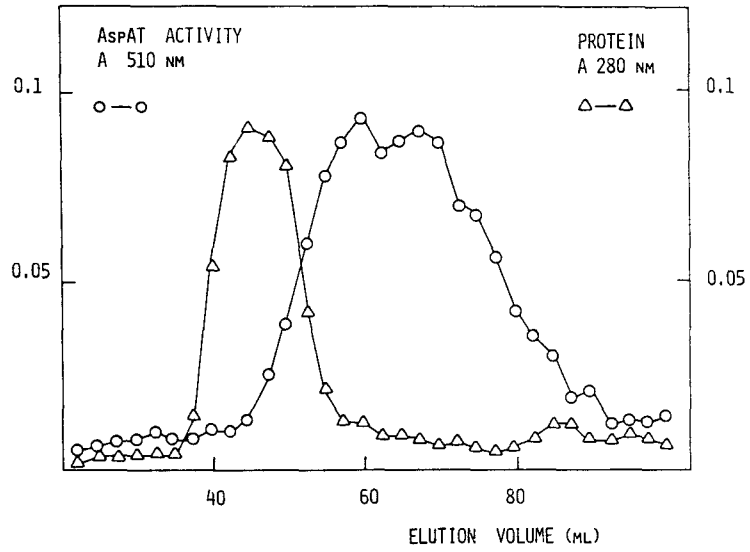

FIG. 1. Chromatography of pig heart aspartate aminotransferase and bovine albumin on 2-oxoglutarate-AE-agarose (see gel number 1 in Table 1). The elution conditions are given in Table 1.

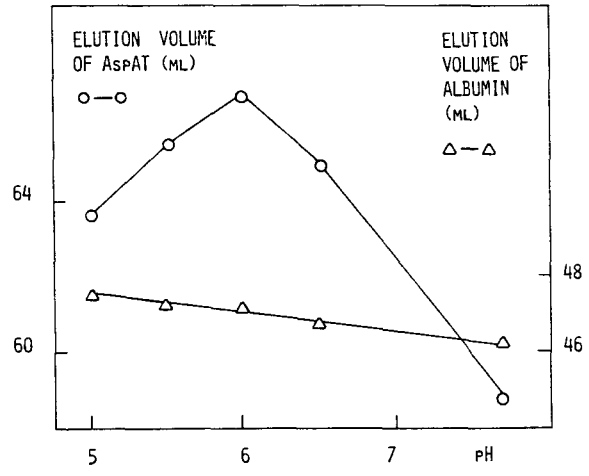

FIG. 2. The effect of the pH of the elution buffer (25 mM sodium phosphate plus 0.1 M NaCl) on elution volumes of aspartate aminotransferase and albumin chromatographed on 2-oxoglutarate-AE-agarose (gel number 1 in Table 1). Details of the elution conditions are given in Table 1. Protein, absorbance at 280 nm; AspAT, absorbance at 510 nm.

TABLE 2. **Elution Volumes (v_e) of Proteins Having Different Isoelectric Points (IP) on 2-oxoglutarate-AE-agarose Under Conditions Given in the Legend of Table 1**

Protein	Source	IP[a]	v_e (ml)
AspAT	Pig heart	5.7	66
Albumin	Bovine serum	5.1	46
Alkaline phosphatase	*E. coli*	5.3	47
Alkaline phosphatase	Calf intestine	4.4	47
Acid phosphatase	Wheat germ	—[b]	48
Cytochrome *c*	Horse heart	9.4	53

[a]From ref. 18.
[b]Not known to us.

preparate from calf intestine apparently contained proteins having very low isoelectric points which caused retardation of bulk protein ($v_e = 57$ ml). This was supported by the fact that a pepsin preparation (IP = 1–2) was not elutable from the column in a moderate volume with the buffer used. Consequently, these results indicate that 2-oxoglutaric-AE-agarose behaved as a mixed-function ion-exchanger, but that ion-exchange was significant for separation purposes, under the conditions used, only when the protein had a very high positive or negative charge. Thus, albumin appeared to have been the correct reference protein for studies of the present work as well.

If we do not suppose a rare situation in which the AspAT molecule has a local area possessing a heavy charge density ("wrong-side" adsorption, ref. 10), the separation of AspAT is not explainable by ion-exchange.

DISCUSSION

Ligands for the purification of AspAT were selected on the basis of their K_i- of K_m-values as given in the literature (11–13). 2-Oxoglutarate is a substrate of AspAT and has a K_m value of about 10^{-4} M (14,15). It is well known that other mono- and dicarboxylic acids also bind tightly to AspAT (16). Because the conditions of synthesis were chosen so that, on the average, only one carboxyl of citrate was attached to AE-agarose, the resulting gel (number 2 in Table 1) might possess affinity due to two free carboxyls imitating the substrates. Low pH favors the binding (15).

When comparing the elution volumes of albumin (headed v_{alb} in Table 1), one can see that they lie in a rather narrow range (6 ml) on AE-agarose and its derivatives because of the different charges and chain lengths of the

ligands. We believe that variation of elution volumes on different gels is, in reality, smaller than 6 ml because of the difficulties in accurately measuring the gel volumes. This narrow range is understandable because the degree of substitution of AE-groups was only 5 to 10%, and because the positive charge of the isourea linkage additionally buffers the gels to maintain a positive charge. As stated under Results, the contribution of ion-exchange to separation is small and, therefore, an increase of about 20 ml in the elution volume of AspAT when 10% of the AE-groups were substituted with carboxylic ligands seem to us most probably explainable as being due to substrate recognition or carboxylic inhibition.

It is interesting that AspAT and AlaAT behaved so differently on various affinity gels, although they catalyze closely similar reactions. One explanation is that their mode of recognizing substrates is different. AspAT seems to recognize them preferable by carboxylic groups and AlaAT by amino groups. Since transaminases have been supposed to possess separate binding sites for keto and amino substrates, it is possible that the site topographically most available to ligands is reversed with AlaAT and AspAT (5,17).

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