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AFFINITY CHROMATOGRAPHY OF B_6-VITAMIN-DEPENDENT ENZYMES: PURIFICATION OF PIG-HEART BRANCHED-CHAIN AMINO ACID TRANSAMINASE

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SUMMARY

Pig-heart branched-chain amino acid transaminase (EC 2.6.1.42) was purified to near homogeneity with a yield of 27%. A prepurification was performed by heat treatment, gel chromatography and DEAE-Sepharose methods. For the final step, several affinity gels were tested and the one containing cycloserine coupled to CNBr-activated Sepharose 4B was selected. This effected an additional five-fold purification with a yield of 60%. The present affinity results are compared with corresponding studies with other aminotransferases in an attempt to find possible universal techniques for their purification.

INTRODUCTION

We have earlier studied the affinity chromatographic purification of alanine aminotransferase (AlaAT)[1–3] and aspartate aminotransferase (AspAT)[4] on various agaroses derivatized with substrates or inhibitors of the enzymes. These studies were designed to search for common parameters needed for the affinity purification of B_6-vitamin-dependent enzymes. We report here affinity results from a new B_6-enzyme, branched-chain amino acid transaminase (BCAT; EC 2.6.1.42) from pig heart.

BCAT is widely distributed in different organisms; it catalyses both biosynthetic and catabolic reactions of branched-chain amino acids. Animals cannot synthesize them and thus their BCAT performs only the catabolic functions[5]. The enzyme from pig heart has been studied in detail[6–10]: it has a molecular weight of 75,000 and is probably formed from two subunits[8,11]. BCATs from prokaryotes are more complex, at least as far as the biosynthetic enzyme species are concerned[5,12–15].

Mercaptoethanol usually activates BCAT manifold and the activation is dependent on the purity and the age of the preparation[10]. Caproate significantly protects the enzyme, and whenever this agent is omitted from the isolation buffer the enzyme degenerates to a form having a higher K_m and a reduced k_{cat}[8]. Alanine aminotransferase and BCAT resemble each other at least in the respect that they both contain essential thiol groups and very tightly bound coenzyme pyridoxal 5'-phosphate (PLP). Because BCAT readily loses its activity in crude preparations, it is clear that short and effective affinity methods are especially beneficial in its purification.

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Our earlier studies with other aminotransferases[1–4] were made chiefly with relatively pure enzyme preparations. To facilitate a comparison of the affinity results with the previous findings and to increase the capacity of the affinity gels, the enzyme BCAT was prepurified. These prepurification steps were a modernized version of the method of Jenkins and Taylor[8,16], which was developed before the present, more convenient, materials were available.

EXPERIMENTAL

Reagents

D-Cycloserine, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, α-ketoisocaproic acid and pyridoxal 5'-phosphate were purchased from Sigma (U.S.A.). Caproic acid and 6-aminocaproic acid were from E. Merck (Darmstadt, F.R.G.). Bis-(3-aminopropyl)amine was from Fluka (Buchs, Switzerland) and 1,2,4,5-benzenetetracarboxylic acid anhydride from Aldrich-Europe (Beerse, Belgium). Ultrogel AcA 44 was purchased from LKB-Producter (Bromma, Sweden) and DEAE-Sepharose CL-6B and Sepharose 4B were from Pharmacia, (Uppsala, Sweden). O-Aminoserine (aminooxyalanine) was synthesized as described elsewhere[17].

Preparation of the affinity gels

The ligands were similar to those used in our earlier studies[1–4], and correspondingly similar synthetic methods were used. Synthesis of the gels commenced with the activation of Sepharose 4B by using 0.15 g of cyanogen bromide (dissolved in acetonitrile) per millilitre of packed gel. The diamines (1 *M*) were allowed to react with the activated gel at pH 10.0 (0.1 *M* sodium tetraborate) at 10°C for 16 h. For monoalkylamines an 0.5 *M* ligand solution was applied, whereas cycloserine was reacted at a concentration of 0.25 *M* at pH 9.5. Valine and amino-oxyalanine were coupled onto aminated gels by the carbodiimide reaction: 1 mmol of valine or amino-oxyalanine was employed per 60 ml of the spacer gel. After washing, the gels were stored at +2°C.

For coupling of pyromellitic acid dianhydride to to the aminated gels, 0.1 g of the anhydride per millilitre of packed gel was mixed in water to get appropriate slurry. It was stirred at 6–10°C while the pH was held at *ca.* 6 with 6 *M* sodium hydroxide for as long as substantial decrease in the pH was observed. The gel was then rinsed with water and the preceding treatment was repeated. Finally the gel was thoroughly washed.

Table II includes the chemical structures of the prepared gels at pH *ca.* 6.0. We have previously measured the ligand concentrations of several synthesized gels[1,4]. Because we used closely similar methods here, it can be supposed that there are roughly similar concentrations of bound ligands on the present gels.

Measurement of the enzyme activity

The method for the determination of BCAT activity was a modified version from the literature[18]. The substrate solution contained 30 m*M* L-leucine, 30 m*M* α-ketoglutarate, 15 μ*M* PLP and 80 m*M* Tris–HCl (pH 8.0). This solution (0.4 ml) was incubated at 37°C for 5 min, and then 0.1 ml of enzyme sample was added. After 30 min the reaction was stopped with 0.1 ml of 5 *M* sulphuric acid. 2,4-Dinitrophenyl-

hydrazine (1 ml, a 15 m*M* solution in 2 *M* hydrochloric acid) was added, and after 5 min 2.5 ml of toluene were applied to extract the hydrazone of α-ketoisocaproic acid into the toluene phase. For this purpose the test-tubes were stoppered and continuously inverted in a mechanical device for 2 min. The phases were separated by centrifugation for 2 min at 2000 *g*. This procedure also eliminated the disturbing effects of precipitated hydrazone of α-ketoglutarate. The absorbance of the toluene layer was measured at 348 nm. The blank solution contained water in place of the enzyme, and a 2.5 m*M* solution of α-ketoisocaproate in 0.1 *M* Tris–HCl served as a standard and control solution which yielded absorbance of *ca.* 0.86. The unit of enzyme activity used here (Table I) is defined as the change of absorbance units at 348 nm and 37°C within 30 min.

Protein determination

Absorbance at 280 nm was usually employed to indicate protein during chromatographic runs. The success of the purification procedure was also followed by measuring protein from the pooled fractions with the Coomassie Brilliant blue method[19]. The standard solutions were made from bovine serum albumin.

RESULTS

Prepurification

The purification procedure initially followed the outline described by Jenkins and Taylor[8,16] until the ammonium sulphate precipitation. Because we failed to obtain adequate purification with the salt fractionation this step was used only to concentrate the solution. The salt was added to 50% saturation and after 30 min (a longer time lowered the recovery drastically) in an ice-bath the precipitate was centrifuged off (10,000 *g*, 10 min). The enzyme was dissolved in a minimum of 20 m*M* Tris–caproate buffer (pH 6.0) supplemented with 2 m*M* EDTA. The opalescent solution thus obtained was again centrifuged, and the supernatant was dialysed against the same buffer overnight. If the last centrifugation was omitted the dialysate became gelatinous, which hampered the subsequent step.

TABLE I

PURIFICATION OF BRANCHED-CHAIN AMINO ACID TRANSAMINASE FROM 2 kg OF PIG HEARTS

For Sepharose 4B-CS, see Table II. The enzyme unit is defined in the Experimental Section.

Procedure	*Volume (ml)*	*Total activity (units)*	*Protein (mg/ml)*	*Specific activity (units/mg)*	*Yield (%)*	*Purification*
Extraction	4000	53,000	12.3	1.1	–	–
Heat	1940	122,000	6.8	9.3	100	1
$(NH_4)_2SO_4$ precipitation	43	105,000	53.7	45.5	86	5
Dialysis	60	87,000	31.6	45.9	71	5
Ultrogel AcA 44	149	71,400	2.0	240	58	26
DEAE-Sepharose CL-6B	68	55,900	0.9	913	46	98
Sepharose 4B-CS	240	33,500	0.03	4660	27	501

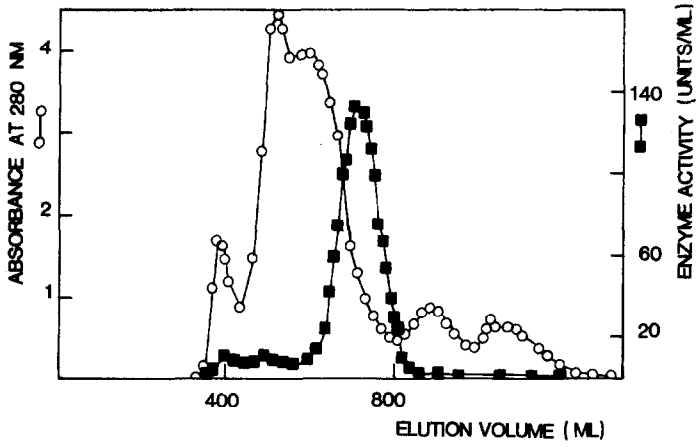


Fig. 1. Chromatography of a preparation of BCAT on Ultrogel AcA 44 gel. The sample solution (Table I) contained 660 mg of protein and 19,300 units of BCAT. The experiment was performed at a flow-rate of 70 ml/h in a 110-cm column of 3.4 cm I.D. The elution was carried out with 20 m*M* Tris-caproate (pH 6.0), including 2 m*M* EDTA.

Gel chromatography of the dialysate on Ultrogel AcA 44 yielded a 5.5-fold purification with excellent recovery of the enzyme (Fig. 1). The tubes containing BCAT were pooled and concentrated with an Amicon membrane filter (PM 30) for the following ion-exchange step.

CM-Sepharose Cl-6B did not purify BCAT when the elution was carried out with a gradient from 20 m*M* to 100 m*M* sodium caproate (pH 6.1). When DEAE-Sepharose CL-6B was used (Fig. 2), a 4.1-fold purification was achieved. After these procedures the specific activity of BCAT was *ca.* 900 units per milligram of protein (Table I). This preparation was used in the final affinity purifications.

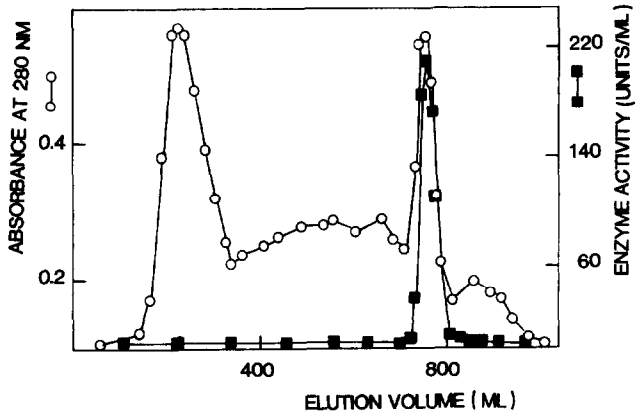


Fig. 2. Chromatography of a preparation of BCAT on DEAE-Sepharose CL-6B gel. The sample solution (Table I) contained 70 mg of protein and 10,200 units of BCAT. The elution was carried out with 50 m*M* Tris-caproate buffer (pH 8.5) supplemented with 5 m*M* EDTA by using a linear gradient from 0 to 0.5 *M* sodium chloride in the buffer (total volume 1300 ml) in a 30-cm column of 4.1 cm I.D.

Studies on affinity gels

Various substituted agaroses were tested to find appropriate gels for the purification of BCAT. The selected affinants involved substrate-resembling structural elements as well as effectors of the enzyme (Table II, the column headed Sepharose derivative). Table II shows the ratios of the elution volumes of the enzyme divided by that of protein (V_e^E / V_e^P) which relate to the effectiveness of the affinity gel.

All the prepared gels purified BCAT to some extent. However, the resolution on *S*-caproate gel was negligible unless an elution buffer of quite a low ionic strength was used. When the carboxyl groups of *S*-caproate gel were masked by the peptide bond to the amino group of valine, the separation increased as measured under the standard conditions (Table II) despite the fact that the negative character of the ligand remained. Similar separation was found on *S*-BAPA-PMA gel, which is also a cation exchanger. These two gels apparently are considerably hydrophobic in nature.

The most effective gels were *S*-Lys, *S*-BAPA-AO-Ala, *S*-BAPA-Val and *S*-CS. The elution patterns on the last two gels are exemplified in Figs. 3 and 4. Some UV-absorbing material was usually observed after 1 *M* sodium chloride had been applied (indicated by the arrows in the figures). The gel *S*-Lys separated best BCAT (V_e^E / V_e^P = 2.0). This gel comprises amino and carboxyl functions (a mixed ion-exchanger) and, in addition, a rather extensive alkyl chain links these groups to the agarose. Lysine was attached mainly via its ε-amino group because the reaction was performed at pH 10.0. If we assume that the alkyl group is sterically able to interact with the enzyme binding site, *S*-Lys can offer several recognition points for the enzyme. An alkylamino gel, *S*-BAPA, of corresponding chain length but without the carboxyl function, was clearly less effective. Free cycloserine is known to be a potent inhibitor of many B_6-enzymes[20]. Even though *S*-CS had no spacer arm it appeared to be one of the best gels for the separation of BCAT from other proteins. Since the

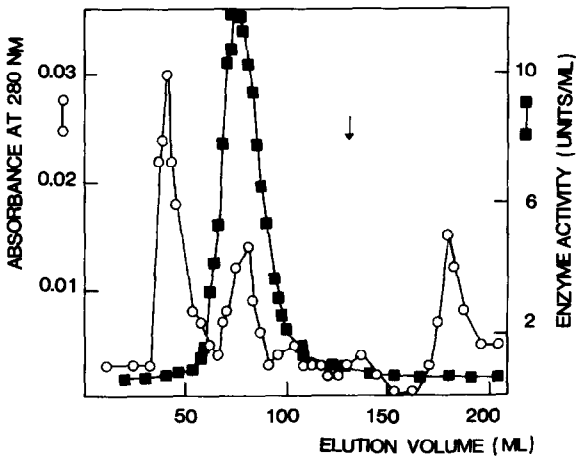
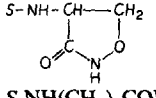
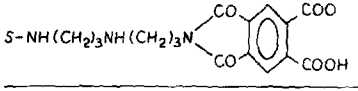


Fig. 3. Chromatography of a preparation of BCAT on *S*-BAPA-Val affinity gel (see Table II). The BCAT sample contained 530 units of the enzyme and the experiment was performed in a 53-cm column of 1.1 cm I.D. The elution conditions are described in Table II. At the point indicated by the arrow, 1 *M* sodium chloride was applied.

TABLE II

SEPARATION OF PIG HEART BRANCHED-CHAIN AMINO ACID TRANSAMINASE ON DERIVATIZED AGAROSES

The chromatographic runs were performed at +6°C with a column height of about 40 cm and flow-rate of about 25 ml/h. The abbreviations V_e^E and V_e^P refer to the elution volumes of the enzyme and protein, respectively. The column headed "activity recovery" apprises of how many per cents of the enzyme activity was detected after the run. The eluents were as follows: (A) 25 m*M* sodium phosphate, pH 6.0, plus 0.1 *M* NaCl and 1 m*M* 2-mercaptoethanol; (B) 25 m*M* sodium phosphate, pH 6.0, plus 1 m*M* mercaptoethanol and 0–0.1 *M* NaCl; (C) 25 m*M* sodium phosphate, pH 6.0, plus 1 m*M* mercaptoethanol; when the protein peak had come out of the column, the enzyme was eluted with 10 m*M* α-ketoglutarate, pH 6.0; (D) 25 m*M* sodium phosphate, pH 6.0, plus 1 m*M* mercaptoethanol, 0.1 *M* NaCl and 0.5 m*M* EDTA. Ca+E = into the test tubes on the fraction collector sodium caproate plus EDTA, pH 6.0, were pipetted beforehand to bring the final fraction 5 m*M* in respect to caproate and 1 m*M* to EDTA.

Sepharose 4B derivative	*Abbreviation*	*Gel volume (ml)*	V_e^P *(ml)*	V_e^E/V_e^P	*Activity recovery (%)*	*Elution conditions*
S-$NH(CH_2)_5COO^-$	*S*-Caproate	48	44	1.1	47	A
			48	1.6	22	B
			46	1.8	17	C
S-$NH(CH_2)_3NH(CH_2)_3NH_3^+$	*S*-BAPA	48	52	1.6	36	A
S-$NH(CH_2)_4CH(NH_3^+)COO^-$	*S*-Lys	49	44	2.0	33	A^{Ca+E}
						
S-$NH(CH_2)_5CONHCH(COO^-)CH(CH_3)_2$	*S*-Caproate-Val	47	48	1.5	62	D^{Ca+E}
S-$NH(CH_2)_3NH(CH_2)_3NHCOCH(NH_3^+)CH(CH_3)_2$	*S*-BAPA-Val	50	44	1.8	51	D^{Ca+E}
S-$NH(CH_2)_3NH(CH_2)_3NHCOCH(NH_3^+)CH_2$-O-$NH_2$	*S*-BAPA-AO-Ala	50	44	1.9	54	D^{Ca+E}
	*S*-BAPA-PMA	52	44	1.5	58	D^{Ca+E}

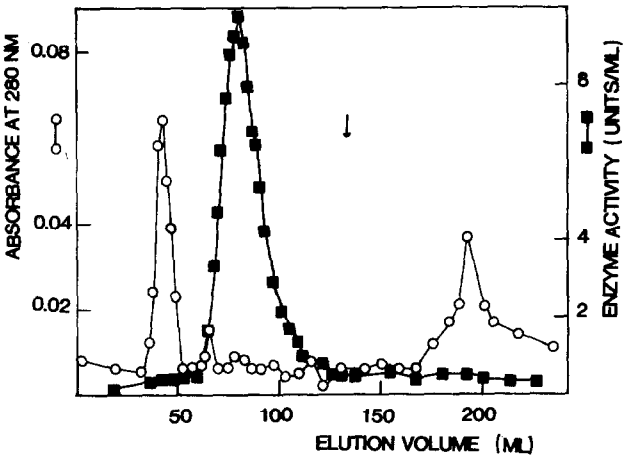


Fig. 4. Chromatography of a preparation of BCAT on *S*-CS affinity gel (see Table II). The experiment was carried out as in Fig. 3, except that 370 units of the enzyme were used.

pK_a of the amino-oxy function of amino-oxyalanine is 3.2[17], at pH 6.0 the ligand assembly carries only one positive group.

Table II also lists the recoveries of the enzyme activities. They were generally lower (30–60%) than in the prepurification steps (*ca.* 80%). The low protein concentration may cause this in part. The elution conditions of the affinity runs were varied, but results indicated that the decreased recoveries were a property of the gel itself rather than of the elution conditions.

If the enzyme recovery is regarded as the important criterion, *S*-Lys is no longer the gel of choice among those in Table II. Whereas *S*-CS and *S*-BAPA-AO-Ala gels produced nearly the same resolution as *S*-Lys, their recoveries were double that of *S*-Lys. Because *S*-CS is simple to prepare it was selected for the purification of BCAT described in Table I. In the preparative run the column cross-section was increased from that given in Fig. 4. Purification of the affinity step was 5.1-fold with a yield of 60%, and the total purification of BCAT was 500-fold. When the enzyme fractions were concentrated by ultrafiltration to *ca.* 5 mg/ml protein, the preparation was 75% active after 4 months at +2°C.

DISCUSSION

Progress of purification

The method described for the purification of BCAT is considerably shorter and apparently more resproducible than the earlier ones. The specific activity of the product corresponds to that resulting from the technique of Taylor and Jenkins[8], and the purification coefficient is also the same. The yield (27%) is excellent, especially in the light of the easy denaturation of BCAT. Mercaptoethanol increased the activity by less than 1.2-fold, and in the earlier stages of purification the increment was at least two. In the previous work[8] it was also larger with final preparation. Therefore our product was almost free from degenerated enzyme forms and may represent the most homogenous BCAT obtained to date.

Comparison of purification of BCAT with other transaminases on affinity gels

The mechanisms of various transaminases are widely accepted to differ only in minor details[21] which correlate to the structures of their substrates. So far more than 50 transaminases have been described differing in substrate specificity and biological origin. This enzyme family could thus serve as a continuous series of test objects for affinity studies.

In our previous investigation[1] we found that AlaAT separated best on similar aminic gels as BCAT. On the contrary, the same gels did not retard AspAT which was affected by gels containing carboxyl groups[4]. This difference may reflect the possibility that AspAT belongs to another subgroup of the family. AlaAT and BCAT have aminic substrates with hydrophobic side-chains whereas the substrates of AspAT carry a carboxylic one. It is self-evident that the enzyme must recognize only the side-chain because the common amino acids are structurally different only in that respect.

Because of the mechanistic uniformity of vitamin B_6-dependent enzymes, it may well be that there could be available a common group-specific "general ligand" for their chromatography. To probe this possibility, we studied affinants that contain aminooxy functions (*e.g. S*-BAPA-AO-Ala in Table II). The aminooxy compounds are potent inhibitors of B_6-enzymes because they covelently bind to their PLP coenzyme more tightly than common amines[20]. The aminooxy gels purified AlaAT[1] and BCAT (Table II) but in the purification of AspAT they performed poorly[4]. It appeared in later experiments (not published) that aminooxy gels can release PLP from AspAT holoenzyme. Most probably the coenzyme is attached to the gel as an oxime. This removal must be very fast and one-step process, because the apoenzyme does not separate from the bulk of proteins. The inability of aminooxybutyl-cellulose to sorb AspAT from a solution was also observed by Nedospasov and Khomutov[22]. Passage of AlaAT or BCAT through the aminooxy columns yielded high recoveries in the fractions, and the activities were not increased by treatment with PLP. Therefore these two enzymes bind their coenzymes tightly or the resolution is not sterically possible. This is in accordance with the fact that their PLP is very hard to remove and explains why reconstitutable apoenzyme has not been described in connection with AlaAT or BCAT[23].

Aspects of the affinity separation of B_6-enzymes

It can be envisaged that a sufficient binding of B_6-enzymes may occur to affinity gels either by formation of a Schiff base linkage or by the non-covalent attractions at the apoprotein sites that recognize the substrates. In fact, there were indications in an earlier affinity study that all amino ligands would possess a group specificity toward B_6-enzymes[24]. We observed that inhibition constants of various amines for leucine aminotransferase from *Escherichia coli* did not markedly differ from K_m of leucine (0.3 m*M*), even though the structures of the tested inhibitors poorly resembled those of the substrates[25]. This implies that the Schiff base as a covalent bond is energetically most important in the binding. On the scale normally used in affinity chromatography, Schiff base formation (presumably corresponding to a K_i value of *ca.* 1 m*M*) is weak, and only in favourable conditions can lead to a sufficient separation. Therefore all weaker biospecific and even non-biospecific attractions should be put to use.

The chromatographically observed interaction between affinant and enzyme is always compounded of both biospecific and non-biospecific effects. The amount of immobilized ligand affects both these factors in a complex way and is therefore a significant parameter[26]. We discussed in a previous report[1] a special case of this kind of compound affinity which could cause even a relatively weak specificity element to result in an adequate separation. The model postulates that a biospecific contact will rapidly open or locally labilize the enzyme structure, allowing the respective functional groups to be exposed and reaccommodated with the functional groups on the gel surface. Without the biospecific element the adsorption would be slow, but in its presence the interaction is catalysed sufficiently to be chromatographically observable. This phenomenon would be thus related to syncatalytic enzyme inhibition or slow protein adsorption on some chromatographic supports. The reverse situation probably applies in the affinity elution.

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